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Characterization of Exon and Intron of *Defensin* 1 Gene in *Apis cerana* and *Apis dorsata*

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Abstract

Honey bee *defensin* 1 gene belongs to the class of immunity genes in this social insect. The peptide acts as a defensive mechanism against infections caused by Gram-positive bacteria. The aim of this study was to characterize exon 2, intron 2, and exon 3 of *defensin* 1 gene in both the Indonesian honey bees *Apis cerana* and *A. dorsata*. First, *defensin* 1 genes of *A. cerana* and *A. dorsata* were sequenced, after which a bioinformatic analysis was conducted. The amplified length of these *defensin* 1 genes of *A. cerana* and *A. dorsata* were 479 and 458 bp, respectively, and their putative amino acid sequences comprised 66 and 65 amino acids, respectively, with 6 cysteine residues. The cysteine residues formed a disulfide bond and then linked the three domains in the defensin peptide with each other, thereby allowing the lysis of the bacterial membrane through pore formation. Intron 2 of the *defensin* gene demonstrated nucleotide variations between *A. cerana* from Indonesia and that from Korea and between *A. dorsata* from Indonesia and that from Malaysia; the latter species also demonstrated variations in exon 3. Phylogenetic tree topology of the bee, which was constructed based on *defensin* 1 gene, was compatible with a previous study showing that *A. cerana* and *A. dorsata* are more closely related to *A. mellifera* than to *A. florea*.

Keywords: cysteine residues, defensin 1 gene, honey bee phylogeny, immunity genes, intron variations

Introduction

Honey bees that belong to the genus *Apis* exhibit a highly social behavior and live in colonies with numerous individuals [1], which are categorized into three castes, viz., queen, drone, and worker. Worker honey bees perform grooming and some other tasks related to their antiseptic behavior to protect the nest from disease vectors [2]. These tasks are important for the honey bees that live in a large population to prevent the spread of various infectious diseases [2] caused by viruses, bacteria, fungi, and protists [3]. In addition to their hygienic behavior, honey bees possess certain defense systems against pathogen attack through the evolution of adaptive immunity genes [4]. There are four genes in honey bees that are involved in the innate immune system. All these four genes are used to attack the pathogen and encode antimicrobial peptides and are generally considered to be essential to fight against infectious Gram-positive bacteria [5, 6]. One of these four genes is the *defensin* gene.

Apis mellifera, one of the most extensively investigated honey bee species, has two types of *defensin* genes, *defensin* 1 (AY496432) and 2 (AY588474) [6]. The size of *defensin* 1 gene is 2012 bp, consisting of two introns and three exons. The lengths of introns 1 and 2 are 571 and 278 bp, respectively, while those of exons 1, 2, and 3 are 67, 181, and 40 bp, respectively, with the 5 UTR (untranslated region) and the 3 UTR ends measuring 706 and 169 bp in length, respectively [6]. In addition to the *defensin* 1 gene of *A. mellifera*, there are some other *Apis defensin* 1 genes that are available in GenBank, including those of *A. florea* (NW_003790532), Korean *A. cerana* (NW_016018233), and Malaysian *A. dorsata* (NW_006263879). The *defensin* 1 gene of these three *Apis* species, like the *defensin* 1 gene of *A. mellifera*, also consists of two introns and three exons but has a different length. The lengths of *defensin* 1 gene of *A. florea* (NW_003790532), Korean *A. cerana* (NW_016018233), and Malaysian *A. dorsata* (NW_006263879) are 1181, 1228, and 1067 bp, respectively.

It is worth noting that defensin 1 protein has potential applications in the pharmaceutical field. In fact, the defensin protein found in honey was able to reduce the viability of wound-causing pathogenic bacteria, including *Staphylococcus aureus*, *Streptococcus agalactiae*, and

Pseudomonas aeruginosa [7], as well as other pathogenic bacteria such as *Escherichia coli* [8]. Furthermore, another study reported that bee-derived defensin 1 may promote cutaneous wound closure both *in vivo* and *in vitro* [9]. However, there is still a lack of data regarding *defensin* genes from Indonesian honey bees such as *A. cerana* and *A. dorsata*. Therefore, we intended to fill this gap of unavailable data of *defensin* 1 gene from Indonesian honey bees through the results of our study. Moreover, it is important to further elaborate the application of *defensin* gene and protein, especially for both health and medical purposes. Hence, we conducted this study to characterize exon 2, intron 2, and exon 3 of the *defensin* 1 gene of *A. cerana* and *A. dorsata* from Indonesia, the results of which could be used as a comparison material in immune-related analyses in honey bee species, especially those belonging to the genus *Apis*.

Materials and Methods

Biological samples. The honey bees of the species *A. dorsata* used in this study were obtained from the collections of Dr. Rika Raffiudin and Desmina Hutabarat from Sampai Mountain, Sukabumi, West Java. Dr. Rika Raffiudin also provided the worker honey bees of *A. cerana* collected from Bogor, West Java (Table 1).

DNA Extraction, amplification, and sequencing. DNA was extracted from the honey bee thorax samples using a standard phenol–chloroform extraction method and ethanol precipitation [10] with few modifications [11]. The targets of the *defensin* 1 gene, including exon 2, intron 2, and exon 3 (hereafter, *defensin* gene 1), were amplified using forward and reverse primers (Table 2, Figure 1) that were designed based on the DNA sequences of the *defensin* 1 gene of *A. mellifera* (AY496432). The conditions for the gene amplification procedure were initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C (1 min), annealing at 48 °C and 45 °C for *A. cerana* and *A. dorsata defensin* 1 gene, respectively (30 s), elongation at 72 °C (1 min), and postelongation at 72 °C (2 min). The PCR products were then separated by 1.5% agarose gel electrophoresis and stained using Diamond^{TM} Nucleic Acid Dye (Promega, US). DNA sequencing was conducted at 1st Base Company, Malaysia.

Table 1. Primers Used for the Amplification of the *Defensin* 1 Gene

No	Gene target	Primer name	Nucleotide sequence (5 -3)
1	*Defensin* 1 exon 2, intron 2, and exon 3	Amel_def1.2_F	**GATGAATTCGAGCCACTTGAGC**
		Amel_def1.2_R	**TAACCGAAACGTTTGTCCCAGA**

Table 2. Ingroup and Outgroup Data for Nucleotide Variation and Phylogenetic Analysis

No	Species	Accession number	Origin	Reference
Ingroup				
1	*Apis cerana* (sample)	LC331613	Bogor	This study
2	*A. dorsata* (sample)	LC331614	Sukabumi	This study
3	*A. mellifera*	AY496432	Slovakia	[6]
4	*A. cerana*	NW_016018233	Korea	[32]
5	*A. dorsata*	NW_006263879	Malaysia	Rueppell *et al.* 2013
6	*A. florea*	NW_003790532	*	Qu *et al.* 2010
Outgroup				
1	*Bombus terrestris*	NC_015767	Switzerland	[33]
2	*B. ardens-ardens*	FJ172506	Korea	[34]

* = data were unknown

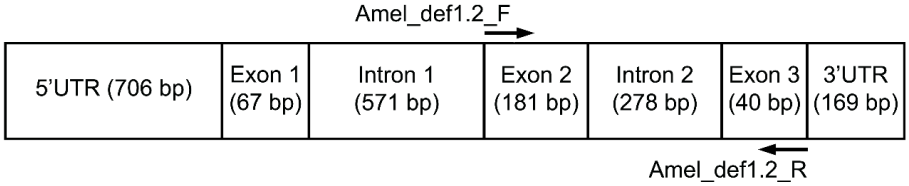

Figure 1. Schematic Position of Primers for Amplification of *Defensin* 1 Gene; Primers Were Designed Based on *A. mellifera Defensin* 1 Gene (AY496432); the Formation Of UTR, Exon and Intron *Defensin* 1 Gene of *A. mellifera* (AY496432) Based on Klaudiny *et al.* (2005)

DNA Extraction, amplification, and sequencing. DNA was extracted from the honey bee thorax samples using a standard phenol–chloroform extraction method and ethanol precipitation [10] with few modifications [11]. The targets of the *defensin* 1 gene, including exon 2, intron 2, and exon 3 (hereafter, *defensin* gene 1), were amplified using forward and reverse primers (Table 2, Figure 1) that were designed based on the DNA sequences of the *defensin* 1 gene of *A. mellifera* (AY496432). The conditions for the gene amplification procedure were initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C (1 min), annealing at 48 °C and 45 °C for *A. cerana* and *A. dorsata defensin* 1 gene, respectively (30 s), elongation at 72 °C (1 min), and postelongation at 72 °C (2 min). The PCR products were then separated by 1.5% agarose gel electrophoresis and stained using Diamond™ Nucleic Acid Dye (Promega, US). DNA sequencing was conducted at 1st Base Company, Malaysia.

Bioinformatics analyses of *defensin* 1 gene. The *defensin* 1 gene sequences obtained from *A. cerana* and *A. dorsata* were analyzed using a bioinformatics approach covering the putative amino acid sequences and analyses of nucleotide variations, homology, phylogeny, and protein motif of putative amino acid sequences. The putative amino acid sequences were obtained using the Genetyx-Win 4.0 program (www.genetyx.co.jp), while the homology analysis was performed using the BLAST program [12] in the NCBI website (https://blast.ncbi.nlm.nih.gov) by selecting the option "Refseq_genomic" in the "Database" column of BLAST-N and "Refseq_protein" in BLAST-X and BLAST-P, filling in "Apis (taxid: 7459)" in the "Organism" column in BLAST-N, BLAST-X, and BLAST-P. Protein motif searches of putative amino acid sequences were done using PROSITE (http://prosite.expasy.org/) [13]. DNA sequences of the *defensin* 1 gene of *A. cerana* and *A. dorsata* samples were aligned using the Clustal-X program [14] with the sequences of the *defensin* 1 gene obtained from other species (Table 2) and were then used for analyzing the nucleotide sequence and phylogeny. Nucleotide variations were analyzed using the MEGA 5 program [15]. The phylogenetic analysis was performed in MEGA 5.0 [15] using the neighbor-joining approach with the Kimura two-parameter model using 1000× bootstrap values. The DDBJ accession number of the *defensin* 1 gene sequence reported in this study is LC331613 for *A. cerana* and LC331614 for *A. dorsata*.

Results and Discussion

***Defensin* 1 gene sequence and putative amino acid sequence.** We successfully amplified exon 2, intron 2, and exon 3 of the *defensin* 1 gene of *A. cerana* and *A. dorsata* samples collected from West Java, Indonesia, with the sequence length of each sample being 479 and 458 bp, respectively (Figure 2 and 3). Both sequences were found to be AT-rich by 70.56% and 70.96%, respectively. The reason for using the regions of exon 2, intron 2, and exon 3 in this study was that these regions can be amplified and provide several nucleotide variations that can be discriminated at the species level of *A. cerana* and *A. dorsata*. In addition, these regions exhibit the gene signature of the cysteine residues of *A. cerana* and *A. dorsata* [16].

```
5′-GAGCCACTTGAGCATCCTGAGAACGAAGAACGTACCGATAGACATAGAAGAGTAACTT 58
     E  P  L  E  H  P  E  N  E  E  R  T  D  R  H  R  R  V  T
GTGACCTTCTCTCATTCAAAGGACAAGTCAATGACAGTGCTTGCGCTGCTAACTGTCTTAG 119
 C  D  L  L  S  F  K  G  Q  V  N  D  S  A  C  A  A  N  C  L
TTTGGGTAAAGCTGGAGGTCATTGCAAGAACGGAGTTTGTATTTGTCGAAAgtatgtgatt 180
S  L  G  K  A  G  G  H  C  K  N  G  V  C  I  C  R
tttcaaatttaagttttaaaatttttgaatttaactaaatcttgaatactttcatgaat 241

tcatttaatattatcgattgaaaattttcatgaataaatgaataactggtcacaatcatgt 302

catttaaatatgttcaaaattatttgcttatttatattactcgattaagaattaatttcct 363

tcgacattttctttttattatttatcaaatttagaatgattttataaagaaaagtataaaa 424

tttcatgctattattcctatttcagAACCAGTTTCAAAGATCTCTGGGACAAACG-3′    479
                        K  T  S  F  K  D  L  W  D  K
```

Figure 2. ***Defensin* 1 Gene Sequence and Putative Amino Acid Sequence of Exon 2 and 3 of *A. cerana* Samples; Capital Words: Exon 2; Capital and Uderline Words: Exon 3; Non-Capital Words: Intron 2; Putative Amino Acid Sequence: Below Exon 2 and 3**

```
5′-GCCACTTGAGCATTTTGAGACCGAAGAACGTACCGACAGACATAGAAGAGTAACTTGT 58
      P  L  E  H  F  E  T  E  E  R  T  D  R  H  R  R  V  T  C
GACCTTCTTTCGTTCAAAGGACAAATTAATGACAGTGCTTGCGCTGCTAACTGTCTCAGTT 119
  D  L  L  S  F  K  G  Q  I  N  D  S  A  C  A  A  N  C  L  S
TGGGTAAAGCTGGAGGCCATTGCAAGAACGGAGTTTGTATTTGTCGAAAgtatgtgatttc 180
 L  G  K  A  G  G  H  C  K  N  G  V  C  I  C  R
tcaaatttaagttttctaaatttttttgaattcaattaaatcttgaatactttcatgaatcc 241

atttaatattattkaaaattttcatgaataagtgrataactggtcacatcatttaaatata 302

ttcaaaattatttgcttatttatattattcgattaagaattaattttcttcaacatttttt 363

ttttattatttaggatgatttcataaagaaaaatataaaattttatactattatttctatt 424

tcagAACCAGTTTTAAGGAACTCTGGGACAAACG-3′                          458
    K  T  S  F  K  E  L  W  D  K
```

Figure 3. ***Defensin*** **1 Gene Sequence and Putative Amino Acid Sequence of Exon 2 and 3 of** ***A. dorsata*** **Samples; Capital Words: Exon 2; Capital and Uderline Words: Exon 3; Non-Capital Words: Intron 2; Putative Amino Acid Sequence: Below Exon 2 and 3**

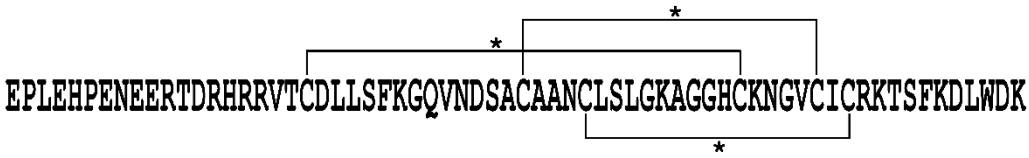

Figure 4. Schematic Illustration of Protein Motif of Putative Amino Acid Sequence of ***A. cerana Defensin 1*** **Gene Based on PROSITE Analysis; Asterisk (*): Disulfide Bond that Formed by Two Cycteine Residues**

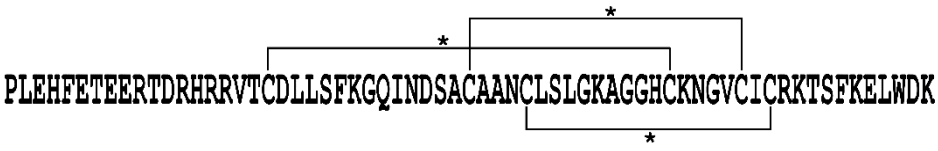

Figure 5. Schematic Illustration of Protein Motif of Putative Amino Acid Sequence of ***A. dorsata Defensin 1*** **Gene Based on PROSITE Analysis; Asterisk (*): Disulfide Bond that Formed by Two Cycteine Residues**

The lengths of exon 2 (168 bp) and exon 3 (30 bp) of both *A. dorsata* and *A. cerana* were similar; however, two additional nucleotides were found in exon 2 of *A. cerana*. On the other hand, the length of intron 2 of *A. dorsata* (260 bp) was shorter than that of *A. cerana* (279 bp). The putative amino acid length of *defensin* 1 gene was 66 and 65 residues for *A. cerana* (Figure 2) and A. *dorsata*, respectively (Figure 3), and both sequences had six cysteine residues, which is consistent with the results of a previous study on the *defensin* 1 gene of *A. mellifera* [6]. Protein motif searches revealed that six cysteine residues, in both *A. cerana* and *A. dorsata*, formed three disulfide bonds (Figure 4 and 5). The first cysteine residue formed a disulfide bond with the fourth cysteine residue, the second residue formed a disulfide bond with the fifth residue, and the third cysteine residue formed a disulfide bond with the sixth residue.

Defensins are cationic antimicrobial peptides rich in cysteine residues [16]. In this study, the number of positively charged amino acids in the putative amino acid sequences of the *defensin* 1 gene of *A. cerana* and *A. dorsata* samples exceeded the number of negatively charged amino acids. The positively charged amino acids of the two honey bee samples consisted of 14 amino acids, including five arginine (R), six lysine (K), and three histidine (H) residues. These numbers were found to be similar to those of the positively charged amino acids of the *defensin* 1 gene of *A. mellifera* (AY496432), *A. florea* (NW_003790532), and *A. cerana* (NW_016018233) and that of the amino acid sequence of *A. dorsata* (NW_006263879), which included a total of 16 amino acids comprising six arginine (R), seven lysine (K), and three histidine (H) residues.

Nucleotide variations in *defensin* 1 gene. Numerous nucleotide substitution variations were found in exon 2 (Table 3 and 7), exon 3 (Table 4 and 8), and intron 2 (Table 5 and 9) among the analyzed samples of the *defensin* 1 gene of *A. cerana* and *A. dorsata* compared with the *defensin* 1 gene sequences of *A. cerana* (NW_016018233), *A. mellifera* (AY496432), *A. dorsata* (NW_006263879), and *A. florea* (NW_003790532). The number of nucleotide substitution variations in exon 2 was higher than that in exon 3, whereas insertion and deletion of nucleotides occurred only in intron 2 (Table 6 and 10) in *A. cerana* and *A. dorsata*. *A. cerana* samples collected from West Java, Indonesia, revealed two nucleotide variations compared with the *defensin* gene of Korean *A. cerana* in intron 2. A similar number of nucleotide variations were also found between *A. dorsata* collected from West Java, Indonesia, compared with that of *A. dorsata* collected from Malaysia in introns 2 and 1 and synonymous mutations in exon 3. The probability of the occurrence of synonymous mutations in the first and third codons was 5% and 72%, respectively, whereas nucleotide variations in the second codon altered the amino acid (100%) [17].

The exon and intron regions of the *defensin* 1 gene sequences of *A. cerana* and *A. dorsata* samples were determined based on the alignment of the *defensin* 1 gene sequences of both samples with the *defensin* 1 gene sequences of honey bees in the ingroup species (Table 2). The intron sequence was determined using the conserved sequences of GT and AG in the upstream and downstream positions, respectively [18]. The estimated intron in the *defensin* 1 gene sequences of both samples were flanked by GT and AG in the upstream and downstream positions, respectively. Therefore, the upstream and downstream regions of the estimated intron 2 were estimated as exons 2 and 3, respectively.

Table 3. Substitution Nucleotide Variation in Exon 2 of *Defensin* 1 Gene of *A. cerana* Sample

No	Species	Nucleotide site											Variation number (bp)
		16	17	23	24	29	34	36	39	69	72	84	
1	*A. cerana* (sample)	C	C	A	C	A	A	C	T	C	A	A	0
2	*A. cerana*	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	T	T	.	T	.	G	.	C	.	.	.	5
4	*A. dorsata*	T	T	C	.	.	.	.	C	T	G	.	6
5	*A. florea*	.	.	C	.	G	T	T	.	.	G	G	6

Table 3. (*Continue*)

No	Species	Nucleotide site											Variation number (bp)
		85	87	90	102	115	117	119	138	145	148	150	
1	*A. cerana* (sample)	G	C	T	C	C	T	G	T	A	A	C	0
2	*A. cerana*	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	.	T	.	.	.	C	.	.	G	.	A	4
4	*A. dorsata*	A	T	.	.	.	C	.	C	.	.	.	4
5	*A. florea*	A	T	C	T	A	C	C	.	.	G	.	8

Table 4. Substitution Nucleotide Variation in Exon 3 of *Defensin* 1 Gene of *A. cerana* Sample

No	Species	Nucleotide site							Variation number (bp)
		453	455	459	462	463	465	468	
1	*A. cerana* (sample)	C	G	C	A	G	T	C	0
2	*A. cerana*	.	.	.	.	.	.	.	0
3	*A. mellifera*	.	.	.	.	.	.	.	0
4	*A. dorsata*	.	.	T	G	.	A	T	4
5	*A. florea*	T	C	.	G	A	.	T	5

Table 5. Substitution Nucleotide Variation in Intron 2 of *Defensin* 1 Gene of *A. cerana* Sample

No	Species	Nucleotide site																Variation Number (bp)
		177	178	182	184	185	197	198	199	214	217	223	235	240	242	243	257	
1	*A. cerana* (sample)	G	A	T	C	A	T	T	A	T	C	C	C	A	T	C	G	0
2	*A. cerana*	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	2
3	*A. mellifera*	A	T	C	.	.	C	C	C	.	T	T	.	.	C	.	.	9
4	*A. dorsata*	.	.	C	.	.	.	C	T	C	T	.	.	.	C	.	T	7
5	*A. florea*	A	.	C	.	G	A	.	T	.	.	.	T	T	C	T	.	9

Table 5. (*Continue*)

No	Species	Nucleotide site																Variation number (bp)
		279	287	291	292	293	294	295	298	301	303	314	317	320	321	342	350	
1	*A. cerana* (sample)	A	C	T	C	A	C	A	C	G	C	G	C	A	A	C	A	0
2	*A. cerana*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	-	1
4	*A. dorsata*	G	.	.	.	.	.	.	-	-	.	A	.	.	.	T	.	3
5	*A. florea*	G	T	C	T	C	T	G	A	A	T	.	A	G	T	.	G	14

Table 5. (*Continue*)

No	Species	Nucleotide															Variation Number (bp)
		361	366	368	374	390	398	406	417	419	428	431	432	438	440	443	
1	*A. cerana* (sample)	C	G	C	C	A	A	T	G	A	C	G	C	T	C	A	0
2	*A. cerana*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	.	.	T	.	.	.	.	C	G	.	.	.	.	.	.	3
4	*A. dorsata*	T	A	.	T	-	G	C	A	.	T	A	.	.	T	.	9
5	*A. florea*	.	.	.	-	G	.	.	-	-	T	A	T	C	.	T	6

Table 6. Insertion/Deletion in Intron 2 of Defensin 1 Gene of *A. cerana* Samples

No	Species	Insertion/deletion number (nucleotide)	Nucleotide site of insertion/deletion
1	*A. cerana*	0	-
2	*A. mellifera*	29	236-237; 252-253; 316-317; 344-358
3	*A. dorsata*	19	253-256; 296-301; 385-393
4	*A. florea*	28	197-198; 252-253; 316-317; 350-351; 289-290; 372-375; 415-421

Table 7. Substitution Nucleotide Variation in Exon 2 of *Defensin* 1 Gene of *A. dorsata* Samples

No	Species	Nucleotide site											Variation Number (bp)
		14	15	21	22	27	32	34	37	67	70	82	
1	*A. dorsata* (sampel)	T	T	C	C	A	A	C	C	T	G	A	0
2	*A. dorsata*	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	.	.	A	T	.	G	.	.	C	A	.	5
4	*A. cerana*	C	C	A	.	.	.	.	T	C	A	.	6
5	*A. florea*	C	C	.	.	G	T	T	T	C	.	G	8

Table 7. (*Continue*)

No	Species	Nucleotide site											Variation Number (bp)
		83	85	88	100	113	115	117	136	143	146	148	
1	*A. dorsata* (sample)	A	T	T	C	C	C	G	C	A	A	C	0
2	*A. dorsata*	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	G	.	.	.	.	.	.	T	G	.	A	4
4	*A. cerana*	G	C	.	.	.	T	.	T	.	.	.	4
5	*A. florea*	.	.	C	T	A	.	C	T	.	G	.	6

Table 8. Substitution Nucleotide Variation in Exon 3 of Defensin 1 Gene of *A. dorsata* Samples

No	Species	Nucleotide site							Variation number (bp)
		453	455	459	462	463	465	468	
1	*A. dorsata* (sample)	C	G	T	G	G	A	C	0
2	*A. dorsata*	.	.	.	.	.	.	T	1
3	*A. mellifera*	.	.	C	A	.	T	.	3
4	*A. cerana*	.	.	C	A	.	T	.	3
5	*A. florea*	T	C	C	.	A	T	T	6

Table 9. Substition Nucleotide Variation in Intron 2 of Defensin 1 Gene *A. dorsata* Samples

No	Species	Nucleotide site																Variation Number (bp)
		175	176	180	182	183	195	196	197	212	215	221	233	238	241	251	255	
1	*A. dorsata* (sample)	G	A	C	C	A	T	C	T	C	T	C	C	A	C	T	K	0
2	*A. dorsata*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	1
3	*A. mellifera*	A	T	.	.	.	C	.	C	T	.	T	.	.	.	G	G	8
4	*A. cerana*	.	.	T	T	.	.	T	A	T	C	.	.	.	.	G	G	8
5	*A. florea*	A	.	.	.	G	A	T	.	T	C	.	T	T	T	G	G	11

Table 9. (*Continue*)

No	Species	Nucleotide site																Variation Number (bp)
		273	276	281	285	286	287	288	289	291	302	305	308	309	330	338	349	
1	*A. dorsata* (sample)	G	R	C	T	C	A	C	A	C	A	C	A	A	T	A	T	0
2	*A. dorsata*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
3	*A. mellifera*	A	A	T	.	.	.	.	.	.	G	.	.	.	C	-	C	6
4	*A. cerana*	A	A	.	.	.	.	.	.	.	G	.	.	.	C	.	C	5
5	*A. florea*	.	A	T	C	T	C	T	G	T	G	A	G	T	C	G	C	15

Table 9. (*Continue*)

No	Species	Nucleotide site													Variation Number (bp)
		354	356	362	377	385	396	398	407	410	411	417	419	422	
1	*A. dorsata* (sample)	A	C	T	G	C	A	A	T	A	C	T	T	A	0
2	*A. dorsata*	.	.	.	.	.	.	.	.	.	.	.	.	.	0
3	*A. mellifera*	G	T	C	A	T	C	G	C	G	.	.	C	.	10
4	*A. cerana*	G	.	C	A	T	G	.	C	G	.	.	C	.	8
5	*A. florea*	G	.	-	A	T	-	-	.	.	T	C	C	T	7

Table 10. Insertion/Deletion in Intron 2 of *Defensin* 1 Gene of *A. dorsata* Samples

No	Species	Insertion/deletion number (nucleotide)	Nucleotide site of insertion/deletion
1	*A. dorsata*	0	-
2	*A. mellifera*	48	234-235; 250-251; 289-290; 304-305; 332-346; 375-376
3	*A. cerana*	19	250-251; 289-290; 375-376
4	*A. florea*	36	195-196; 250-251; 283-284; 289-290; 304-305; 338-339; 360-363; 375-376; 394-400

Table 11. BLAST-N Result in Homology Analysis of *Defensin* 1 Gene Sequence of *A. cerana* and *A. dorsata* Samples

Species sample	Description	*Query cover*	*E-value*	*Identity*	Accession number
Apis cerana	*Apis cerana* strain Korean unplaced genomic scaffold, ACSNU-2.0 scaffold_17, whole genome shotgun sequence	100%	0.0	99%	NW_016018233
Apis dorsata	*Apis dorsata* unplaced genomic scaffold, Apis dorsata 1.3 scaffold_708, whole genome shotgun sequence	100%	0.0	99%	NW_006263879

Table 12. BLAST-P Result in Homology Analysis of Amino Acid Sequence of *Defensin* 1 Gene of *A. cerana* and *A. dorsata* Samples

Species sample	Description	*Query cover*	*E-value*	*Identity*	Accession number
A. cerana	PREDICTED: defensin-1 [*Apis cerana*]	100%	1e-45	100%	XP_016905914
A. dorsata	PREDICTED: defensin-1-like [*Apis dorsata*]	100%	1e-44	100%	XP_006622575

Table 13. BLAST-X Result in Homology Analysis of Amino Acid Sequence of *Defensin* 1 Gene of *A. cerana* and *A. dorsata* Samples

Species sample	Description	*Query cover*	*E-value*	*Identity*	Accession number
A. cerana	PREDICTED: defensin-1 [Apis cerana]	99%	1e-45	100%	XP_016905914
A. dorsata	PREDICTED: defensin-1-like [Apis dorsata]	98%	1e-44	100%	XP_006622575

In addition to the nucleotide variations found in the exon and intron of *defensin* 1 genes among the species, several variations were found between *defensin* 1 and 2 genes in one individual among the honey bees. These variations could be observed based on the size of the gene, the number of exons and introns, promoters, regulatory sequences, the position where the genes are expressed, and the molecular weight of both *defensin* peptides [6].

Homology analysis of *defensin* 1 gene and putative amino acid sequence. Homology analysis of the *defensin* 1 gene sequences using BLAST-N (Table 11) resulted in the following two findings: first, the *defensin* 1 gene of *A. cerana* samples from Bogor, Indonesia, was homologous to that of Korean *A. cerana* (NW_016018233), and second, the *A. dorsata* samples collected from West Java, Indonesia, were homologous to *A. dorsata* samples collected from Malaysia (NW_006263879), with an identity value of 99% for both species. Similar results were also observed in the homology analysis of the amino acid sequence of the *defensin* 1 gene using BLAST-P (Table 12) and BLAST-X (Table 13). The putative amino acid sequence of the *defensin* 1 gene of *A. cerana* samples from West Java, Indonesia, was homologous to that of Korean *A. cerana* (XP_016905914), and the *A. dorsata* samples collected from West Java, Indonesia, were homologous to *A. dorsata* (XP_006622575) samples collected from Malaysia, with an identity value of 100% for both species in BLAST-P and BLAST-X. Nucleotides or amino acid sequences are considered to be homologous when the E-value is close to 0 or the identity value is > 70% for nucleotide sequence and 25% for amino acid sequence data compared with query data [19]. The honey bee genome evolves with a low rate of mutations compared with the genomes of the fruit fly and the malaria-causing mosquito [2]. Therefore, it is

possible that the *defensin* 1 gene of *A. cerana* and *A. dorsata* samples has a high identity value compared to that of the *defensin* 1 gene of both honey bee species from the GenBank database (NW_016018233 and NW_006263879).

The positively charged defensin peptide has three domains (amino-terminal loop, amphipathic α-helix, and carboxyl-terminal antiparallel β-sheet) and three disulfide bonds formed by six cysteine amino acids, which mediate the defense system of bees against bacterial pathogens [20]. The three disulfide bonds link the three domains with each other, thereby allowing the lysis of the bacterial membrane through pore formation [20]. Defensin peptides have the ability to fight against infections caused by both pathogenic Gram-positive and Gram-negative bacteria [21] and also fungal pathogens [22]. Defensin recognizes pathogens through molecular receptors on the pathogen cell surface, lipopolysaccharide molecules and lipoteichoic acid in bacterial cells [23], and glucosylceramide in fungal cells [24]. After identifying the target cells, the defensin protein lyses the target cells through pore formation in the cell membrane, as a result of the bonds formed between the positively charged defensin molecules and the negatively charged components in the cell membrane [25]. The formation of pores increases the permeability of the cell membrane, thereby causing cell lysis in both bacteria and fungi [26].

Despite the important role of defensin peptide, the expression of *defensin*s in the body of the honey bee tends to be low and delayed [5, 6]. This is presumably due to the fact that *defensin* may act as the final resort to fight against Gram-positive bacteria that can still persist after 24 h of infection. The concentration of defensin peptide in the body after 24 h is probably sufficient to attack Gram-positive bacteria that are still persistent in the bee body [5]. This phenomenon occurs because the honey bee possesses other defensive mechanisms such as "social immunity" through hygienic behaviors and the use of the antimicrobial resin that is collected to line the nest crack cavity [27]. The other defensive mechanisms include increasing the intracolonial genetic diversity through multiple polyandry mating [28]; therefore, the colonies have a high rate of genetic recombination [29] and are thus able to increase the resistance level of the colony.

In addition to defensin, honey bees have some other antimicrobial peptides, including apidaecin [30], abaecin [31], and hymenoptaecin [32]. Apidaecin is encoded by the *apidaecin* gene and has a broad-spectrum activity against both Gram-negative and Gram-positive bacteria when its concentration is high [30], as well as against some other human pathogenic bacteria [31]. Abaecin is encoded by the *abaecin* gene and is active against both Gram-positive and Gram-negative bacteria; however, its activity against Gram-negative bacteria is lower than that of apidaecin [31]. Hymenoptaecin is encoded by the *hymenoptaecin* gene and has the ability to inhibit the viability of both Gram-positive and Gram-negative bacteria and some bacterial pathogens in humans [32].

Genetic distance analysis and construction of phylogenetic tree. The relationship between *A. cerana* and *A. dorsata* samples with ingroup and outgroup species could be observed, according to the genetic distances (Table 14) and the phylogenetic tree (Figure 6) based on the *defensin* gene. The genetic distance analysis revealed that the highest genetic distance exists between *A. florea* and *A. mellifera* (0.154). Two pairs show the lowest genetic distance (0.002), i.e. between *A. cerana* samples collected from West Java, Indonesia, and Korean *A. cerana* as well as between *A. dorsata* samples collected from West Java, Indonesia, and *A. dorsata* from Malaysia. It was observed that the greater the genetic distance between two species, the more distant was the relationship.

Table 14. Genetic Distance Based on Defensin 1 Gene Exon 2, Intron 2 and Exon 3 Ingroup and Outgroup Specieses

	[1	2	3	4	5	6	7	8]
[1]								
[2]	0.107							
[3]	0.104	0.002						
[4]	0.127	0.069	0.067					
[5]	0.124	0.072	0.069	0.002				
[6]	0.154	0.082	0.079	0.098	0.101			
[7]	0.557	0.526	0.531	0.550	0.550	0.534		
[8]	0.554	0.529	0.534	0.531	0.531	0.536	0.085	

[1] = *Apis florea*; [2] = *Apis cerana*; [3] = *Apis cerana* (sample); [4] = *Apis dorsata* (sample); [5] = *Apis dorsata*; [6] = *Apis mellifera*; [7] = *Bombus terrestris*; [8] = *Bombus ardens-ardens*

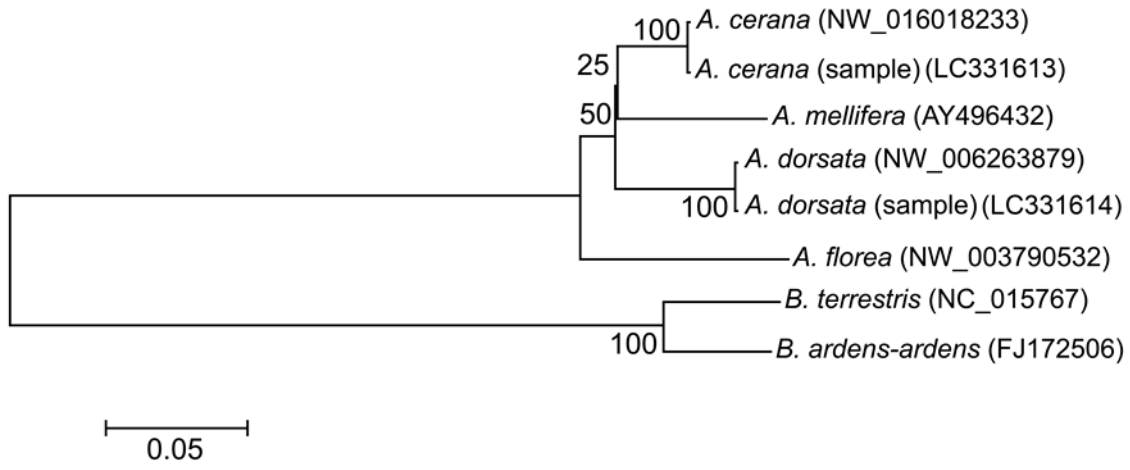

Figure 6. Phylogenetic Tree Based on Defensin 1 Sequence Eon 2, Intron 2 and Exon 3 in Genus *Apis* and *Bombus*

Genetic distance analysis and construction of phylogenetic tree. The relationship between *A. cerana* and *A. dorsata* samples with ingroup and outgroup species could be observed, according to the genetic distances (Table 14) and the phylogenetic tree (Figure 6) based on the *defensin* gene. The genetic distance analysis revealed that the highest genetic distance exists between *A. florea* and *A. mellifera* (0.154). Two pairs show the lowest genetic distance (0.002), i.e. between *A. cerana* samples collected from West Java, Indonesia, and Korean *A. cerana* as well as between *A. dorsata* samples collected from West Java, Indonesia, and *A. dorsata* from Malaysia. It was observed that the greater the genetic distance between two species, the more distant was the relationship.

Based on the bee phylogenetic tree topology constructed using exon 2, intron 2, and exon 3 of the *defensin* 1 gene in accordance with a previous study that was based on the genes *rrnL*, *cox*2, *NAD*2, and *itpr* [11], it was found out that *A. cerana* was closely related to *A. mellifera* and *A. dorsata* was more closely related to *A. cerana* and *A. mellifera* compared with *A. florea*.

Conclusions

We characterized exon 2, intron 2, and exon 3 of the *defensin* 1 gene of the Indonesian *A. cerana* and *A. dorsata* honey bee samples, which resulted in amplified sequences of 479 and 458 bp in length, respectively, and both sequences were found to be AT-rich. The putative amino acid sequence of both honey bee samples had six cysteine residues that were similar to those of *A. mellifera*. The nucleotide and the amino acid sequences of exon 2, intron 2, and exon 3 of the *defensin* 1 gene of *A. cerana* and *A. dorsata* exhibited a slow evolutionary rate that was indicated by the high identity value and the low E-value in BLAST-N and BLAST-P of this region compared with those in GenBank database. The results of the phylogenetic tree analysis, which was constructed based on exon 2, intron 2, and exon 3 of the *defensin* 1 gene sequence, were similar to those of a previous study that was based on the genes *rrnL*, *cox*2, *NAD*2, and *itpr*.

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