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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™ 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 | GATGAAATTCGAGCCACTTGAGC |
|    |                          | Amel_def1.2_R | TAAACCAGAAGCTTGTGCCCAGA |

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

| Ingroup | Species                  | Accession number | Origin     | Reference |
|---------|--------------------------|------------------|------------|-----------|
| 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      |           |
| 5       | *A. dorsata*             | NW_006263879     | Malaysia   | Rueppel et al. 2013 |
| 6       | *A. florea*              | NW_003790532     | *          | Qu et al. 2010 |

| Outgroup | Species                  | Accession number | Origin     | Reference |
|----------|--------------------------|------------------|------------|-----------|
| 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].

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 Underline Words: Exon 3; Non-Capital Words: Introns 2; Putative Amino Acid Sequence: Below Exon 2 and 3
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 of *A. dorsata* (216 amino acids, including five arginine (R), six lysine (K), and three histidine (H) residues) was 66 and 65 residues for *A. cerana* samples, respectively (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) |
|----|--------------------|-----------------|-----------------------|
| 1  | A. cerana (sample) | C   A   A   A   T   C   A   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) |
|----|--------------------|-----------------|-----------------------|
| 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) |
|----|------------------|-----------------|-----------------------|
| 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) |
|----|-----------------------|----------------|-----------------------|
|    |                       |                |                       |
| 1  | *A. cerana* (sample)  | G A T C A 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 . T T . C . . T  | 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) |
|----|-----------------------|----------------|-----------------------|
|    |                       |                |                       |
| 1  | *A. cerana* (sample)  | A C T C A C A G C G C A A C A  | 0                     |
| 2  | *A. cerana*          | . . . . . . . . . . . . . . .  | 0                     |
| 3  | *A. mellifera*       | . T . . . . . . . C G . . . .  | 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 site | Variation Number (bp) |
|----|-----------------------|----------------|-----------------------|
|    |                       |                |                       |
| 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) |
|----|--------------|----------------|-----------------------|
|    |              |                |                       |
| 1  | *A. dorsata* (sample) | 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) |
|----|-----------------|-----------------|-----------------------|
| 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) |
|----|-----------------|-----------------|-----------------------|
| 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. Substitution Nucleotide Variation in Intron 2 of Defensin 1 Gene *A. dorsata* Samples

| No | Species         | Nucleotide site | Variation Number (bp) |
|----|-----------------|-----------------|-----------------------|
| 1  | *A. dorsata* (sample) | G | A | C | C | A | T | C | T | C | 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 | G | G | 11 |

Table 9. (Continue)

| No | Species         | Nucleotide site | Variation Number (bp) |
|----|-----------------|-----------------|-----------------------|
| 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) |
|----|-----------------|-----------------|-----------------------|
| 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 sample | 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; 322-346; 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 |
|----------------|------------------------------------------------------------------------------|-------------|---------|----------|------------------|
| A. cerana      | Apis cerana strain Korean unplaced genomic scaffold, ACSNU-2.0 scaffold_17, whole genome shotgun sequence | 100%        | 0.0     | 99%      | NW_016018233     |
| A. dorsata     | Apis dorsata unplaced genomic scaffold, A. 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 defensins 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] | 0.107 | | | | | | |
| [2] | 0.104 | 0.002 | | | | | |
| [3] | 0.127 | 0.069 | 0.067 | | | | |
| [4] | 0.124 | 0.072 | 0.069 | 0.002 | | | |
| [5] | 0.154 | 0.082 | 0.079 | 0.098 | 0.101 | | |
| [6] | 0.557 | 0.526 | 0.531 | 0.531 | 0.531 | 0.536 | 0.085 |
| [7] | 0.554 | 0.529 | 0.534 | 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
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 *rRNA*, *cox2*, *NAD2*, and *itpr* [11], it was found 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 *rRNA*, *cox2*, *NAD2*, and *itpr*.

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