

Isolation and characterization of an envelope protein (VP19) of a White Spot Syndrome Virus from diseased vannamei (*Litopenaeus vannamei*) in Indonesia

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Abstract. This study aimed to isolate and characterize White spot syndrome virus (WSSV) viral protein 19 (VP19) from Indonesian vannamei (*Litopenaeus vannamei*) isolates. Juvenile vannamei were collected from infected vannamei located at Pangkep, Barru and Pinrang regency of Indonesia. Total genomic DNA was isolated from the walking legs and telson of shrimp by DTAB-CTAB DNA extraction procedure. The length of DNA sequences of WSSV VP19 from Indonesian isolate was 387 bp. The homology of the Barru isolates with the other isolates from Pangkep and Pinrang was between 96-99%. Closer relationships were found between isolates from Barru and Pangkep. There could be a genetic flow between shrimp populations in Pangkep and Barru since these two areas are geographically close. BLAST-N (Basic Local Alignment Search Tool-Nucleotide) analysis showed that the highest similarity of WSSV VP19 was found between Indonesian and Mexican isolates. These findings reveal that all geographical isolates of WSSV VP19 are not completely identical.

Key Words: VP19, shrimp, virus, BLASTN, South Sulawesi.

Introduction. White spot syndrome virus (WSSV) is the cause of a viral disease which affects mainly cultured shrimp (Lightner 1996). It is a double-stranded rod-shaped DNA virus, and belongs to the Nimaviridae family (Wang et al 1995; Mayo 2002; Yi et al 2003). WSSV is the most pathogenic virus and causes high mortality among the penaeid shrimp (Sun et al 2015; Jariyapong et al 2015). The virus exists in an extremely wide range of potential hosts, including shrimps and other crustaceans such as crabs and crayfish (Wang et al 1998). WSSV was first detected in Asia, with occurrences in Taiwan (Chou et al 1995), Japan (Nakano et al 1994), and Korea (Park et al 1998). Since these first events, the disease has spread among most Asian countries, as well as in the Americas (Lightner 1996; O.I.E. 2003).

One of the known shrimp hosts of WSSV are *Litopenaeus vannamei*, which is one of the most commonly cultured species in Indonesia and is one of the most commercially important species comprising the wild shrimp fishery along the South Sulawesi coast of the Indonesia (Zainuddin et al 2014). Because WSSV has been catastrophic to the shrimp farming industry and may be a threat to the wild shrimp fisheries, a thorough study of its molecular biology is still urgently needed for a better understanding of its nature and replication strategy and the molecular mechanisms of its pathogenesis. Tsai et al (2006) have identified the protein components of the WSSV virion by proteomic methods. VP26, VP36A, VP39A, and VP95 were all identified as tegument proteins distinct from the envelope proteins (VP19, VP28, VP31, VP36B, VP38A, VP51B, VP53A) and nucleocapsid proteins (VP664, VP51C, VP60B, VP15). More than 58 structural proteins had been identified, now, the envelope protein of WSSV has become the focus of numerous studies (Seok et al 2004; Tang & Hew 2007; Tsai et al 2004; van Hulten et al 2001; Wu et al 2005; Xu et al 2009; Liang et al 2011; Huang et al 2013). Viral envelope proteins play an important role in virus entry and assembly (Chazal & Gerlier 2003).

To our knowledge, there is no available information regarding an envelope protein (VP19) of a WSSV isolate from Indonesian *Litopenaeus vannamei*. Therefore, the objective of this work was to characterize WSSV VP19 from Indonesian *L. vannamei* isolate.

Material and Method

Sources of samples. Juvenile *L. vannamei* were collected from shrimp ponds located at Pangkep, Barru and Pinrang, South of Sulawesi, Indonesia from June–September 2014. Moribund shrimp in infected ponds were collected for PCR assays according to the methods described by Lightner (1996). Prior to DNA isolation, the walking legs and telson of shrimp were preserved in 70% ethanol and subjected to PCR analysis in laboratory following the method of Corriveau et al (2010).

DNA isolation and PCR assay in shrimp. Total genomic DNA was isolated from the walking legs and telson of shrimp by DTAB-CTAB DNA extraction procedure with PCR kits (IQ2000, Taiwan) and subsequently precipitated with ethanol (Hoa et al 2011). The isolated DNA pellet was dissolved in DEPC ddH₂O and quantified using NANODROP spectrophotometer (Hoa et al 2011). The nested PCR was carried out in a 0.2 mL thin wall PCR tube with 50 ng of genomic DNA, 10 pM of each primers, 50 μM of dNTPs, 1X PCR buffer, 1.5 mM MgCl₂, and 1.0 U Ampli Taq Gold (Perkin Elmer, USA). Amplification conditions were followed 39 cycles at 94°C for 4 min, 55°C for 1 min, 72°C for 3 min and final extension at 72°C for 5 min in a PCR thermocycler. Positive and negative controls were included in all tests. PCR products were subjected to electrophoresis in 2% agarose gel and quantified as per the method of Hoa et al (2011).

PCR amplification and sequence analyses. PCR was performed to amplify the complete Open Reading Frames (ORFs) of structural viral protein of WSSV VP19. Primers for PCR were designed from nucleotide sequences in the GenBank/EMBL databases of WSSV (AY160771 for VP19). The PCR primers were as follows: VP19 upstream, 5'-GTC TTT ACG TTA CAT TGA CGT ACC-3'; VP19 downstream, 5'-CTG CCT CCT CTT GGG GTA-3'. The template used was genomic DNA isolated from the infected tissues of *L. vannamei*. Briefly, the PCR mixture consisted of 2 mL of each primer (5 pmol), 2 mL of 10x PCR buffer, 1.5 μL of 2 mM dNTP, 1 μL of 15 mM MgCl₂, 0.1 μL of *Taq* DNA polymerase (100 units) (Invitrogen, U.S.A.), 2 μL of the DNA template (1 μg mL⁻¹) and scaled up to 20 μL using distilled water. PCR amplification was carried out using the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 sec and elongation at 72°C for 1 min; then a final elongation at 72°C for 5 min. PCR products (5 μL) were electrophoresed on a 0.8% agarose gel with 1% TBE electrophoresis buffer (pH 8.0) for 30 min, visualized using a hand-held densitometer and photographed. The PCR products were sequenced using Big Dye Terminator ver 3.1 (Applied Biosystems, CA, USA) and comparative sequence analyses were performed by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/>). Multiple alignments of the sequences of VP19 were done using CLUSTAL W (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Phylogenetic tree was constructed using the Neighbor Joining Method with 1000 bootstrap values of MEGA 4 (Tamura et al 2007).

Results and Discussion. Using one primer set to amplify a fragment of the VP19 genes, we were able to obtain sequences of the VP19 of an Indonesian isolate of WSSV. The sequence was 387 bp in length. Alignment of the sequences of the Barru isolate together with the isolates from Pangkep and from Pinrang is shown in Figure 1. The homology of the Barru isolates with the other isolates from Pangkep and Pinrang was between 96-99%.

A.txt	1	AGTTCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	60
B.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
C.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
D.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
E.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
F.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
G.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
H.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
I.txt	1	---TCGCGGATCCGATGGCCACCACGACTAACACTCTTCCTTTGGGCAGGACCGGAGCCCC	57
A.txt	61	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	120
B.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
C.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
D.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
E.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
F.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
G.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
H.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
I.txt	58	AGGCCGCTGGCCCTTCTTACACCATGGAAGATCTTGAAGGCTCCATGTCTATGGCTCGCA	117
A.txt	121	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	180
B.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
C.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
D.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
E.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
F.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
G.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
H.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
I.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
A.txt	121	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	180
B.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
C.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
D.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
E.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
F.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
G.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
H.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
I.txt	118	TGGGTCTCTTTTTGATCGTTGCTATCTCAATTGGTATCCTCGTCCGCGGCCGTCATGAATG	177
A.txt	181	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	240
B.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
C.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
D.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
E.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
F.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
G.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
H.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
I.txt	178	TATGGATGGGACCAAAGAAAGGACAGCGATTCTGACACTGATAAGGACACCGGTGATGATG	237
A.txt	241	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	300
B.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
C.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
D.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
E.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
F.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
G.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
H.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
I.txt	238	ACGACACTGCCAACGATAACGATGATGAGGACAAATATAAGAACAGGACCAGGGATATGA	297
A.txt	301	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	360
B.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
C.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
D.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
E.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
F.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
G.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
H.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
I.txt	298	TGCTTCTGGCTGGGTCCGCTCTTCTGTTTCCTCGTTTCGCGGCCACCGTTTTTATGSCCTT	357
A.txt	361	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	390
B.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	387
C.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	387
D.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	387
E.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	387
F.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	380
G.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGAAA	389
H.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGGA--	387
I.txt	358	ACCCCAAGAGGAGGCAGTAAGAAATCCGGG---	386

Figure 1. Nucleotide sequence alignment results of VP19 gene of WSSV isolates from Barru (A, B, C); Pangkep (D, E, F); and Pinrang (G, H, I).

In the phylogenetic tree the different places of isolates are all present in clades which are well bootstrap-supported. The computer-generated dendrogram revealed that all isolates were clustered into two distinct groups as shown in Figure 2. The Barru and Pangkep isolates clustered in one group as well as the Pinrang isolates. Closer relationships were found between Barru and Pangkep isolates than between Pinrang isolates. It can be hypothesized that there could be a genetic flow between shrimp populations in Pangkep and Barru since these two areas are geographically close.

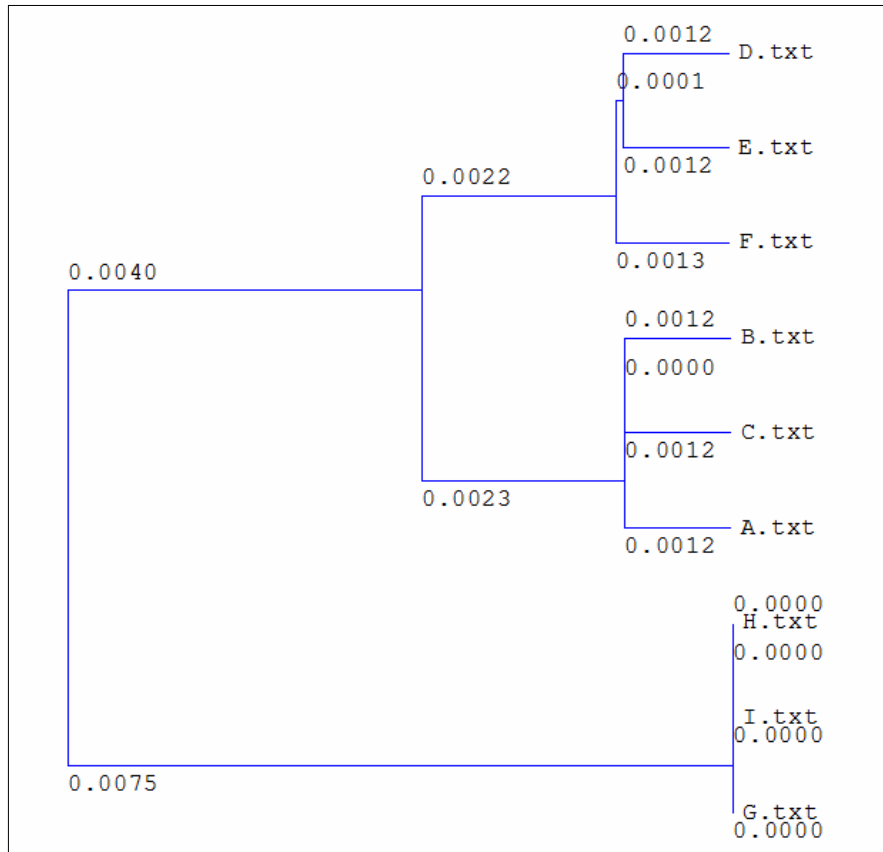


Figure 2. Phylogenetic tree of VP19 gene of WSSV isolates from Barru (A, B, C); Pangkep (D, E, F); and Pinrang (G, H, I). The Neighbor Joining method with 1000 bootstrap values was used for the analysis.

The amplified VP19 sequences were used as BLASTN queries against the NCBI database. The sequences of the PCR amplicons from Barru, Pangkep and Pinrang isolates were found to be 98-99% similar to the sequences of VP19 from other country isolates (Figure 3). The VP19 sequence amplified from Barru and Pangkep isolates showed 98% identity with VP19 from China (AY245790.1) and Xiang shan (DQ007314.1) isolates. These two isolates (Barru and Pangkep) also showed 99% identities with South Korea (JX515788.1), India (DQ681071.1), China (AY249445.1), China (AY249444.1), Netherland (AF369029.2), Taiwan (AF440570.1), India (GU734035.1), Cochin (EU012447.1), Vietnam (AY160771.1), China (AF332093.2), Korea (GQ328028.1), Korea (AY316119.1), China (AY220744.1), India (AY873786.1), Mexico (AY713372.1), India (AY422227.1), Singapore (AF402997.1), India (DQ902655.1), and Mexican Isolates (AJ937860.1). Similar patterns were observed amongst isolates of Pinrang that also showed 98% identities to isolates of China (AY245790.1), Xiang shan (DQ007314.1), South Korea (JX515788.1), India (DQ681071.1), China (AY220744.1), Netherland (AF369029.2), Taiwan (AF440570.1), India (GU734035.1), China (AF332093.2), Korea (AY316119.1), India (DQ902655.1) and Singapore (AF402997.1). Additionally, Pinrang isolates showed 99% identity to isolates of Cochin (EU012447.1), Vietnam (AY160771.1), Korea

(GQ328028.1), India (AY873786.1), Mexico (AY713372.1), India (AY422227.1), and Mexico (AJ937860.1) (Figure 3).

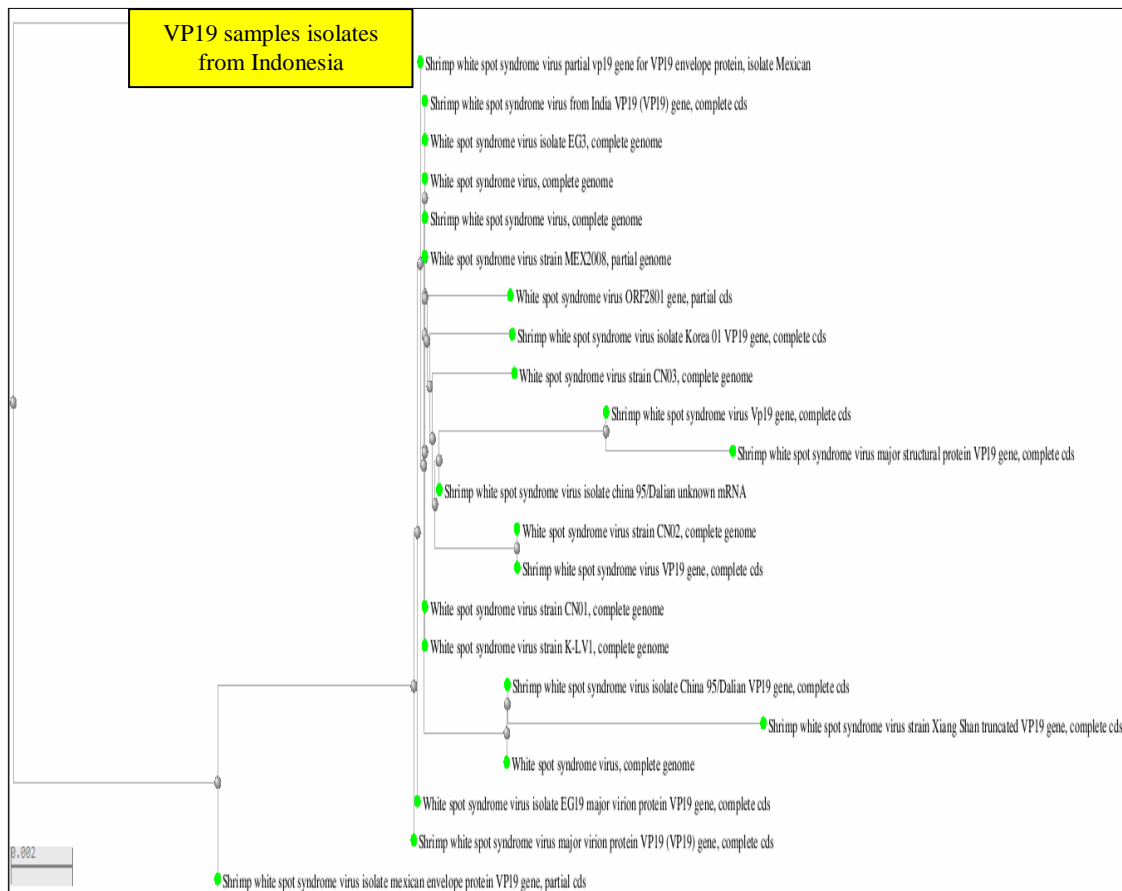


Figure 3. Consensus phylogenetic tree of VP19 gene of WSSV isolates from Indonesia.

In the present study, we have shown that BLASTn analysis of these sequences revealed high similarity to those of WSSV virion protein 19 genes reported from Mexico, China, South Korea, India, Netherland, Taiwan, and Vietnam. The highest similarity was found between isolates from Indonesia and Mexico. The results confirm previous observations that there is little genetic variation in geographic isolates of WSSV from China, India, Thailand and the United States (Lo et al 1999).

WSSV is an enveloped DNA virus. More than 30 proteins were classified as envelope proteins within the structural proteins (Huang et al 2013). Viral envelope proteins play a crucial role in viral infection. Seok et al (2004) demonstrated that at the nucleotide level, VP19, VP28 and VP15 sequences of Korean isolates were, respectively, 99, 100 and 100% identical to those of China, Indonesia, Japan and the United States. Molina-Garza et al (2008) revealed that VP19 may have application as a genetic marker. They found that VP19 of a WSSV isolate from Mexican *L. vannamei* was distinguishable from an American strain of WSSV, although homology was found with isolates from Taiwan and India. Furthermore, the major envelope proteins, VP19, VP26 and VP28 have been characterized from diseased *Penaeus vannamei* and moribund *Procambarus clarkii*. Homologous contrast showed that the sequences of VP19, VP26 and VP28 in the study possess over 97% DNA and 100% amino acid sequence similarity to WSSV in the NCBI database (Liang et al 2011).

Conclusions. Based on sequences of the genomic DNA of WSSV VP 19 and its phylogenetic analysis, there is likely a genetic flow between *L. vannamei* populations in Pangkep and Barru. BLASTN analysis showed that the highest similarity of WSSV VP19 was found between Indonesian and Mexican isolates. These findings reveal that all geographical isolates of WSSV VP19 are not completely identical.

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- *** <http://blast.ncbi.nlm.nih.gov/>.
- *** <http://www.ebi.ac.uk/Tools/msa/clustalw2/>.

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