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Variability of White Spot Syndrome Virus (WSSV) Envelope Protein VP28 from Diseased Shrimp (*Litopenaeus vannamei*) in Indonesia

(Kepelbagaian Virus Sindrom Bintik Putih (WSSV) Protein Sampul VP28 daripada Udang (*Litopenaeus vannamei*) Berpenyakit di Indonesia)

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ABSTRACT

White Spot Disease (WSD) is a viral disease affecting crustaceans. Caused by the White Spot Syndrome Virus (WSSV), this disease has caused significant mortality in commercially cultivated marine shrimp species with severe impacts on the shrimp farming industry and may be a threat to wild shrimp stocks. Thorough studies on the molecular biology of this pathogen are urgently needed to improve understanding of the virus at a molecular level, including variation in key viral protein (VP) components of the WSSV virion. This study aimed to isolate and characterize WSSV VP28 gene encoding envelope proteins from Indonesian Pacific white shrimp (*Litopenaeus vannamei*) isolates. Infected juvenile shrimp were collected from Pangkep, Barru, and Pinrang Districts in South Sulawesi, Indonesia. Genomic DNA was isolated from infected shrimp muscle tissue using a DTAB-CTAB (dodecyle trimethyl ammonium bromide-hexadecyl trimethyl ammonium bromide) DNA extraction procedure. The WSSV VP28 DNA sequences from Pangkep, Barru, and Pinrang isolates were 640-680 bp in length. Homology of Pangkep isolates with isolates from Barru and Pinrang was 97-99%. BLAST-N (Basic Local Alignment Search Tool-Nucleotide) analysis showed isolates from all three sites clustered with WSSV VP28 accessions from China, Indonesia, Japan, South Carolina and Vietnam. These results increase the geographic spread and host taxon coverage of WSSV VP28 sequence data for Indonesia.

Keywords: *Litopenaeus vannamei*; South Sulawesi; VP28; viral protein variability; WSSV

ABSTRAK

Penyakit Bintik Putih (WSD) adalah penyakit virus yang menjejaskan krustasea. Disebabkan oleh Virus Sindrom Bintik Putih (WSSV), penyakit ini telah menyebabkan kematian yang ketara dalam spesies udang marin yang diusahakan secara komersial dengan kesan yang teruk terhadap industri penternakan udang dan mungkin menjadi ancaman kepada stok udang liar. Kajian menyeluruh tentang biologi molekul patogen ini amat diperlukan untuk meningkatkan pemahaman virus pada tahap molekul, termasuk variasi dalam komponen protein virus (VP) utama virion WSSV. Kajian ini bertujuan untuk mengasingkan dan mencirikan gen WSSV VP28 pengekodan protein sampul surat daripada pencilan udang putih Pasifik Indonesia (*Litopenaeus vannamei*). Udang juvana yang dijangkiti dikumpul dari Daerah Pangkep, Barru dan Pinrang di Sulawesi Selatan, Indonesia. DNA genom telah diasingkan daripada tisu otot udang yang dijangkiti menggunakan prosedur pengekstrakan DNA DTAB-CTAB (*dodecyle trimethyl ammonium bromide-hexadecyl trimethyl ammonium bromide*). Urutan DNA WSSV VP28 daripada pencilan Pangkep, Barru dan Pinrang adalah 640-680 bp panjang. Homologi pencilan Pangkep dengan pencilan Barru dan Pinrang adalah 97-99%. Analisis BLAST-N (Alat Carian Penjajaran Tempatan Asas-Nukleotida) menunjukkan pencilan daripada ketiga-tiga tapak berkelompok dengan aksesi WSSV VP28dari China, Indonesia, Jepun, Carolina Selatan dan Vietnam. Hasil ini meningkatkan sebaran geografi dan liputan takson hos bagi data jujukan WSSV VP28 untuk Indonesia.

Kata kunci: Kebolehubahan protein virus; *Litopenaeus vannamei*; Sulawesi Selatan; VP28; WSSV

Introduction

Pacific white shrimp (*Litopenaeus vannamei*) have been cultivated in Indonesia since they were first introduced around 2000 as a substitute for the giant tiger shrimp (*Penaeus monodon*) (Amelia et al. 2021; Hukom et al. 2020). Pacific white shrimp are widely considered as having several advantages for aquaculture: They can be cultivated at high stocking densities, can grow quickly, have a relatively high tolerance to low salinity conditions, lower feed protein requirements, and are more easily bred in captivity with higher larval survival. The development of specific pathogen free (SPF) and specific pathogen-resistant (SPR) strains meant that these shrimp strains were initially considered more resistant to disease (Briggs et al. 2004; Taukhid et al. 2008). However, as aquaculture has developed over time, Pacific white shrimp have also been attacked by a variety of pathogens including White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Infectious Myo Necrosis Virus (IMNV), and *Vibrio* spp., while Early Mortality Syndrome (EMS), later called Acute Hepatopancreas Necrosis Disease (AHPND), is also a concern (Amelia et al. 2021; Kokarkin & Supito 2011; Sugama et al. 2006; Vaiyapuri et al. 2021).

WSSV is the primary pathogen causing outbreaks of white spot disease (WSD) which has been a significant cause of the decline in shrimp production (Amelia et al. 2021; Koesharyani et al. 2019; Manoppo et al. 2010; Vaiyapuri et al. 2021). Initially detected in 3 Asian countries (Taiwan, Japan, and Korea), this disease subsequently spread to most shrimp producing countries in Asia and the Americas (Hidayani et al. 2016). WSSV infection is characterized by the appearance of white spots on the inner surface of the exoskeleton, especially the carapace, so that it became known as white spot disease (Parenrengi et al. 2018).

Other than the tiger prawn *P. monodon* and the Pacific white shrimp *L. vannamei*, penaeid shrimp known to be susceptible to WSSV include the kuruma shrimp *Marsupenaeus japonicus* (Caipang et al. 2008; You et al. 2010), the Indian prawn *Fenneropenaeus indicus* (Onihary et al. 2021), the Chinese shrimp *Fenneropenaeus chinensis* (Moon et al. 2003) and at least 12 other species (Sánchez-Paz 2010; Seibert & Pinto 2012). Many tropical and temperate marine and freshwater decapod taxa other than penaeid shrimps can be infected (Escobedo-Bonilla et al. 2007; Sánchez-Paz 2010), including caridean shrimps such as *Macrobrachium* prawns (Yoganandhan & Sahul Hameed 2007), crayfish such as *Procambarus clarkii* (Jha et al. 2006; Jiang et al. 2017) and *Cherax quadricarinatus* (Liu et al. 2018), crabs (Hossain et al. 2001; Sánchez-Paz 2010; Seibert & Pinto 2012) including the valuable *Scylla* mudcrabs (Ravi et al. 2020), panulurid spiny lobsters (Syed Musthaq et al. 2006) and stomatopod mantis shrimps (Hossain et al. 2001). While penaeid shrimps are particularly susceptible to WSD, some taxa are relatively resistant but can act as carriers of the disease, including wild populations of *Macrobrachium rosenbergii* (Yoganandhan & Sahul Hameed 2007), *Squilla mantis* and some crabs (Hossain et al. 2001).

Efforts to prevent WSSV disease in shrimp include the use of chemicals and antibiotics, but the continuous use of antibiotics with inappropriate doses can result in increased pathogen resistance (Thornber et al. 2020; Vaiyapuri et al. 2021). Furthermore, chemical and antibiotic residues can pollute the environment and damage aquatic ecosystems (Chen et al. 2020; Henriksson et al. 2018; Rico et al. 2012; Sargenti et al. 2020). Potentially more environmentally friendly approaches to preventing WSSV disease include the use of immunostimulants and vaccines (Feng et al. 2017; Haryanti et al. 2014; Manoppo et al. 2010; Rajkumar et al. 2017). Several significant genes have been found in the WSSV virus, including the viral protein (VP) coding genes labelled as VP15, VP19, VP24, VP26, and VP28 (Boonyakida et al. 2020; Chai et al. 2013; Feng et al. 2017; Li et al. 2019; Yi et al. 2004). Of these, three virion proteins (VP15, VP24, and VP26) are found in the nucleocapsid while two (VP28 and VP19) are envelope proteins found on the surface of the virion (van Hulten et al. 2001). The WSSV VP28 gene is thought to be involved in attachment to and entry into host cells (van Hulten et al. 2001; Yi et al. 2004; Zheng et al. 2019). Involved in systemic infection, VP28 can stimulate or activate elements of the crustacean immune system, making it a potential vaccine candidate (Feng et al. 2017; Jha et al. 2006; Namikoshi et al. 2004; Seethalakshmi et al. 2021; Solís-Lucero et al. 2016; van Hulten et al. 2001; van Hulten et al. 2000; Witteveldt et al. 2006, 2004). Studies on the development of recombinant DNA vaccines in shrimp have suggested the characterization and cloning of the VP28 gene, in particular from tiger shrimp (*Penaeus monodon*) infected with WSSV (Joseph et al. 2015).

Further study of the VP28 gene and envelope protein is therefore important for protection against the WSSV virus, including the development of vaccines (Feng et al. 2014; Lanh et al. 2021), and with respect to the defence mechanisms against WSSV infection in Pacific white shrimp (Hirono et al. 2011). Considered relatively conserved, VP28 is nonetheless one of the WSSV VPs that can vary between infected host populations (Hernández-

montiel et al. 2021; Joseph et al. 2015; Siddique et al. 2018). Therefore, the purpose of this research was to identify and characterise the WSSV gene that encodes the VP28 surface protein, specifically in WSSV-infected Pacific white shrimp populations from major shrimp producing centres such as Pangkep, Barru, and Pinrang Districts in South Sulawesi Province, Indonesia.

Materials and Methods

Pacific White Shrimp Samples

Samples of the Pacific white shrimp *Litopenaeus vannamei* were collected from the waters around Pangkep, Barru, and Pinrang Districts, South Sulawesi Province, Indonesia in 2014. Three samples were collected from each study location with the following codes: Pa = Pangkep District; B = Barru District; and Pi = Pinrang District. Each sample comprised shrimp legs and part of the tail. The samples were placed in 1.5 mL Eppendorf tubes filled with 96% absolute ethanol and taken to the Maros Biotechnology Laboratory of the Brackish Water Aquaculture Research and Development Centre (BPPBAP Maros) in Maros District, South Sulawesi.

Extraction of Genomic DNA

Genomic DNA was isolated from Pacific white shrimp *L. vannamei* infected with WSSV following the Dodecyl Hexadecyl Trimethyl Ammonium Bromide -Cationic Hexadecyl Trimethyl Ammonium Bromide (DTAB-CTAB) method. A 30 mg sample of shrimp leg taken from each specimen was ground using a plastic mortar and pestle and placed in a 1.5 mL sterile Eppendorf tube to which 0.6 mL DTAB solution was added. The mixture was vortexed for 1 min, incubated at 75 °C for 5 min, and then allowed to cool to room temperature. After the addition of 0.7 mL chloroform, the sample was vortexed again for about 20 s and centrifuged at 12000 rpm for 5 min. The supernatant was transferred to a new Eppendorf tube and 100 µL CTAB and 900 µL ddHO were added before vortexing for 30 s then incubating for 5 min at a temperature of 75 °C. After cooling to room temperature, the sample was centrifuged at 12000 rpm for 10 min, the supernatant was discarded, and 150 mL lysis solution (IQ-2000, Taiwan) was added. The sample was incubated at 75 °C for 5 min, then cooled to room temperature, centrifuged at 12000 rpm for 5 min, and the supernatant was transferred to a 0.5 mL Eppendorf tube filled with 300 µL of 95% chilled ethanol to precipitate the DNA. The sample was centrifuged again at 12000 rpm for 5 min and the ethanol was then discarded, leaving a DNA pellet in the Eppendorf tube. Ethanol 70% was added and the sample was centrifuged at 12000 rpm for 5 min, then the ethanol was discarded. The tube with the DNA pellet was dried for 2 h, then 10 µL TE buffer was added and the DNA template extracted from the sample was stored in a refrigerator at -20 °C.

Detection of WSSV by Polymerase Chain Reaction (PCR)

The presence of WSSV virus in the Pacific white shrimp samples was detected through Polymerase Chain Reaction (PCR) Applied Biosystems 2720 Thermal Cycler, USA) using the IQ2000™ WSSV Detection and Prevention System kit (GeneReach Biotechnology Corp.). After the DNA extraction process, the PCR process was carried out twice (first PCR for gene amplification, followed by nested PCR). The first PCR profile comprised 5 cycles with 30 s denaturation at 94 °C, 30 s annealing at 62 °C and 30 s extension at 72 °C followed by 15 cycles with 15 s denaturation at 94 °C, 15 s annealing at 62 °C and 20 s extension at 72 °C; then extension at 72 °C for 30 s, 20 °C for 30 s and final extension at 4 °C. The nested PCR profile was: 25 cycles with 20 s denaturation at 94 °C, 30 s annealing at 62 °C and 30 s extension at 72 °C with final extension for 30 s at 72 °C and 30 s at 20 °C.

The presence of WSSV in the amplified PCR product was verified through electrophoresis (BioRad FIGE Mapper Cell, USA) on 2% agarose gel prestained with nucleic acid gel stain (Gelred Biotium, USA). For each sample, 7 µL PCR product was mixed with 3 µL loading dye and 1 µL DNA ladder marker (50 bp DNA Step Ladder, Sigma-Aldrich, Germany) in a clean Eppendorf tube and pipetted into a well. Positive and negative controls were also run using a proprietary kit (IQ-2000, Taiwan). The electrophoresis was run in 1 × TBE buffer (pH 8.0) for 25 min at 100 V. Electrophoresis results were observed under a UV transilluminator.

Isolation of the VP28 Gene from WSSV Virus Extracted DNA Product

The WSSV virus VP28 gene was isolated following the methods developed by Sarathi et al. (2008). The genomic DNA extracted from the infected Pacific white shrimp was used as DNA template using a specific primer pair for VP28 gene isolation: VP-28F AND VP-28R. The primer sequences were: VP-28F (forward): 5'-GTTCGATAAAGAAAAAAACTCG-3' and VP-28R (reverse): 5'- CCCTATCTATATAAAAGCACG -3".

The WSSV VP-28 gene was amplified through PCR (GeneAmp PCR System, Model: 2700, Applied

Biosystems, USA) using the PureTaq Ready-To-Go PCR Beads (GE Healthcare) kit. Each 25 µL reaction contained 1 µL (50 µmol/µL) of each primer, 1.5 µL Pacific white shrimp genomic DNA (template) and 21.5 µL of aquamilliQ. After centrifuging for ±10-15 s, the sample tubes were placed in the PCR machine with the following PCR profile: pre-denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 30 s; final extension at 72 °C for 7 min then resting at 4 °C.

The presence of amplified PCR product was verified through electrophoresis on 2% agarose gel using the previously described setup with 3 µL of each PCR product and 1 µL 100 bp DNA Ladder placed in the wells. A Gel Documentation System (Biometra) was used to observe and record the single clear band indicating VP-28 gene amplification.

PCR AMPLICON SEQUENCING AND NUCLEOTIDE SEQUENCE ASSEMBLY

The final PCR product from each sample was sent to First Base Laboratory in Singapore for Sanger nucleotide sequencing (ABI PRISM 310). Sequencing results were viewed manually using the sequence navigator (Applied Biosystems) program. The resulting sequences were analysed in Mega X (Kumar et al. 2018). The sequences were trimmed to remove ambiguous base reads and aligned to obtain a consensus sequence from the forward and reverse sequences for each sample. Genetic distances between the sequences were calculated in MEGA X using the Maximum Composite Likelihood model (Tamura et al. 2004).

DATA ANALYSIS

To confirm the identity of the WSSV VP28 gene sequences isolated and evaluate their similarity with WSSV VP28 isolates from other locations in Indonesia and worldwide, the NCBI BLAST-n (Basic Local Alignment Search Tool-nucleotide) was used. The BLAST-n parameters were set to align the VP28 sequences obtained from the 3 study locations with NCBI GenBank accession sequences and display the 100 closest matches. Of these, aligned GenBank accessions sequences with 90% or higher coverage were selected and downloaded in FASTA format for further analysis. Details of individual accessions were retrieved from GenBank including country of origin and, where available, the published reference for the accession.

The results of the BLAST-n routine were analysed in Mega X (Kumar et al. 2018). Sequences were aligned using the ClustalW routine with default settings and phylogenetic trees were constructed for the South Sulawesi sequences alone and for the full data set (South Sulawesi sequences & GenBank accessions). A phylogenetic tree was constructed using the Maximum Likelihood routine in MEGA X with Kimura 2-parameter model (Kimura 1980), exported as Newick files. The tree was edited in the interactive Tree of Life (iTOL) online tool (Letunic & Bork 2019, 2016).

The start and stop codons were identified and a data set trimmed to this coding region was produced. These sequences were translated into proteins in the MEGA X alignment tool. To characterise the WSSV VP28 sequences from South Sulawesi, the base composition and amino-acid composition were obtained. The genetic distances between the South Sulawesi and GenBank accession VP28 sequences were calculated in MEGA X using the Maximum Composite Likelihood model (Tamura et al. 2004).

RESULTS AND DISCUSSION

ISOLATION OF WSSV FROM PACIFIC WHITE SHRIMP SAMPLES

The PCR using the IQ2000™ WSSV Detection and Prevention System kit extracted and detected WWSV DNA from all Pacific white shrimp samples (Figure 1). The first three columns in Figure 1 are the WSSV specific DNA ladder (848, 630 & 333 bp), the negative and positive controls; the remaining columns are PCR product from infected Pacific white shrimp. The assay results indicate moderate to severe levels of infection.

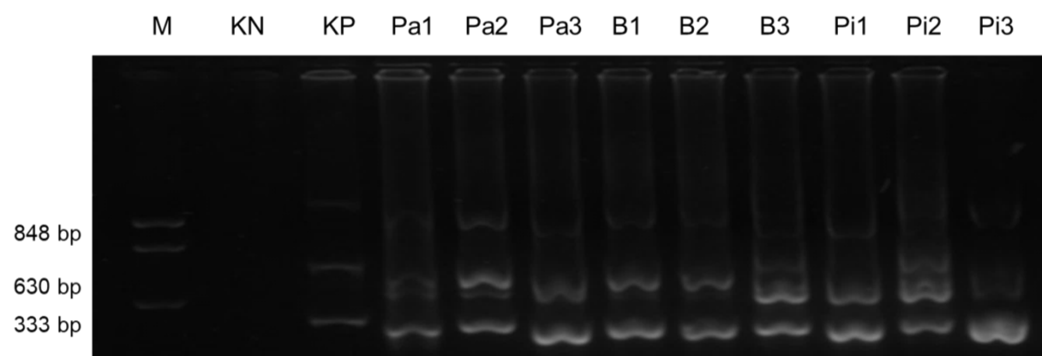
AMPLIFICATION AND SEQUENCING OF THE WSSV VP28 GENE

The coding gene for the WSSV viral protein VP28 was successfully amplified and sequenced for seven out of the nine Pacific white shrimp samples. The electrophoresis visualisation showed amplicon bands with lengths around 600-700 bp (Figure 2). Only one sample from Barru was successfully sequenced. The unedited nucleotide sequence lengths were: Pangkep = 669-672 bp; Barru = 672 bp; and Pinrang = 668-672 bp. After trimming for removal of ambiguous nucleotide readings, the resulting VP28 gene sequences comprised 634 nucleotides (634 bp).

HOMOLOGY BETWEEN SAMPLES

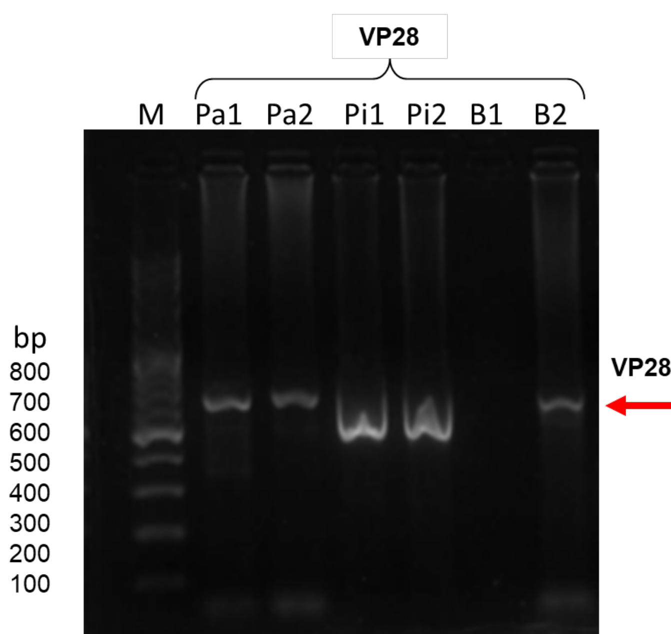
The WSSV VP28 samples of Pacific white shrimp from the three locations in South Sulawesi were closely related,

with five out of seven identical to each other and to the only WSSV VP28 GenBank accession from Indonesia (AY249441), isolated from *Penaeus monodon*. The greatest genetic distance was between the two non-identical isolates (Barru and Pangkep3) (Table 1). The high proportion of identical isolates is not surprising in view of the low geographical distance between these three districts. Furthermore, shrimp seeds are commonly transported between regions within Indonesia, often with relatively low levels of biosecurity, raising the likelihood of transfer of potentially infected material and hence viral strains between sites (Koesharyani et al. 2019).



Lanes: M = DNA ladder; KN = negative control; KP = positive control; Pa = samples from Pangkep District; B = samples from Barru District; Pi = samples from Pinrang District

FIGURE 1. Electrophoresis of the PCR product from the IQ2000™ WSSV assay of Pacific white shrimp from South Sulawesi, Indonesia



Lane legend: M = molecular weight ladder; Pa = samples from Pangkep District; B = samples from Barru District

FIGURE 2. Electrophoresis visualisation of PCR product from the amplification of the WWSV VP28 gene from WSSV infected Pacific white shrimp from South Sulawesi, Indonesia

TABLE 1. Genetic distances between seven WSSV VP28 sequences isolated from infected Pacific white shrimp in South Sulawesi and a GenBank accession from Indonesia

Sequence	AY249441	Pinrang1	Pinrang2	Pinrang3	Pangkep1	Pangkep2	Pangkep3
Pinrang1	0.00000						
Pinrang2	0.00000	0.00000					
Pinrang3	0.00000	0.00000	0.00000				
Pangkep1	0.00000	0.00000	0.00000	0.00000			
Pangkep2	0.00000	0.00000	0.00000	0.00000	0.00000		
Pangkep3	0.00808	0.00808	0.00808	0.00808	0.00845	0.00808	
Barru	0.00847	0.00847	0.00889	0.00847	0.00847	0.00847	0.01720

The BLAST-n results for the partial VP28 gene structural protein encoding sequences obtained from Barru, Pangkep, and Pinrang, South Sulawesi identified 100 oligonucleotide sequence GenBank accessions with a percentage query cover range of 10-99% and percentage identity of 94.67-100%. The accessions with the highest query cover and identity percentages are shown in Table 2. The BLAST-n results indicate that the partial oligonucleotide sequences obtained are highly similar to many other sequences for the WSSV VP28 gene sequences registered in GenBank and confirm the identity of the gene sequenced.

The 67 accessions with a query cover of 90% or higher were used to construct a phylogenetic tree (Figure 3). The accessions not listed in Table 1 came from Australia (Oakey & Smith 2018); Bangladesh (Siddique et al. 2018; unpublished); China (Joseph et al. 2015; Li et al. 2017; Li et al. 2016; unpublished); Egypt (unpublished); India (Sathish et al. 2004; Valappil et al. 2021; Vinaya Kumar et al. 2018; unpublished); Iran (Vinaya Kumar et al. 2018); Mexico (Parrilla-Taylor et al. 2018); Thailand (Joseph et al. 2015), and Vietnam (unpublished).

The phylogenetic tree (Figure 3) shows a predominantly south Asian (India and Bangladesh) subclade (lower left-hand region), also comprising sequences from Egypt, Australia, China, and Vietnam. The variation between accessions from the 16 countries could be (at least in part) an artefact of sampling effort, as the greatest variation is seen in the countries with the most accessions, as well as the highest number of different accession sources, i.e., India with 16 accessions, followed by China (13 accessions). However, Mexico, the country with the third highest number of accessions (10), and indeed all accessions from the Americas, had very low diversity, despite the wide geographical spread of the isolate sources (Dantas et al. 2018; Parrilla-Taylor et al. 2018; Pereira et al. 2019; Restrepo et al. 2018; Rodriguez-Anaya et al. 2016). Conversely, of the four accessions from Vietnam only two are similar to each other. In comparison to most countries or regions, the diversity within the Sulawesian isolates appear quite large, although one appears identical to the only Indonesian accession returned by the BLAST-n search and four are identical or very similar to 29 accessions including the 15 accessions from the Americas,13 accessions from Southeast and East Asia, and one accession from India.

SOUTH SULAWESI WSSC VP28 CODING REGION CHARACTERISATION

The initial codon (genetic code) or translation start codon codes for the amino acid methionine which initiates the structure of a polypeptide (protein), whereas the translation termination or stop codon in a gene is generally one of three codon sequences (TAA, TAG, TGA) (van Hulten et al. 2001). Analysis of the alignment in MEGA X showed that the stop codon was TAA and the coding region comprised 157 codons. The amino-acid composition (Table 3) was identical for all seven South Sulawesi isolates with a nucleotide composition of T(U) 23.4%; C 24.9%; A 26.6% and G 25.1% and CG content 50%.

TABLE 2. Gen Bank accessions of partial WSSV VP28 gene oligonucleotide sequences with query cover > 90% and percentage identity > 99.8% to sequences isolated from WSSV infected Pacific white shrimp from Barru, Pangkep, and Pinrang Districts, South Sulawesi Province, Indonesia

Country of Origin	*GenBank* Accession	Query Cover (%)	Percentage Identity (%)	Host taxon	Reference
Japan	AY249443.1	97	100	*Marsupenaeus japonicus*	Unpublished
USA	AY249442.1	97	100	*Litopenaeus setiferus*	Unpublished
Vietnam	AJ551447.1	96	100	Unknown	Unpublished
China	AY249440.1	96	100	*Fenneropenaeus chinensis*	Unpublished
Indonesia	AY249441.1	95	100	*Penaeus monodon*	Unpublished
Vietnam	AY168644.1	95	100	Unknown	Unpublished
USA	MN840357.1	99	99.84	*L. vannamei*	Cruz-Flores et al. (2020)
Mexico	KU216744.2	99	99.84	*L. vannamei*	Rodriguez-Anaya et al. (2016)
Mexico	MG432474.1 MG432476.1 MG432479.1 MG432480.1 MG432481.1 MG432482.1	99	99.84	*L. vannamei* *L. vannamei* *L. vannamei* *L. vannamei* *L. vannamei* *L. vannamei*	Parrilla-Taylor et al. (2018)
Thailand	AF173993.1	99	99.84	*P. monodon*	van Hulten et al. (2000)
Taiwan	AF440570.1 AF272979.1	99	99.84	*P. monodon*	Tsai et al. (2000)
China	AF227911.1	99	99.84	*P. monodon*	Zhang et al. (2001)
China	AF332093.3	99	99.84	*M. japonicus*	Yang et al. (2001)
Thailand	AF369029.2	99	99.84	*P. monodon*	van Hulten et al. (2001)
Korea	JX515788.1	99	99.84	*L. vannamei*	Chai et al. (2013)
Mexico	KT748521.1	99	99.84	*L. vannamei*	Unpublished
China	KT995472.1	99	99.84	*M. japonicus*	Li et al. (2017)
China	KX686117.1	99	99.84	*Procambarus clarkii*	Jiang et al. (2017) Tsai et al. (2000) Tsai et al. (2000)
Brazil	MF784752.1			*L. vannamei*	Pereira et al. (2019)
Brazil	MG264599.1			*L. vannamei*	Dantas et al. (2018)
Ecuador	MH090824.1			*L. vannamei*	Restrepo et al. (2018)
India	MW248106.1	99	99.84	*L. vannamei*	Valappil et al. (2021)
Korea	AF380842.1	95	99.83	*F. chinensis*	Moon et al. (2003)
China	AF502435.1	95	99.83	Unknown	Joseph et al. (2015)
China	AY682926.1	95	99.83	Unknown	
India	DQ013883.1	95	99.83	*P. monodon*	
Korea	GQ328029.1	95	99.83	Unknown	Unpublished
Vietnam	JX444993.1	95	99.83	*P. monodon*	Unpublished
India	KT963933.1	95	99.83	*P. monodon*	Unpublished
Bangladesh	MF489077.1	95	99.82	*P. monodon*	Unpublished

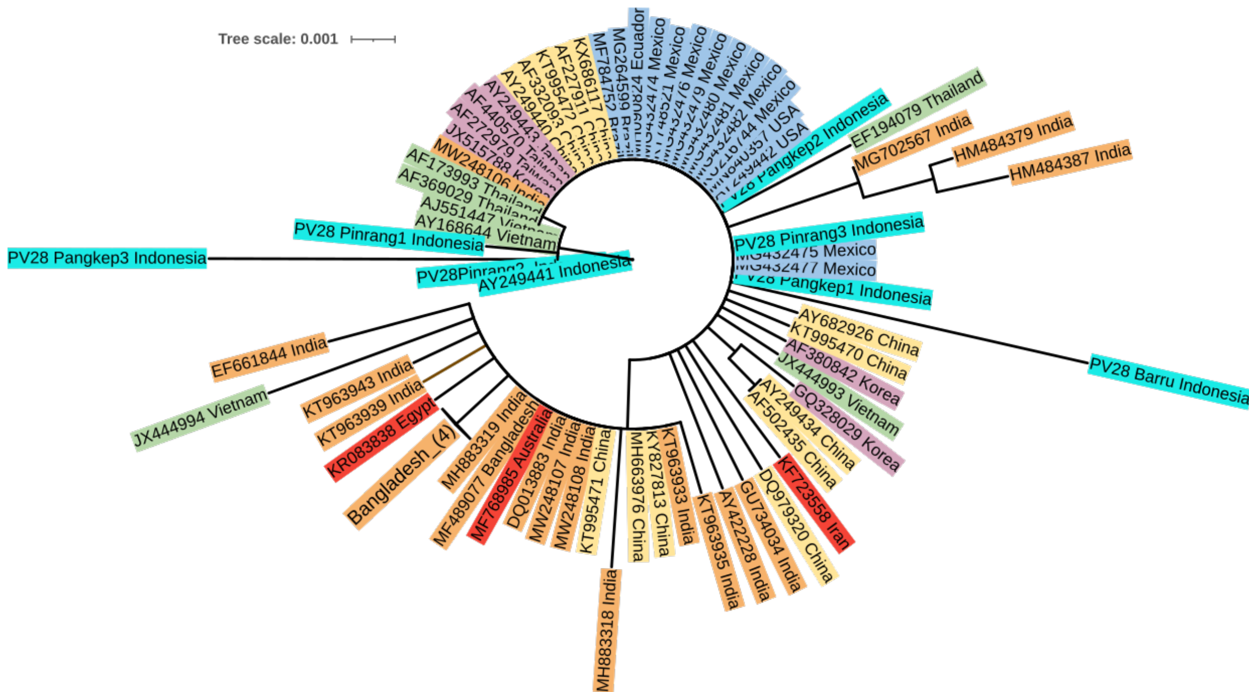


FIGURE 3. Phylogenetic tree of 7 WSSV VP28 gene isolates from Barru, Pangkep, and Pinrang Districts in South Sulawesi Province, Indonesia with 67 GenBank accessions from several countries (634 nucleotide positions). Evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model (Kimura 1980) in MEGA X (Kumar et al. 2018) and edited in the interactive Tree of Life (iTOL) online tool (Letunic & Bork 2019, 2016). Colour code: turquoise = Indonesia; green = Southeast Asia; yellow = China; purple = Northeast Asia; brown = South Asia; blue = Americas; red = other regions

TABLE 3. Protein composition of the WSSV VP28 coding region of isolates from infected Pacific white shrimp in South Sulawesi, Indonesia

Code	%	Code	%	Code	%	Code	%
Ala	5.732	Gly	9.554	Met	3.822	Ser	7.643
Cys	0.637	His	0.637	Asn	5.732	Thr	11.465
Asp	6.369	Ile	5.732	Pro	3.822	Val	9.554
Glu	7.006	Lys	5.732	Gln	1.911	Trp	0.637
Phe	5.732	Leu	3.822	Arg	2.548	Tyr	1.911

GENETIC DISTANCE (CODING REGION)

The genetic distance between coding regions of VP28 WSSV samples of Pacific white shrimp from the three locations in South Sulawesi was zero. However, genetic distance (including the presence of functional mutations) from the South Sulawesi isolates for the coding region varied between the 67 GenBank accessions included in the analysis (Table 4). The absence of functional mutations in the VP28 sequences from South Sulawesi indicate that the WSSV samples isolated in this study were most likely closely related and came from one virus population, despite the differences in the full sequences

TABLE 4. Origin, publication status and genetic distance of the 67 GenBank accessions used in the phylogenetic analysis from the VP28 consensus coding sequence isolated from WSSV infected Pacific white shrimp in South Sulawesi, Indonesia

Country	GenBank Accessions		Unpublished		Genetic distance		
	No.	%	No	%	0	0.002-0.004	> 0.004
Australia	1	1.49%	0	0.00%	1	0	0
Bangladesh	6	8.96%	4	66.67%	1	5	0
Brazil	2	2.99%	0	0.00%	2	0	0
China	13	19.40%	2	15.38%	9	2	2
Ecuador	1	1.49%	0	0.00%	1	0	0
Egypt	1	1.49%	1	100.00%	0	1	0
India	16	23.88%	10	62.50%	6	3	7
Indonesia	1	1.49%	1	100.00%	1	0	0
Iran	1	1.49%	0	0.00%	0	0	1
Japan	1	1.49%	1	100.00%	1	0	0
Korea	3	4.48%	1	33.33%	2	1	0
Mexico	10	14.93%	1	10.00%	8	0	2
Taiwan	2	2.99%	0	0.00%	2	0	0
Thailand	3	4.48%	0	0.00%	2	1	0
USA	2	2.99%	1	50.00%	2	0	0
Vietnam	4	5.97%	0	0.00%	2	1	1
Total	67	100%	22	32.84%	40	14	13

obtained (Figures 3). This result is not surprising in view of the low geographical distance between these three districts and the likelihood of transfer of potentially infected material between these three locations.

Of the 67 GenBank accessions with the highest percentage identity and query cover percentages, 60% had identical coding region sequences (genetic distance = 0). The accessions with null genetic distance came from 14 of the 16 countries, the exceptions being Egypt and Iran. This indicates that the WSSV virus strains present in South Sulawesi may have had a recent common ancestor with over half of the isolates in GenBank, even though the latter were obtained from widespread geographical regions. WSSV was first described in cultured crustaceans around 1991-1992 and appears to be a relatively recently evolved virus within the poorly researched viral family Nimaviridae (Kawato et al. 2019; Lightner 1999). WSSV can affect several crustaceans including almost all shrimps, crabs and crayfish (Zheng et al. 2019), including farmed and wild populations (Onihary et al. 2021). The expansion of crustacean aquaculture and international movement of broodstock and seed (potential carriers) resulted in a rapid spread of the virus during the 1990's (Lightner 1999); therefore, such close evolutionary relationships are possible and even likely.

However, 40% (27) of the GenBank accessions had VP28 coding region sequences non-identical to the South Sulawesi isolates. Of these, 14 had pairwise genetic distances (number of base substitutions per site) just over 0.002 and 13 had genetic distances over 0.004. Seven countries had accessions in more than one distance category, indicating a relatively high diversity of WSSV strains in these countries. Accessions from three countries, China, India and Vietnam, were present in all three categories. This result indicates that, in contrast to the seven sequences from South Sulawesi, the relatively

high diversity in the total homologous sequences from these countries shown in Figure 3 includes variations (mutations) in the coding region.

A viral protein or VP is a structural protein found in viruses that has an important function in the process of infecting a host (Liu et al. 2014). In WSSV, the host infection process (WSSV gene expression) is divided into two phases; the early phase occurs before the viral DNA replicates, while the late phase occurs during the initiation of viral DNA replication or later. The WSSV genes transcribed in the late phase include genes encoding the main WSSV structural proteins: VP-28, VP-26, VP-24, VP-19 and VP-15 (Tenriulo et al. 2015). According to Parenrengi et al. (2017), viral proteins VP-28 and VP-19 are located in the nucleocapsid envelope whereas VP-26, VP-24 and VP-15 are located within the nucleocapsid.

The VP19 and VP28 envelope proteins are not only involved in the morphogenesis process but also the first molecules that interact with the host Chang et al. (2008). In general, the WSSV VP28 is considered to be highly conserved even across considerable geographical distances (Li et al. 2017; Marks et al. 2004; Moon et al. 2003). However, mutations do occur, and non-conserved nucleotide positions (mutations) in the VP28 gene may contribute to the virulence of certain WSSV strains (Sathish et al. 2004; Zheng et al. 2019).

Highly conserved WSSV VP28 between isolates have been reported from several countries, including Korea (Moon et al. 2003; Park & Shin 2009) and Mexico (Parrilla-Taylor et al. 2018). It is difficult to estimate the diversity of WSSV strains in Indonesia, specifically with respect to mutations within the VP28 envelope protein coding region, as only one Indonesian isolate (accession AY249441) of unknown origin was found in GenBank. However, a study on VP28 from WSSV infected shrimp in Situbondo, Java reported a coding nucleotide sequence identical to AY249441 (Ali et al. 2015). Similar to this study, Ali et al. (2015) reported identity or very close similarity to WSSV VP28 accessions from several other countries, including China, Japan, Korea, and Mexico; they also recorded differences with accessions from China, India, Iran, Korea Mexico, Thailand, and Vietnam. They concluded that the VP28 gene appears relatively highly conserved in general, and specifically within Indonesia, and should therefore be a good candidate for vaccine development. While one sequence in this study (from the Barru location) differed considerably from the others (Figure 3), the differences were outside the coding region (before the start codon) and therefore should not alter the protein coding function of the gene (Figure 3, Table 2). The geographical distance between Situbondo in Java and South Sulawesi is similar to or greater than that between countries or sites within other countries in Table 2 from which VP28 WSSV accessions had different coding sequences. The results of this study therefore support the conclusions of Ali et al. (2015) based on isolates from *P. monodon* that the WSSV VP28 gene seems highly conserved in Indonesia and seems a promising candidate target for the development of an Indonesian WSSV vaccine.

VARIATION IN VP28 FROM DIFFERENT HOSTS

Of the 191 whole or partial WSSV VP28 accessions returned by a search of the NCBI GenBank database, the majority of those where the host was identified were isolated from penaeid shrimps, with 107 from the tiger shrimp *Penaeus monodon*, 22 from the Pacific white shrimp *L. vannamei*, eight from the Indian prawn *Fenneropenaeus indicus*, four from the kuruma shrimp *Marsupenaeus japonicus*, two from the Chinese shrimp *Fenneropenaeus chinensis* (accessions AY249440, AF380842), and one from *Penaeus setiferus* (AY249442). Accessions from other decapods include three from mudcrabs *Scylla serrata*/*Scylla* sp. (accessions MZ383194, MZ383199 and KP219388 from Bangladesh), three from the Louisiana crawfish *Procambarus clarkii* (accessions KX686117, KT995470 and DQ007315 from China), and one each from the giant freshwater prawn *Macrobrachium rosenbergii* (accession DQ979320 from China), the red claw crayfish *Cherax quadricarinatus* (accession MG148351, from China) and the scalloped spiny lobster *Panulirus homarus*. The remaining accessions simply refer to the host organism as 'shrimp', 'penaeid shrimp', or do not identify the host organism.

Of the 67 accessions with over 90% coverage included in the phylogenetic analysis, the host organisms of the six accessions with VP28 sequences identical to the isolates obtained from *L. vannamei* in South Sulawesi, Indonesia were penaeid shrimp (one each of *M. japonicus*, *L. setiferus*, *F. chinensis*, *P. monodon*) or unknown (2 isolates) (Table 2). Among these 67 accessions, three had percentage identity less than 99%: two Indian isolates from *L. vannamei*: MG702567 ((97.45%; Vinaya Kumar et al. 2018) and MH883318 (98.58%; unpublished) and HM484379, an Indian isolate from *P. monodon* (98.16%, unpublished). Percentage identity with other 20 VP28 sequences isolated from *L. vannamei* ranged from 99.68% to 99.84%. The remaining

41 accessions from other or unknown hosts had over 99% identity, ranging from 99.33% to 99. 4%. Host organisms of accessions with 99.8 to 99.9% identity (Table 2) included *F. chinensis* (1), *M. japonicus* (2), *P. monodon* (9), *P. clarkii* (1) and unknown (3) while those with 99.3 to 99.7% identity included *F. indicus* (1), *M. japonicus* (2), *P. monodon* (14), *P. clarkii* (2), *M. rosenbergii* (1) and unknown (2). These results indicate that VP28 is, in general, highly conserved both within and between host species, with no clear patterns related to geographical origin or host. Therefore, it is likely that vaccines developed for one species or region could be effective for other taxa and populations, especially within the penaeid shrimp group.

Conclusion

The VP28 WSSP protein-coding gene fragments isolated from Pacific white shrimp *L. vannamei* in South Sulawesi were around 670 bp long, yielding unambiguous sequences of 634 nucleotides and a coding region with 474 nucleotides. Homology between the sampled regions in South Sulawesi was from 93-100%; however, the coding region was identical for all locations and replicates. Reference VP28 WSSP sequences with the closest kinship (over 90% coverage and 100% sequence homology) originated from a variety of host taxa, including penaeid shrimps (but not *L. vannamei*), and from widespread geographical origins (Indonesia, Vietnam, China, Japan, and the USA). The phylogenetic analysis indicates relatively high genetic variation within South Sulawesi compared to regional or global variation in VP28 WSSV accessions, but with a highly conserved coding region, identical to that to other known isolates from Indonesia and to homologous isolates from 13 other countries. In view of the number of potential host species present and geographic spread of the country, sequencing of VP28 isolates from other regions and a wider range of host species in Indonesia is recommended. However, these results increase the geographic and host organism range of WSSV VP28 sequence data for Indonesia and could support further research on this promising candidate target for Indonesian WSSV vaccine development.

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