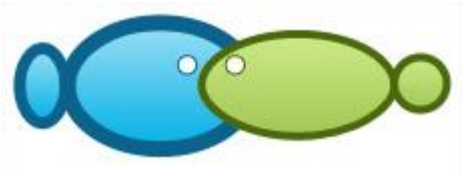

# Molecular characteristics of Indonesian *Enterocytozoon hepatopenaei* isolates based on sequence analysis of spore wall protein genes

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**Abstract**. Disease outbreaks caused by *Enterocytozoon hepatopenaei* (EHP) in Indonesia have been reported in *Litopenaeus vannamei* farms in North Sumatra, Lampung, West Java, Central Java, East Java, Bali, Lombok and Sulawesi. So far, reports on the molecular characteristics of EHP in Indonesia were limited. Characters of EHP from various sites were very important to describe their epidemiological relationship. The aims of this study were to determine the character of DNA, and to review the phylogeny relationship of EHP originating from various locations in Indonesia based on the Spore Wall Protein coding gene (SWP). 12 EHP isolates were successfully sampled from infected vannamei shrimp from farms, using purposive sampling in Lampung, Karawang, Pangandaran, Sidoarjo, Banyuwangi, Probolinggo, Blitar and Makassar. The EHP isolates were molecularly diagnosed using PCR with pairs of specific SWP_1F and SWP_1R primers, DNA sequencing, nucleotide sequence homology analysis and reconstructed genetic relationship tree. DNA sequence homology analysis demonstrated that the 12 EHP isolates were 100% similar with *Enterocytozoon hepatopenaei* SWP1 from Thailand and India. The alignment results illustrated that all EHP sequences from the 8 locations were identical. The phylogenetic tree topology provided the information that all samples were from the same clade and spread evenly. Indonesian EHP species from different locations were identical to the nucleotide sequence character among them and with the EHP isolates from Thailand and India. Therefore, Indonesian EHP isolates observed in this study most likely came from the same source, Asia. Spore wall protein coding genes have a good capacity as genetic markers for partial identification and characterization of EHP species, but they are less able to distinguish EHP intraspecies genetic diversity. However, further genetic analysis may be needed, especially for genes that encode effector proteins for virulence or genes that play a role in pathogenicity.

**Key Words**: disease, DNA sequencing, *Litopenaeus vannamei*, spore wall protein.

**Introduction**. *Enterocytozoon hepatopenaei* (EHP) has been reported to cause slow growth, a high food conversion ratio and significant losses in shrimp farms in Asia (Ha et al 2010; Sritunyalucksana et al 2014; Thitamadee et al 2016). EHP is an intracellular organism of the microsporidian group, found in tiger shrimp (*Penaeus monodon*) and vannamei shrimp (*Litopenaeus vannamei*). This disease was also reported related to the incidence of white fecal syndrome in Thailand, Vietnam and India (Chayaburakul et al 2004; Tourtip et al 2009; Tangprasittipap et al 2013; Tang et al 2015; Kesavan et al 2016). Chayaburakul et al (2004) further stated that EHP caused slow growth syndrome (MSGS) in black shrimp (*Penaeus monodon*). Rajendran et al (2016) reported that overall prevalence of EHP in the Southeast coast of India was 63.5%, detected by nested PCR. They further explained that the major pathology was severe necrosis of hepatopancreatic tissue. The high prevalence of EHP and its impact in the shrimp industry sustainability encouraged the Network of Aquaculture Centers in Asia-Pacific (NACA) to suggest EHP into the target list of pathogens that must be screened in shrimp juveniles that would be transported for cultivation purposes (Suebsing et al 2013). Therefore, these organisms remain one of the important threats to the shrimp farming industry in the Asia-Pacific region, including Indonesia.

White feces disease (WFD) outbreaks first occurred in 2014 and became an emerging problem for white leg (*L. vannamei*) shrimp farming industry in Indonesia (Faisal & Pancoro 2018). This disease was caused by EHP. They further described that EHP could be detected by PCR method which targeted spore wall protein (SWP). EHP infections in Indonesian shrimp farms further occurred in East Java, West Java, North Sumatra, Lampung, Bali, Lombok and Sulawesi (Tang et al 2016). Report from the shrimp farms indicated that EHP outbreaks were still occurring in the beginning of 2019. So far, very limited reports were found regarding the genotypic characteristics of EHP collected from different locations in Indonesia. Therefore, a study was conducted to determine the identity and molecular characteristics of EHP species from several shrimp farms in Indonesia. The results obtained from this research can provide information related to the genetic identity of EHP species from Indonesia and describe the distribution in the region (biogeography). Furthermore, epidemiological relationships of EHP as a pathogenic organism will also be presented for a possible control policy input.

## Material and Method

***DNA genome samples***. 12 selected EHP isolates collected during 2016-2017 in the form of isolated DNA genomes extracted from whiteleg shrimp were used in this study. The 12 samples were collected from various aquaculture environments in Indonesia, namely Lampung, Karawang, Pangandaran, Sidoarjo, Banyuwangi, Probolinggo, Blitar and Makassar. The sample origins and date of collection are presented in Table 1.

Table 1

The origin and date of isolation of *Enterocytozoon hepatopenaei* DNA genomic samples used in the study

| *No* | *Code/Name* | *Origin/Source* | *Collection and isolation* |
|---|---|---|---|
| 1 | Lampung | Lampung, Sumatera | August 2016 |
| 2 | Karawang | Karawang, West Java | August 2016 |
| 3 | Pangandaran | Pangandaran, West Java | December 2017 |
| 4 | Sidoarjo1 | Sidoarjo, East Java | March 2017 |
| 5 | Sidoarjo2 | Sidoarjo, East Java | March 2017 |
| 6 | Sidoarjo3 | Sidoarjo, East Java | March 2017 |
| 7 | Banyuwangi | Banyuwangi, East Java | March 2017 |
| 8 | Probolinggo1 | Probolinggo, East Java | March 2017 |
| 9 | Probolinggo2 | Probolinggo, East Java | March 2017 |
| 10 | Blitar | Blitar, East Java | March 2017 |
| 11 | Makasar1 | Makasar, Southeast Sulawesi | September 2016 |
| 12 | Makasar2 | Makasar, Southeast Tenggara | September 2016 |

***Methods used***. The study was conducted with a combination of explorative methods (Nazir 1999) and molecular characterization of EHP was based on the spore wall protein coding gene (SWP), using bioinformatics techniques as an analysis instrument. The PCR and sequencing of the EHP DNA isolates were carried out at the Laboratory of Molecular and Genetic Biology, Jepara Brackishwater Aquaculture Center.

The data observed were nucleotide sequences of SWP coding genes as a result of EHP DNA sequencing of the test samples. The identified data regarding the EHP sequence were then matched with available references in the GenBank. The variables observed were nucleotide sequences of EHP genes from various locations. The nucleotide sequence profiles were examined to discover the percentage of homology/similarity using nucleotide sequences of EHP SWP coding genes. Phylogenetic trees were constructed to describe the genetic relationship among the twelve EHP sequences of the test samples.

***PCR SWP coding genes***. PCR amplification of SWP coding genes from genomic DNA from EHP isolates was carried out in the ProFlex thermal cycler PCR System using a specific primer pair, SWP_1F 5'-TTG AGG GTT GTT AAG GGT TT-3 '(forward) and SWP_1R 5'-CAC GAT GTG TCT TTG CAA TTT TC-3' (reverse). The expected PCR (amplicon)

product was 514 bp. Primer and PCR protocol to detect EHP DNA gene were carried out according to the protocol designed by Itsathitphaisarn et al (2016) and Jaroenlak et al (2016). PCR was carried out with a reaction mixture consisting of a Promega commercial reagent kit (0.5 U Taq DNA polymerase enzyme; 200 µM from each dNTP; 10 mM Tris-HCl (pH 9); 50 mM KCl; 1.5 mM MgCl2); 0.2 µM for each primer (forward and reverse) and printed DNA (100 ng $\mu L^{-1}$). The DNA genomes of the samples were obtained by looking at the visualization results using ultraviolet transillumination from an amplicon that has been electrophoresed.

***Amplicon SWP genes fragment sequencing***. Identification of DNA amplicon samples from PCR results of EHP SWP coding genes was determined by sequencing. Samples were purified using Exostar and Clean Up (ExoSAP-IT) following the protocol of the manufacturer prior to sequencing. DNA sequencing analysis was carried out in two directions with the same primer pairs used in the PCR amplification. Sequencing is based on the principle of the Big Dye Terminator Cycle Sequencing method using an automated DNA sequencer model, ABI 3500 Genetic Analyzer (Applied Biosystem, USA). The ABI Prism Big Dye Terminator (Applied Biosystem) sequencing kit was used for reagents sequencing, each DNA strand following the protocol of the manufacturer.

***Analysis of nucleotide sequences of EHP SWP genes***. The identity of the DNA sequence of the EHP samples was confirmed by looking at the level of similarity or compatibility with the reference sequence data in the GenBank database using the Basic Local Alignment Search Tool (Altschul et al 1990), which can be accessed on http://www.blast.ncbi.nlm.nih.gov. Homology analysis was carried out to ensure that the sequence of the sample was in accordance with the target gene analyzed.

Examination and editing of sequence data for the determination of consensus sequences was done visually using the SeqA v6.0 (Sequencing Analysis version 6) software that was integrated in the ABI 3500 genetic analyzer automatic sequencing program. Contig reconstruction is done to produce a core region of DNA sequences that can be analyzed outside the gap.

Multiple sequence alignment analyses of EHP sequence consensus data of the samples and the SWP EHP sequences in the GenBank were performed as a screening to see the nucleotide profile that compiled the sample gene. In this study, alignment was carried out using the CLC Sequence v8 software program. The consensus alignment sequences that have been aligned are used to summarize comparisons between sequences of samples. Verification by reading and cross checking is done manually.

***Phylogenetic analysis***. The alignment data of the SWP coding gene sequences from the sample isolates and several reference sequences from the GenBank were then used for the phylogenetic analysis to determine the genetic relationship. Reconstruction of the phylogenetic tree was based on the UPGMA (Unweighted-Pair Group Method with Arithmetic Means) cluster analysis. This was done using the CLC Sequence v8 software program. Bootstrap re-sampling analysis (Felsenstein 1985) with 1000 replications is used as statistical support for estimating and evaluating the stability of the phylogenetic tree topology.

**Results and Discussion**. Electrophoresis visualization from PCR amplification using SWP_1F and SWP_1R primers produced 12 single bands of DNA fragments at a size of approximately 514 bp amplicons (Figure 1).

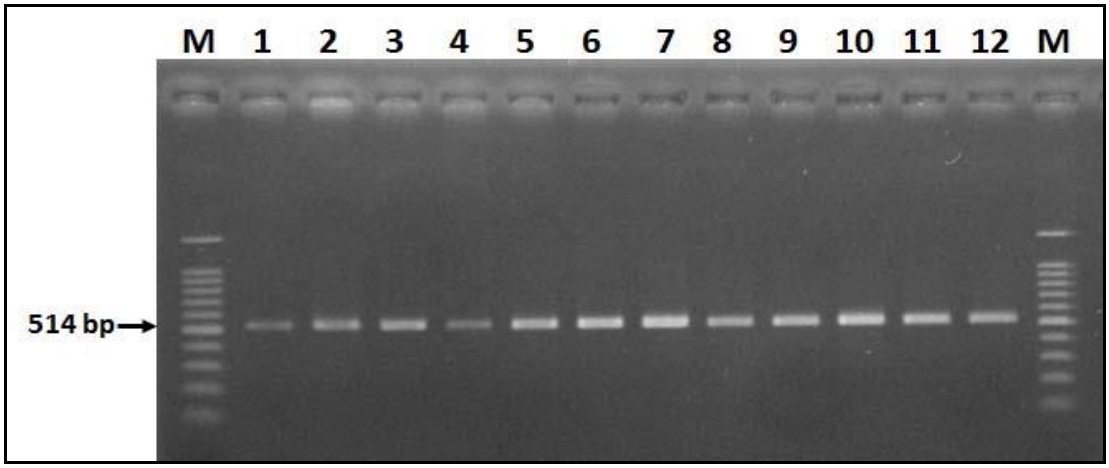


Figure 1. Electrophoregram of PCR DNA of 12 EHP isolates. M - 100 bp ladder DNA markers; 1 to 12 - EHP DNA bands from: (1) Lampung, Sumatera, (2) Karawang, West Java, (3) Pangandaran, West Java, (4) Probolinggo 1, East Java, (5) Probolinggo 2, East Java, (6) Blitar, East Java, (7) Banyuwangi, East Java, (8) Sidoarjo 1, East Java, (9) Sidoarjo 2, East Java, (10) Sidoarjo 3, East Java, (11) Makasar 1, Southeast Sulawesi, (12) Makasar 2, Southeast Sulawesi.

The 12 EHP DNA products using SWP coding genes produced sequences measuring from 503 to 516 base pairs (Table 2). Further alignment of EHP DNA sequences with other EHP gene sequences available in the GenBank database (NCBI) using the BLAST tracking program, indicated that all 12 EHP DNA genes were identical (100%) with *Enterocytozoon hepatopenaei* spore wall protein 1 (SWP1) (Accession Number MG015710.1), which was successfully isolated from infected vannamei shrimp in Thailand.

Table 2

The size of the partial DNA sequence of the twelve *Enterocytozoon hepatopenaei* spore wall protein coding gene and their homology/similarity values with reference available species in the GenBank (NCBI)

| *No* | *Sample* | *Size (bp)* | *Nearby Species (GenBank)* | *Homology/ Similarity (%)* |
|---|---|---|---|---|
| 1 | Sidoarjo1_East Java | 516 | *Enterocytozoon hepatopenaei*, spore wall protein 1 (SWP1) MG015710.1 | 100 |
| 2 | Sidoarjo2_ East Java | 515 | | 100 |
| 3 | Sidoarjo3_ East Java | 515 | | 100 |
| 4 | Blitar_ East Java | 509 | | 100 |
| 5 | Probolinggo1_ East Java | 505 | | 100 |
| 6 | Probolinggo2_ East Java | 513 | | 100 |
| 7 | Banyuwangi_ East Java | 505 | | 100 |
| 8 | Karawang_ West Java | 516 | | 100 |
| 9 | Makasar1_Southeast Sulawesi | 505 | | 100 |
| 10 | Makasar2_ Southeast Sulawesi | 516 | | 100 |
| 11 | Lampung_Sumatera | 503 | | 100 |
| 12 | Pangandaran_ West Java | 505 | | 100 |

Alignments of 12 EHP nucleotide sequences were matched with 4 reference sequences registered and available in the GenBank database, namely EHP infecting vannamei shrimp in India (Accession Number KY674357.1), in Thailand (MG015710.1 and KX258197.1) and in Venezuela, (KY593129.1). Multiple sequence alignment provided constant 486 base pair characters.

The alignment results showed that the DNA EHP consensus sequence of 12 isolates from the samples from Indonesia and 3 EHP isolates, namely KY674357.1 from

India, MG015710.1 and KX258197.1 from Thailand, 100% contained conserved regions without variable sites, meaning that all 486 bp occupied areas that were not easily changed or 100% conserved. Other results were shown by sequences of isolates EHP KY593129.1 from Venezuela, where 38 site variations (polymorphic sites) were found in the form of different nucleotide bases in the sequence (Figure 2).

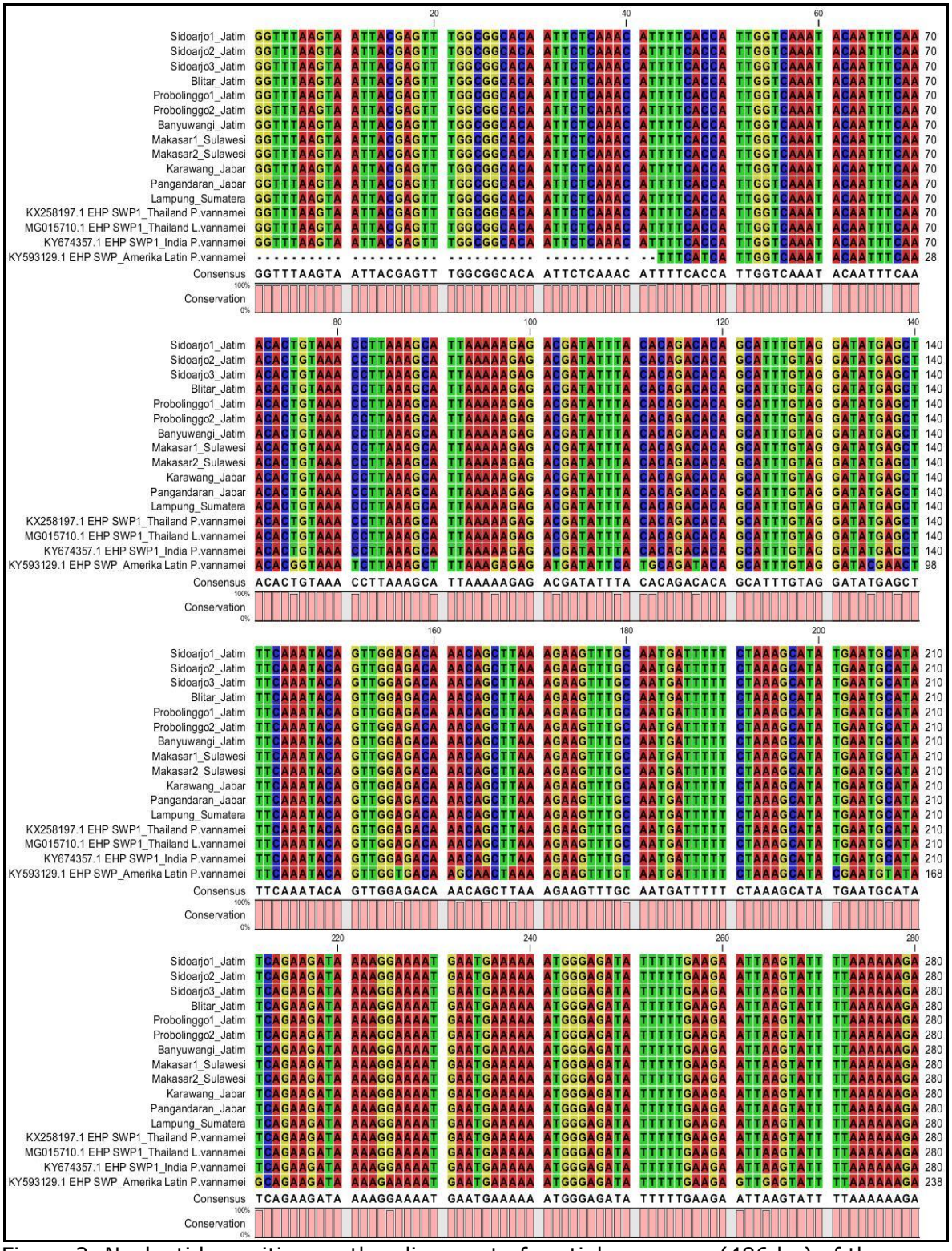


Figure 2. Nucleotide position on the alignment of partial sequence (486 bp) of the spore wall protein gene of 12 *Enterocytozoon hepatopenaei* (EHP) isolates and their comparison with 4 EHP spore wall protein from the GenBank database.

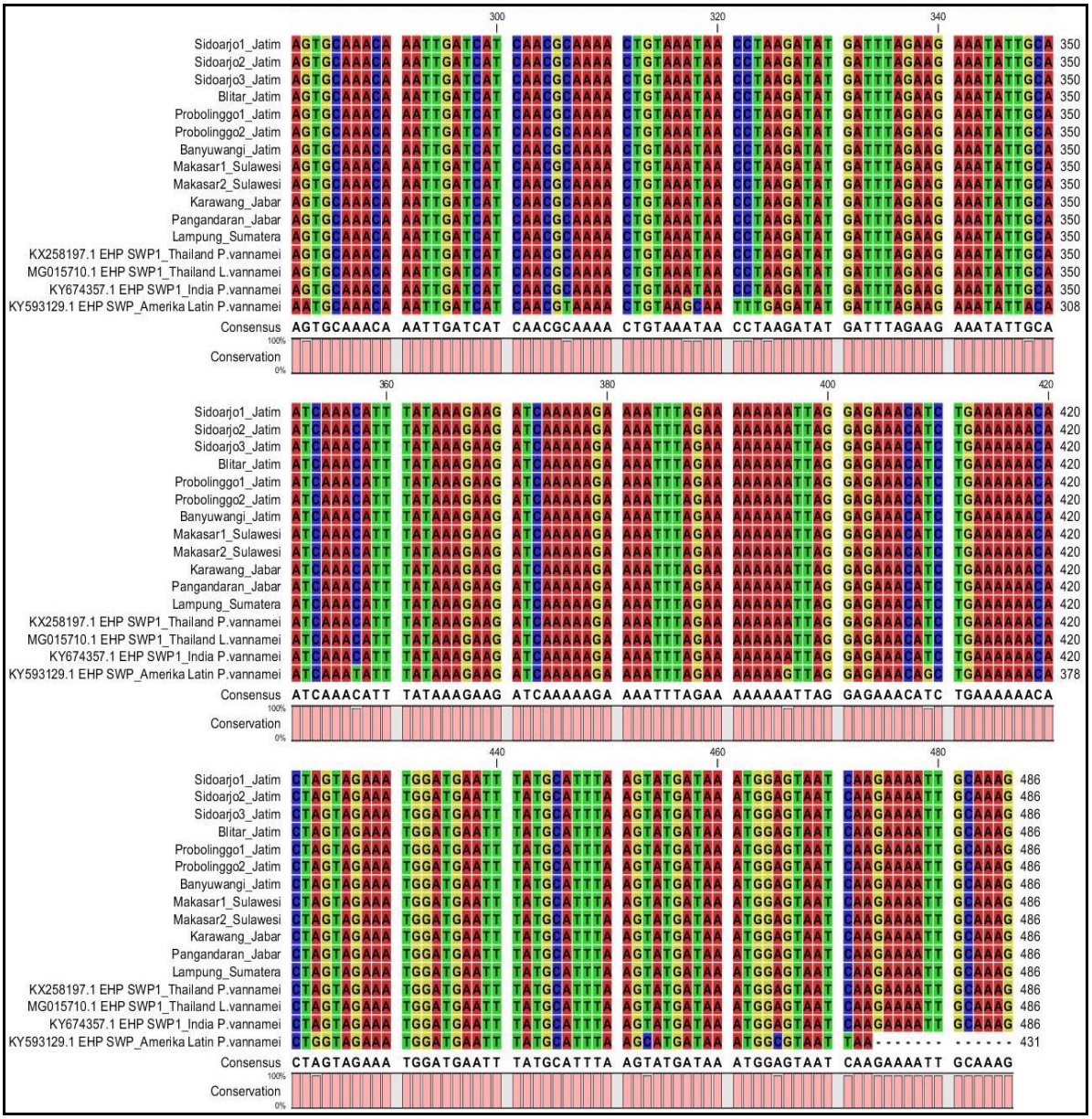


Figure 2. Nucleotide position on the alignment of partial sequence (486 bp) of the spore wall protein gene of 12 *Enterocytozoon hepatopenaei* (EHP) isolates and their comparison with 4 EHP spore wall protein from the GenBank database (continuation).

Reconstruction of the phylogenetic tree showed that the two genetic relationship clades were produced. The statistical validity showed that the clade or branch and building node of the resulting phylogeny tree was stable, with a very high level of trust, reaching 100%. The phylogenetic tree topology revealed that 12 EHP isolates from several Indonesian shrimp culture locations in this study and 3 EHP isolates from India (Accession Number KY674357.1) and Thailand (Accession Numbers MG015710.1 and KX258197.1) were in the same clade. While EHP KY593129.1 accession from Venezuela occupies a separate clade, with different branches from EHP groups from Indonesia, India and Thailand.

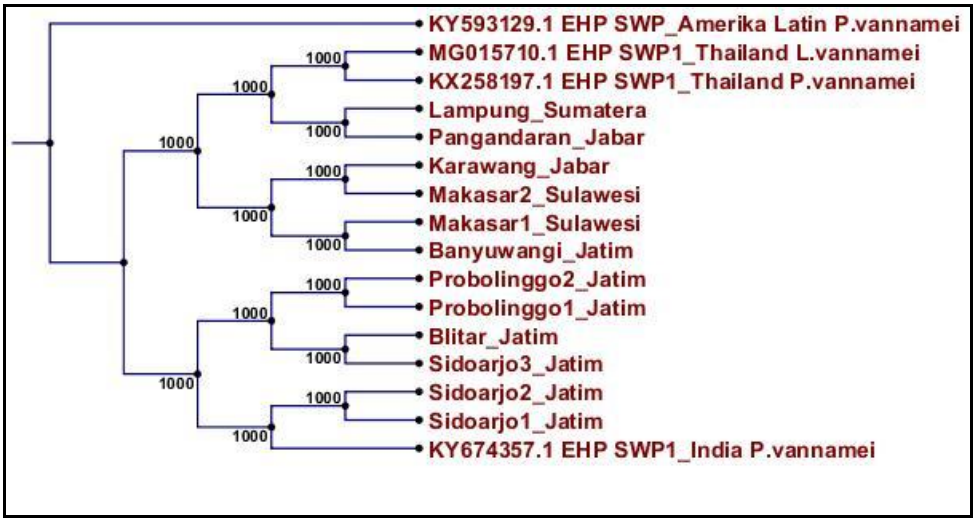


Figure 3. Phylogenetic tree of 12 *Enterocytozoon hepatopenaei* and 4 reference sequencegenes in the GenBank database. The number on the node indicates the percentage of bootstrap replication value.

The single band produced on the PCR results shows that the DNA of EHP isolates was successfully amplified using SWP_1F and SWP_1R primers. These results demonstrated that SWP_1F and SWP_1R primers specifically targeted the SWP coding genes in the EHP. These results confirmed other results (Itsathitphaisarn et al 2016; Jaroenlak et al 2016) which presented SWP_1F and SWP_1R as main PCR primers. The first step of the SWP-PCR in this study was to effectively determine the EHP gene. Furthermore, the SWP-PCR method was able to prevent cross reaction with close related microsporidian compared with the method using SSR rRNA primers. SWP-PCR has better sensitivity compared with SSU-PCR (Jaorenlak et al 2016),

According to Hall (2001), the level of similarity can be determined by the identity value. The higher the identity value, the closer to the reference sequence in the GenBank. In the nucleotide sequence homology analysis, a same species was found when identity similarity values were 99% of the closest organism through rRNA SSU gene sequence (Stackebrandt & Goebel 1994). Based on the comparison of identity, isolates have 100% similarity with the sequence of the EHP SWP1 gene with accession number MG015710.1 from Thailand. This indicated that the Indonesian EHP isolates in this study may have originated from Southeast Asia.

The results of multiple sequence alignment in this study showed that EHP isolates from Indonesia, Thailand and India are genetically identical (100% similar) in the targeted region of 486 characters, using partial SWP gene coding pair primers. The DNA sequence is identical if it has a similarity value of 91-100% (Kolondam et al 2012). Furthermore, multiple sequence alignment with Venezuelan EHP (Accession Number KY593129.1) showed some base site variation (polymorphic sites). Variation occurred in 38 sequence nucleotide base sites due to substitution and transversion. Variation occurring in certain sequence positions indicate an abnormal nucleotide sequences. Substitutions occurred in 31 sites at base numbers 48, 81, 96, 102, 109, 111, 112, 117, 135, 138, 162, 165, 180, 201, 207, 225, 261, 264, 282, 306, 317, 318, 321, 322, 324, 348, 357, 396, 423, 453 and 471. Transversion occurred in 7 sites of nucleotide base position numbers 75, 90, 156, 168, 211, 409 and 465. These differences in alignment might be due to differences in the length of the nucleotides. The Venezuelan EHP SWP gene has complete identity, but the reported sequence length was only 431 bp. Therefore, there were 55 sites of nucleotides lacking compared to others with 486 bp. Thus, there were 93 nucleotide site differences from Indonesian, Thailand and Indian isolates.

The topology of the phylogenetic tree of 12 EHP isolates from different geographical locations in Indonesia and 3 accessions of comparative EHP isolates from India, Thailand and Venezuela indicates that the EHP species were not originating from

separate groups. Furthermore, the dendogram shows that all isolates constructed were in the same main branch. This confirms that EHP species placed in this clade are genetically very close, are identical to each other and consistent with the results of sequence alignment analysis. According to Campbell et al (2010), if two organisms are closely related, then the DNA is very similar. The results of sequencing and phylogenetic analysis confirm that there was no filo-geography and no gene specificity was found in the different geographical regions among EHP isolates from Indonesia, India and Thailand. These findings indicate a potential epidemiological relationship of the EHP strains that affect shrimp cultivated in Indonesia, Thailand and India. The closeness of the genetic relationship becomes an important information to examine the pattern of translocation of this species. In contrast, phylogenetic patterns of Venezuelan EHP isolate demonstrated a different branch and clade, and therefore can be separated from the EHP Asian isolates. This was in line with the results of a study conducted by Tang et al (2017), in which EHP species from infected vannamei shrimp in Venezuela was genetically different from EHP species isolates from Southeast Asia.

Genetic distance among the EHP isolates was analyzed using a pairwise distance calculation. This analysis was used to see the level of transitional substitution and transversion through the number of nucleotide differences per pair. Finkeldey (2005) stated that genetic distance is one of the parameters for measuring genetic diversity between populations, by the differences in genetic structure in a particular gene site. The placement of Venezuelan EHP isolate in the separate clade indicates that there were significant differences in gene sequences and genetic distances compared with Indonesian, Thai and Indian EHP isolates. The differences reach 19.1% or have a genetic similarity level of 80.9% of 486 character comparable nucleotides. Schmitt & Haubrich (2008) stated that the presence of genetic distance indicates the possibility of geographical isolation of a population. Geographical locations far apart allowed the formation of different and specific ecological niches, where these conditions allow significant changes of nucleotide base sequences of Venezuelan EHP. Identical sequences of EHP isolates from Indonesia, India and Thailand showed a wide range geographical distribution of EHP species and potential epidemiological relationships among EHP species that affected the health status of shrimp farming in the Asian region. This geographical distribution pattern explained that EHP was an endemic parasite, and not an exotic parasite (Thitamadee et al 2016). This was also the reason for NACA to suggest the inclusion of EHP in the target list of pathogens that must be screened in shrimp juveniles that would be transported for cultivation purposes (Suebsing et al 2013).

The validity of the phylogenetic tree construction was statistically tested to determine the level of confidence by the method of re-sampling of existing data, known as bootstrap analysis (Efron 1979). The value of the bootstrap analysis indicated the level of confidence in the clade formed and the branching of phylogeny tree accuracy. The greater the bootstrap value, the higher the level of trust in the tree topology from the reconstruction (Hillis et al 1996; Nei & Kumar 2000; Hall 2001). Phylogenetic trees were statistically tested using 1000 bootstraps (Swofford et al 1996). Bootstrapping analysis of phylogenetic trees formed in this study indicated that the clade or branch of the resulting phylogeny tree was stable, with a very high level of trust, reaching 100%. According to Felsenstein (1985) and Osawa et al (2004), a clade or branch that has a bootstrap value or a trust value of 95% or more can be concluded to be a stable clade, where the group arrangement is consistent.

**Conclusions**. The Indonesian EHP species from different locations were identical based on the nucleotide sequence character among them and with EHP isolates from Thailand and India. Therefore, Indonesian EHP isolates observed in this study most likely came from the same source, namely Asia. Spore wall protein coding genes have a good capacity to be used as genetic markers for the identification and characterization of EHP species based on nucleotide sequences. However, they could not be used to distinguish EHP intraspecies genetic diversity. Further genetic analysis may be needed, especially for genes that encode effector proteins for virulence or genes that play a role in pathogenicity.

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