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Molecular Characteristics of Indonesian Isolate *Enterocytozoon hepatopenaei* Based on Sequence Analysis of 18S rRNA Genes

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ABSTRACT

Enterocytozoon hepatopenaei (EHP) infection have been reported as an obstacle of whiteleg shrimp (*Penaeus vannamei*) culture in Indonesia. However, understanding of the molecular characteristics of EHP species in Indonesia is not widely known. The aims of this study were to determine the identity and characters of DNA, and their phylogeny of EHP species from several different locations in Indonesia with specific references to 18S rRNA gene. The EHPs were collected from cultured *P.vannamei* from Lampung, Pangandaran, Sidoarjo, Banyuwangi, Probolinggo, Blitar, Makassar, and Lombok. Thirteen (13) samples were randomly selected to explore their gene characters through 18S rRNA gene sequencing. The primers used were EHP_F and EHP_1R. Parameter observed were DNA sequencing, nucleotide sequence homology with related available genes in the Gen Bank database, multiple sequence alignment, and reconstruction of genetic relationship trees. DNA sequence homology analysis showed that all samples had 99.89-100% similarity to Indian *Enterocytozoon hepatopenaei* (Accession Number MH259890.1 and MH260592.1). The alignment results illustrated that all EHP sequences of Indonesian isolates were 100% identical each other. The phylogenetic tree topology provided information that all sample accessions were in the same clade and spread evenly. The conclusion were that the Indonesian EHP species were identical (100%) and it could be said that they were genetically homogeneous.

Keywords: Enterocytozoon hepatopenaei, 18S rRNA gene, Indonesia.

1. Introduction

The emerging of microsporidian pathogen *Enterocytozoon hepatopenaei* (EHP), the causative agent of *hepatopancreatic microsporidiosis* in shrimp aquaculture that become a critical threat to the shrimp farming industry has been widely reported in shrimp-farming countries like Thailand, China, Indonesia, Malaysia, Vietnam, and India, by showing symptoms of slow growth and increased feed conversion ratios. EHP infection is currently prevalent and is one of the most severe and undesirable problems occurring in shrimp culture. Because even if it only produces a low or even non-fatal mortality rate, shrimp that survive after the EHP attack cannot grow optimally until the end of the harvest, so that resulting in considerable economic losses due to inadequate production performance and increased feed conversion ratio (Tourtip et al., 2009; Ha et al., 2010; Tangprasittipap et al., 2013; Sritunyalucksana et al., 2014; Tang et al., 2015; Rajendran et al., 2016; Kesavan et al., 2016; Thitamadee et al., 2016). Therefore, shrimp fries must be screened prior to stocking and during cultivation period (Suebsing et al., 2013).

EHP outbreaks have been reported to occur in whiteleg shrimp populations in East Java, West Java, North Sumatra, Lampung, Bali, Lombok and Sulawesi (Tang et al., 2016). Based on monitoring until March 2019, cases of EHP infection have been and still occur in almost all waters of Indonesia where shrimps were cultivated. So far, very little observations

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and publication of EHP in Indonesia, causing limited information about the genotypic characteristics of EHP species found in Indonesia. Thus, a study was conducted to determine the identity and molecular characteristics of EHP species in Indonesia. The data obtained from the research will provide information related to the genetic status of EHP species from Indonesia and the description of distribution in a region (biogeography). All of this information was a valuable and essential guide that can be used to describe the epidemiological relationship of EHP as a pathogenic organism and for its control.

The aims of this study were to determine the identity and character of DNA and the relationship of phylogeny of EHP species originating from several different locations in Indonesia, namely Lampung (Sumatra); Pangandaran (West Java); Sidoarjo, Banyuwangi, Probolinggo and Blitar (East Java); Makassar (Sulawesi); and Lombok (West Nusa Tenggara).

2. Materials and Methods

2.1. DNA Genomic Samples

A total of 13 genomic DNA samples collected from the shrimp that infected by EHP from different culture ponds located in (Lampung, Pangandaran, Sidoarjo, Banyuwangi, Probolinggo, Blitar, Lombok and Makassar) during August 2016–March 2019.

The samples (extracted DNA) that suspended in 50µl of nuclease-free water in sterile microtubes collected in a frozen condition in polyethylene bags kept in ice and transported to the laboratory and processed within 24 hours.

2.2. PCR 18S rRNA Gene

PCR amplification was performed using primers targeting the 18S rRNA gene of EHP as reported by Tang et al., (2015) that used for the labeling probe and in situ hybridization reaction. The primers used for amplifying were EHP-F (5'- GGG AAC GAC GAA CGG CTC AG – 3') and EHP-R1 (5'- TGC CTT GAT GAG ACA CTG TT – 3') that generated a 1.1 kb fragment to amplify the rRNA gene region of 18S rRNA.

The PCR reaction was carried out in total volume of 25 µL mixture containing 0.125 µl Taq DNA polymerase (5 U/µl) Promega, 0.5 µl dNTP (10 mM), 1.5 µl $MgCl_2$ (25 mM), 5 µl 5x PCR buffer, 0.5 µL (10 mmol) of each primer and 1 µl of template DNA. Amplification performed in a thermocycler ProFlex PCR System (Applied Biosystems) with the following cycling condition: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension / elongation at 72°C for 30 seconds, and final extension at 72°C for 5 minutes.

PCR products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination using a gel documentation system. The size of fragments were compared with standard 100 bp DNA ladder which contained fragments of known size. The PCR products was purified using Exostar and Clean Up with ExoSAP-IT extraction kit according to the manufacturer's instructions.

Table 1. EHP DNA genomic samples used in the study

No.	Code/Name	Origin / Source	Collection and Isolation
1	Lampung	Lampung, Sumatera	August 2016
2	Pangandaran	Pangandaran, West Java	December 2017
3	Aa_Sidoarjo1	Sidoarjo, East Java	March 2017
4	Ab_Sidoarjo2	Sidoarjo, East Java	March 2017
5	Ac_Sidoarjo3	Sidoarjo, East Java	March 2017
6	Dc_Banyuwangi	Banyuwangi, East Java	March 2017
7	Ca_Probolinggo1	Probolinggo, East Java	March 2017
8	Cb_Probolinggo2	Probolinggo, East Java	March 2017
9	Bd_Blitar	Blitar, East Java	March 2017
10	059_Makasar1	Makasar, Southeast Sulawesi	September 2016
11	061_Makasar2	Makasar, Southeast Tenggara	September 2016
12	D1_Lombok1	Lombok, West Nusa Tenggara	March 2019
13	E1_Lombok2	Lombok, West Nusa Tenggara	March 2019

2.3. Sequencing

To characterize the Indonesian EHP, DNA fragment was amplified from the 18S rRNA gene and sequenced. Sequencing of DNA and PCR products, were carried out at ABI 3500 Genetic Analyzer (Applied Biosystem, USA) with an ABI Prism Big Dye Terminator Kit (Applied Biosystems) according to manufacturer's instruction. Extension product was purified followed by capillary electrophoresis with an ABI Big Dye X Terminator Kit. Bidirectional sequences were done for all of 13 samples. The underlying sequencing protocol involved amplification of the target sequence by PCR and purification.

2.4. Sequence Analysis

Both the forward and reverse sequences were aligned and edited using SeqA v6.0 software. Further, the contigs for each gene sequence were BLAST analyzed using NCBI-BLASTN program facility (http://www.ncbi.nlm.nih.gov/BLAST) for searching significant similarity with reference sequences available in Gen Bank.

To assess genetic diversity among EHP isolates, multiple alignments of their various EHP 18S rRNA genotypes were compared. The 18S rRNA gene sequence of the EHP isolated from Indonesia were aligned with other EHP sequence reported from different countries in the Gen Bank database. Multiple sequence alignments were carried out using CLC Sequence v8 software.

2.5. Phylogenetic Analysis

Phylogenetic relationship of EHP sequences that generated in this study estimated from the aligned evolutionary conserved 18S rRNA gene sequences for each data set, including sequences of representative *E.bieneusi* isolates from the Gen Bank. The phylogenetic tree was constructed with the UPGMA algorithm using CLC Sequence v8 software. Bootstrap values of consensus tree inferred from 1000 replicates were determined to support individual branches (Felsenstein, 1985).

3. Results and Discussion

3.1. EHP PCR Analysis

All amplified samples showed discernible amplification using the primer pair (EHP-F and EHP-R1). The PCR amplification yielded a product size of ~ 1.1 kb 18S rRNA gene of EHP by the expected target area of amplification (**Fig.1**).

3.2. EHP DNA Sequence Analysis

Sequencing of the 18S rRNA gene had generated nucleotide sequences of 874 - 961 bp (**Table 2**). By BLAST analysis, the database search revealed that the sequences of these EHP isolates from *P.vannamei* from Indonesia showed 99.89 - 100% homology and identical with the 18S rRNA gene sequence of EHP reported from India that was previously published and deposited in the Gen Bank (Accession No. MH259890.1 and MH260592.1).

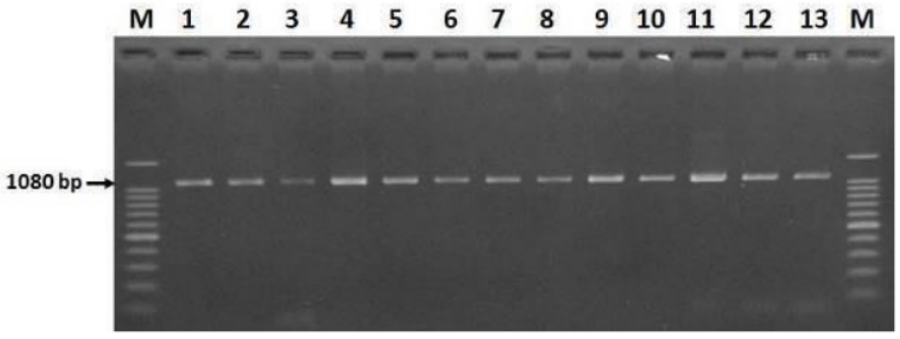


Figure 1. Agarose gel electrophoresis of the PCR products amplified from the samples of EHP genomic DNA. PCR amplification product (~1.1 kb) obtained using primers targeting 18S rRNA of EHP. Lane 1-13: EHP positive; M: 100 bp marker.

3.3. Sequence Comparison

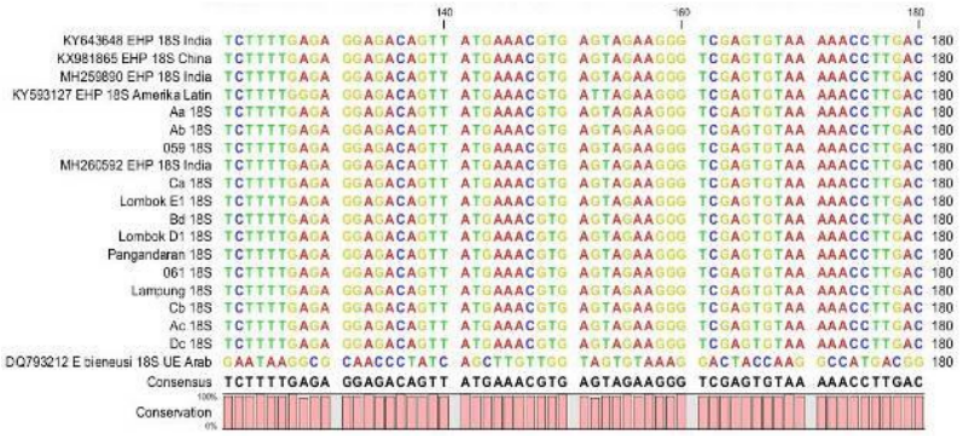
All nucleotide sequences of EHP generated in this study aligned with the EHP 18S rRNA sequences reported from India (MH260592.1, MH259890.1, and KY643648.1), China (KX981865.1) and Latin America (KY593127.1) using CLC Sequence v8 software and compared to each other (**Fig. 2**).

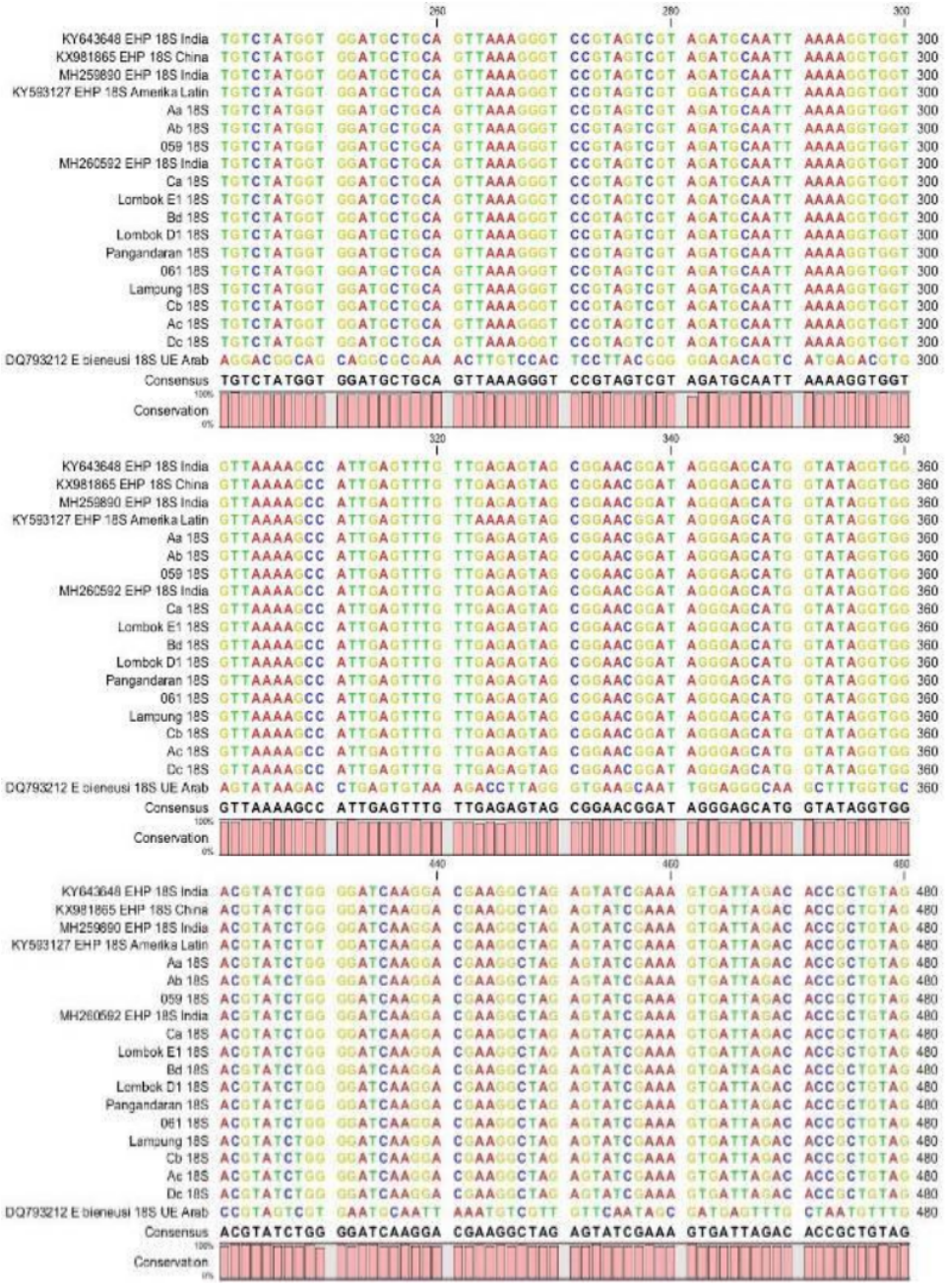
Multiple sequence alignments revealed that the homologous regions were highly conserved. The sequences from all EHP Indonesian isolates were identical to EHP isolates from India and China. Other results were shown by sequences of isolates EHP KY593127.1 from Latin American, where seven site variations (polymorphic sites) were found in the form of different nucleotide bases in the sequence which are variable sites (**Fig. 2**).

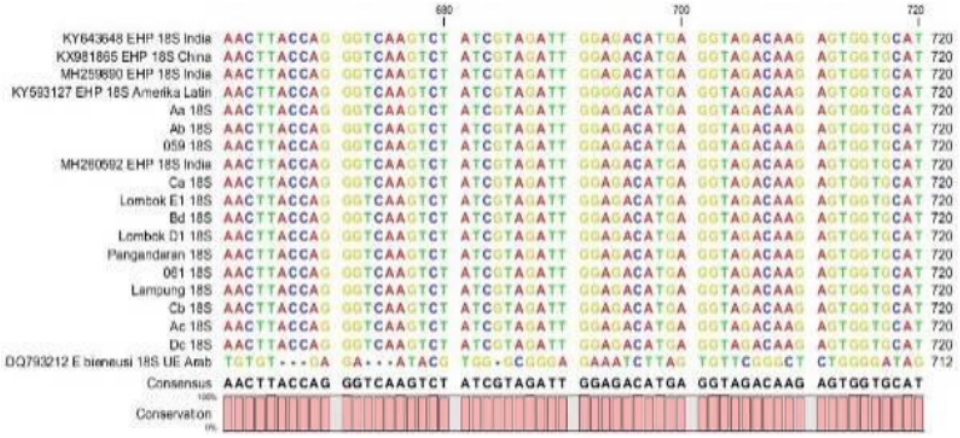
Table 2. The size and identity of the DNA sequence of Indonesian EHP isolates and their homology with nearby species.

No	Sample	Size (bp)	Nearby Species (Gen Bank)	Homology/ Similarity (%)
1	Aa_Sidoarjo1_East Java	958	*Enterocytozoon*	100
2	Ab_Sidoarjo2_ East Java	961	*hepatopenaei*	99.9
3	Ac_Sidoarjo3_ East Java	884	(MH259890.1) India	100
4	Bd_Blitar_ East Java	947		99.89
5	Ca_Probolinggo1_ East Java	956		99.9
6	Cb_Probolinggo2_ East Java	898		99.89
7	Dc_Banyuwangi_ East Java	874		100
8	059_Makasar1_Southeast Sulawesi	960		100
9	061_Makasar2_Southeast Sulawesi	920		100
10	Lampung_Sumatera	907		100
11	Pangandaran_ West Java	936		100
12	D1_Lombok1_West Nusa Tenggara	934		100
13	E1_Lombok2_West Nusa Tenggara	946		100

Figure 2. Nucleotide position on the alignment of a partial sequence of 18S rRNA genes of EHP isolates and their comparison to the Gen Bank database, using the CLC Sequence v8.




KY643648 EHP 18S India
KX981865 EHP 18S China
MH259890 EHP 18S India
KY593127 EHP 18S Amerika Latin
Aa 18S
Ab 18S
059 18S
MH260592 EHP 18S India
Ca 18S
Lombok E1 18S
Bd 18S
Lombok D1 18S
Pangandaran 18S
061 18S
Lampung 18S
Cb 18S
Ac 18S
Dc 18S
DQ793212 E bieneusi 18S UE Arab
Consensus
Conservation



3.4. Phylogenetic Analysis

Phylogenetic trees were used to construct the relationship among EHP isolates. *E.bieneusi* 18S rRNA from UE Arab (DQ793212) was considered as an outgroup for analysis in the present study. The topology of cladogram revealed that 18S rRNA gene of *E.hepatopenaei* isolates of Indonesia was evolutionarily close to MH260592.1, MH259890.1, and KY643648.1-EHP-India and KX981865.1-EHP-China with highest bootstrap value. All of Indonesian EHP 18S rRNA isolates were clustered with India and China isolates. This cluster was supported by a high bootstrap value (**Fig.3**). EHP KY593127.1 accession from Latin America occupied a separate clade with different branches from EHP groups from Indonesia, India, and China.

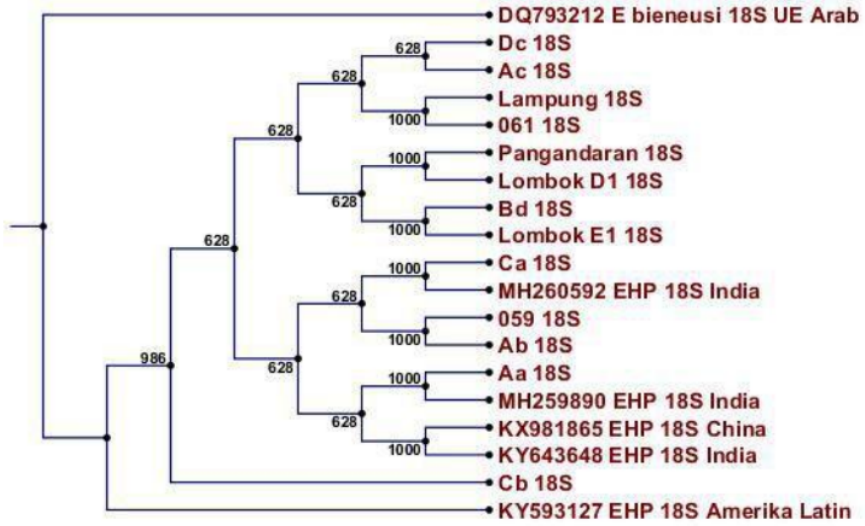


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Figure 3. Phylogenetic tree analysis of EHP based on 18S rRNA nucleotide sequences. Phylogenetic tree was generated using UPGMA method by the CLC Sequence v8 software. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. Bootstrap values are shown at the nodes.

3.5. Discussion

Visualization of the PCR results showed that the DNA of the samples was successfully amplified using EHP_F and EHP_1R primers. All positive samples demonstrated positive results for the 18S rRNA primers using the PCR protocol designed by Tang et al. (2015).

An expected PCR product of 1080 bp was yielded for the EHP positive samples. Based on the BLAST, the sequence of 18S rRNA gene of the collected samples showed 99.89 - 100% homology with EHP 18S rRNA gene sequences submitted in NCBI from different countries viz. India. According to Hall (2001), the level of similarity can be determined by identity values, the higher the identity value, the more similar to reference sequence in the Gen Bank. Furthermore, Stackebrandt and Goebel (1994) stated that the size to be classified in the same species when the similarity value of fragment identity was 99% of closest organism through rRNA SSU gene sequence (Stackebrandt and Goebel, 1994). Based on this guideline, the results of homology analysis of samples isolates, all confirmed as *E.hepatopenaei*.

Nucleotide sequence similarities in the 18S rRNA gene (1080 bp) between the Indonesian EHP, India, China, and Latin America showed that EHP isolates from Indonesia, India and China genetically had 99.89 - 100% identical sequence in the region of the 845 characters partial 18S rRNA gene pairs analyzed. A DNA sequence said to be equal when similarity value was 91-100% (Kolondam et al., 2012). The 100% consensus value formed on the alignment of all EHP sequences of the Indonesian samples compared to EHP sequences from India and China. These explained that the genetic distance between the sequence of EHP isolates of those countries were confirmed to be 0.00 – 0.11.

The results of multiple sequence alignment showed a site variation (polymorphic sites) of fundamental differences with the Latin American EHP nucleotide arrangement (Accession Number KY593127.1). The differences were in particular position in a sequence of 7 characters caused by the substitution of nucleotide sequences which were not regular nucleotide sequences. Changes that occur include transitions found as many as five sites in the position of nucleotide bases number 128, 281, 323, 325, 693, and transversion of 2 locations in nucleotide base positions number 152, 430. This substitution causes changes in nucleotide bases in the Latin American EHP 18S rRNA gene (Accession Number KY593127.1) so that there was a difference in bases. Every sequence difference in a particular base sequence was based on a consensus sequence.

From the topology of phylogenetic trees formed clades, where 13 accessions of EHP isolate samples originating from different geographical locations in Indonesia and 3 accessions of comparative isolates from India (MH260592.1, MH259890.1, KY643648.1), China (KX981865.1), were equally distributed in clusters throughout the tree. The dendrogram showed that all isolates constructed were in the same main branch. There were no different classifications. It's confirmed that EHP species placed in this clade genetically have a very close genetic relationship, identical, and closely related to each other. These results in line with Campbell et al. (2010) that if two organisms were closely related, then the DNA was very similar. The results of sequencing and phylogenetic analysis confirm that there was no filogeography (grouping of genetic relationship based on geography) and no gene specificity found in different geographical regions among EHP isolates from Indonesia, India, and China. These findings indicated a potential epidemiological relationship between EHP strains that affect shrimp cultivated in Indonesia, India, and China. The closeness of the genetic relationship becomes essential information needed for analysis and examination the pattern of translocation of this species.

Different patterns of phylogenetic trees formed in this study demonstrated further genetic relationship with Latin American EHP isolates (KY593127.1), which were placed at different branches, into stand-alone clades and separated from the EHP Asia isolates (Indonesia, India, and China). This description showed that the EHP of Latin American was different from the EHP species in Indonesia, India, and China, although there was genetic relationship. This condition was caused by the existence of site variations (polymorphic sites) in the form of nucleotide base differences in the sequences of 7 characters in the EHP 18S rRNA gene sequences of Latin American species. These results were in line with the findings of the study by Tang et al. (2017) who compared the similarity of nucleotide sequences to spore wall protein gene fragments (431 bp) between the Venezuelan EHP and Southeast Asian isolates collected from Vietnam (in 2014), Thailand and Indonesia (in 2016). Which showed that EHP species isolated from whiteleg shrimp infected in Latin America Venezuela (Accession Number

KY593129.1) was genetically different from EHP species isolates from Southeast Asia. These indicated that EHP from Venezuela was not a result of the introduction or originating from Southeast Asia.

The genetic relationship of Latin American EHP isolates with Indonesia, India, and China isolates was analyzed using a pairwise distance calculation which described the genetic distance between species. This analysis was used to see the level of transitional substitution and transversion through the number of nucleotide differences per pair. Finkeldey (2005) states that genetic distance was one of the parameters that can be used to measure genetic diversity between populations, namely by measuring the differences in genetic structure in a particular gene locus. The pairwise distance calculation was done by phylogenetic tree analysis. The placement of Latin American EHP isolates as a separate clade showed that there were significant differences in gene sequences and genetic distances when compared with Indonesia, India, and China EHP isolates, reaching 0.8% or having a genetic similarity level of 99.2% of 845 characters comparable nucleotides. Based on this calculation, the EHP of Latin America separate from the EHP Asia isolates (Indonesia, India, and China). In the phylogenetic tree if the genetic distance was close, then it was in the same branch, but if the genetic distance was far then, it was in a different chapter. If the organism is closely related, the DNA is very similar. The shorter the genetic distance, the more similar the DNA, the closer the genetic relationship (Campbell et al., 2010). Significant differences in gene sequence and genetic distance from EHP accession in Latin America compared to Asia was likely due to the location and geographical distance that were far apart, this refers to the opinion of Schmitt and Haubrich (2008) which states that the presence of genetic distance indicates the possibility of geographical isolation against a population. Geographic locations that far apart allow for the formation of different and specific ecological niches, where these conditions are associated with genetic factors, allowing for significant changes in the EHP nucleotide base sequence of Latin American.

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 aim was to evaluate branch stability and obtain the appropriate genetic tree branching points. The value of the boost was a measure of the level of confidence in the clade that was formed and the accuracy of the branching phylogeny tree. The greater the bootstrap value, the higher the level of trust in the tree topology from the reconstruction (Hillis et al., 1996; Nei and Kumar, 2000; Hall, 2001). In this study, phylogenetic trees were statistically tested using 1000 bootstrap methods (Swofford et al., 1996). The results of the bootstrapping analysis of phylogenetic trees formed in the study, indicated by the number 1000 on the branching node indicating the bootstrap value (%) with 1000 replications. It showed that the clade or branch of the resulting phylogeny tree was stable with a very high level of trust. 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 said to be a stable clade, where the group arrangement was consistent.

Identical sequences of EHP isolates from Indonesia, India, and China showed a wide geographical distribution range of EHP species and potential epidemiological relationships among EHP species that affected the health status of shrimp farming in the Asian region. The pattern of geographic distribution can explain that EHP was an endemic parasite, not an exotic parasite, as stated by Thitamadee et al., (2016). The widespread occurrence of EHP transmission appears to be due to introduction and translocation through the trajectory of trade in shrimp and shrimp between areas within Indonesia and between countries, which may have been exposed to EHP infection. Another potential possibility caused by the movement of contaminated feed or an unknown intermediary host.

Nucleotide sequencing and analysis of the partial 18S rRNA amplified from EHP showed significantly high similarity with the sequence available in Gen Bank (NCBI). The results confirm that 18S rRNA genes have a good capacity so that they can be used as genetic markers for identification and characterization of EHP species based on nucleotide sequences. However, the genetic analysis carried out was the initial stage of the EHP characterization of Indonesian isolates. To find out differences in pathogenicity or virulence from EHP, further genetic analysis may be needed, especially for genes that encode effector proteins for virulence or genes that play a role in pathogenicity.

Thus, the 18S rRNA-PCR method used here and a more recent one that was also based on the EHP SSU rRNA sequence (Tang et al., 2015) were still appropriate for cultivated shrimp specimens. However, for environmental samples, such as sediments and suspected carriers that previously reported to be SSU-

PCR positive for EHP infection, it was necessary to re-confirm their status by using SWP-PCR method.

4. Conclusion

The conclusion obtained from this study was that the 13 Indonesian isolate EHP species were identical each other. From the phylogenetic analysis, it was found that phylogenetic *E.hepatopenaei* isolates from Indonesia were closely related to EHP strains from India and other parts of China. These findings indicated a close phylogenetic relationship and epidemiological potential between EHP strains that affect shrimp cultivated in Indonesia and Asia. EHP circulating in Indonesia, India and China genetically has the same character, very close and forms one genetic group. All Indonesian EHP isolates observed in this study were likely to come from the same source, Asia.

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