Limited evidence for white spot syndrome virus susceptibility associated with expression of *Pm*VRP15 in local population of giant tiger shrimp (*Penaeus monodon*)

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Abstract

White spot syndrome virus (WSSV) is a devastating viral disease in shrimp aquaculture. Infection of WSSV in penaeid shrimps affects immune defense and changes gene expression. *Pm*VRP15 has been reported as a part of the WSSV propagation pathway that is highly up-regulated in hemocytes at the acute phase of WSSV infection. This study analyzed the expression of *Pm*VRP15 in local populations of giant tiger shrimp (*Penaeus monodon*) to be associated with susceptibility to WSSV. Tested populations consisted of an inbreeding population (G_8) and outbreeding population (G_{8iA}) from Jepara, Indonesia. Susceptibility was determined by cumulative mortality, median lethal time (LT_{50}), and severity of infection at time of death. Though all populations were susceptible to WSSV, the first mortality in G_8 occurred at 18 hours post-infection (hpi) with mild infection, while first mortality of G_{8iA} occurred at 30 hpi with severe infection. The LT_{50} of G_8 was significantly lower than that of G_{8iA}, indicating that G_{8iA} was less susceptible to WSSV than G_8. Relative *Pm*VRP15 transcripts of G_{8iA} were insignificantly down-regulated, whereas relative *Pm*VRP15 transcripts of G_8 were insignificantly up-regulated. Although it's still not conclusive, the results of this study suggest that *Pm*VRP15 has weak potential as a WSSV susceptibility marker in G_8 and G_{8iA} broodstock selection.

Keywords: Disease susceptibility, genetic marker, *Penaeus monodon*, *Pm*VRP15, WSSV susceptibility

Introduction

White spot syndrome virus (WSSV) is one of the pathogens causing high mortality in giant tiger shrimp (*Penaeus monodon*) aquaculture. WSSV has been reported to cause harvest failure with a 100% morbidity and mortality level in three to ten days (Jeswin *et al.*, 2013; Reddy *et al.*, 2013). This virus is an enveloped, double-stranded DNA virus that is classified into genus *Whispovirus* and family Nimaviridae (Tonganunt *et al.*,

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2009). White spot disease can be detected by the appearance of white lesions inside the carapace due to abnormal calcium deposition in the cuticle (Alifuddin *et al.*, 2003; Reddy *et al.*, 2013). Either natural or experimental WSSV susceptibility in penaeid shrimps has been reported in many studies (Alifuddin *et al.*, 2003; Corteel *et al.*, 2012; Escobedo-Bonilla *et al.*, 2006; Hayes *et al.*, 2010; Lo *et al.*, 1996; Lo *et al.*, 1997; Mathew *et al.*, 2007).

Hemocytes play an essential role in the immune system of crustaceans through their molecules which are able to recognize the structure of invading organisms and trigger the removal mechanism (Johansson *et al.*, 2000). A *Penaeus monodon* viral responsive protein, *Pm*VRP15, has been found as a gene that is responsive to the infection of WSSV in

hemocytes by mediating WSSV propagation at the acute phase (Vatanavicharn *et al.*, 2014). Vatanavicharn *et al.* (2014) also conducted RNA interference to alter *Pm*VRP15 expression and elucidated that *Pm*VRP15 was associated with WSSV susceptibility in a *P. monodon* population in Thailand. This signifies the importance of determining the change in expression of *Pm*VRP15 in association with susceptibility to WSSV infection. At the commercial level, change in gene expression, which equates to the effect on fitness, helps to predict and decide the potentially best population or family of *P. monodon* to be used as the broodstock in the next selective breeding program.

In 2005, the Main Center for Brackishwater Aquaculture and Fisheries (MCBAF) Jepara, Central Java, Indonesia, started a selective breeding program to improve the growth rate and survival of *P. monodon*. Regarding the outbreak in 1990, producing a WSSV-tolerant *P. monodon* population was one of the focuses of the MCBAF selective breeding program. However, this existing breeding program does not consider the use of genetic markers to select broodstocks with desired traits. While this study was being conducted, the selective breeding program in MCBAF Jepara reached an eighth generation of an inbreeding (domesticated) population called G_8 and produced an outbreeding population called G_{8iA}. The aims of this study were to determine whether the populations had the potential to adapt to a WSSV infection and to evaluate whether the expression of *Pm*VRP15 could provide a genetic basis to mark WSSV susceptibility in the G_8 and G_{8iA} populations.

Materials and methods
Maintenance of experimental shrimp

Penaeus monodon used in the experiment consisted of 1) an eighth generation of inbreeding (domesticated) population called G_8, and 2) an outbreeding population called G_{8iA}. G_{8iA} was the offspring from introgression of wild type males of an Aceh population to the females of the seventh generation of the inbreeding population (G_7). These shrimps were transferred from NSBC (National Shrimp Broodstock Center) ponds to the Laboratory of Health Management of Aquatic Organism, MCBAF Jepara, Central Java, Indonesia. The mean body weight (MBW) of the experimental *P. monodon* was 20–25 g. Shrimps were fed twice a day with commercial pelleted shrimp feed at a rate of 3% MBW. They were allowed to acclimatize for a week in fiberglass tanks and randomly chosen for WSSV detection using one-step PCR to confirm the absence of WSSV. The WSSV-free *P. monodon* were then randomly maintained in 100-l plastic containers filled with disinfected seawater. Each container was fitted with air-stone aeration and natural photoperiod. Water exchange (50%) was performed daily throughout the trial. Salinity ranged between 20 and 30%, water temperature ranged between 26.3 and 28.5°C, dissolved oxygen ranged between 5 and 7.17 g ml^{-1}, and water pH ranged from 7.34 to 8. This study consisted of two experiments. The first experiment aimed to determine cumulative mortality in order to examine susceptibility of the population to WSSV exposure, while the second experiment aimed to quantify *Pm*VRP15 expression. Each experiment was conducted in both G_8 and G_{8iA} populations. Each population was divided into two groups, a non-infected group (control group) and WSSV-infected group. All experiments were conducted in triplicates. A total of 60 shrimps were used in the first experiment (susceptibility test), while in the second experiment (gene expression assay) 96 shrimps were used (Fig. 1). Each population was maintained in different containers.

Preparation of viral inoculums

Gills from WSSV-reinfected shrimps (positively detected at first step PCR) were homogenized in sterile PBS 1x (1:10 w/v) and then centrifuged at 3,000 rpm for 20 minutes and 8,000 rpm for 30 minutes at
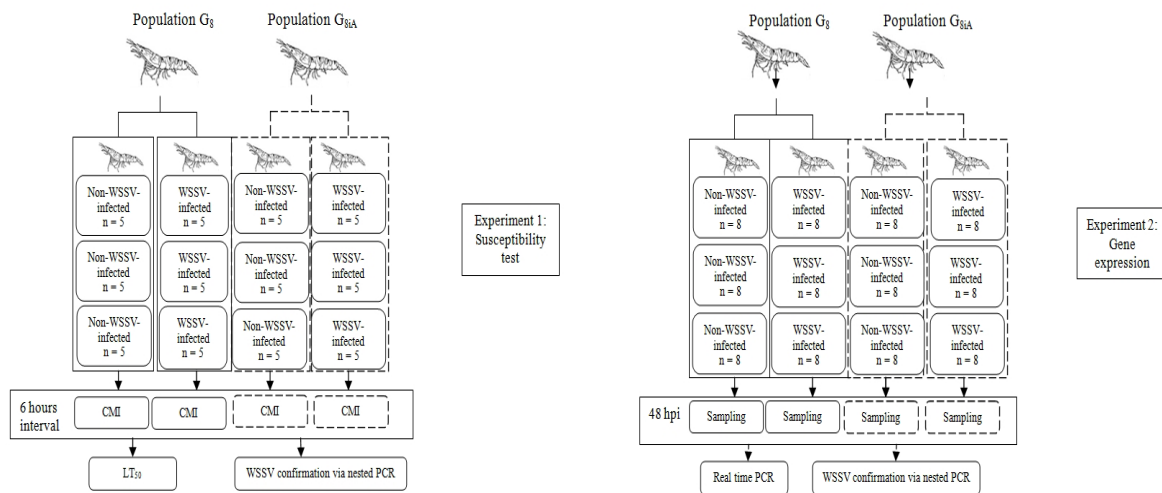
Fig. 1. Experimental design. The first experiment aimed to examine susceptibility within populations (left), whereas the second experiment aimed to measure *Pm*VRP15 expression (right). Acclimated G_8 and G_{8iA} were divided into two subgroups: non-infected groups (control groups) and WSSV-infected groups. All subgroups were in triplicates. Five shrimps were used for each experimental unit in the first experiment, while the second experiment utilized 8 shrimps for each experimental unit. Cumulative mortality was observed every 6 hours to estimate LT_{50}. Hemolymphs were randomly sampled at 48 hpi for *Pm*VRP15 quantification via real-time PCR. All shrimps in the first experiment and random sampled hemolymph in the second experiment were subjected to confirmation of WSSV infection via nested PCR.

4°C. The supernatant was filtered through a 0.45 µm membrane filter. The extract was a 10^{-1} virus extract. This viral inoculum was prepared according to Hameed *et al.* (2006). The median lethal dose (LD_{50}) was determined based on the method described in Gopikrishna *et al.* (2012). The diluted 10^{-2} virus extract was subsequently used for a challenge test in G_8 and G_{8iA}.

Challenge experiment and sampling

Two independent challenge tests were conducted in triplicates to determine the cumulative mortality and to quantify gene expression in sampled hemocytes. Treated shrimps were injected intramuscularly at a rate of 0.02 ml of WSSV extract g^{-1} of body weight into the dorsolateral part of the fourth abdominal segment of the shrimp between the tergal plates into the muscle of the third abdominal segment using a 1 ml syringe with a 20 G 0.9 x 25 mm needle (Escobedo-Bonilla *et al.*, 2006). Control shrimps were injected with PBS 1x at the same rate. Mortality was recorded at 6 hour intervals.

Dead shrimps, which showed no physical response to mechanical stimulation, were removed and the tissues from gills and/or pleopods were preserved in 95% alcohol for WSSV detection. Cumulative mortality was used to estimate the median lethal time (LT_{50}). For the expression profile, random sampling of hemocytes was conducted at 48 hpi by collecting 0.2 ml of hemolymph from the ventral sinus in the second abdominal segment using a 1 ml syringe provided with a 20 G 0.9 x 25 mm needle previously filled with 0.2 ml of 10% tri-sodium citrate (Andrade, 2011; Peraza-Gomez *et al.*, 2014). Hemolymph was then poured into a microtube and kept on ice. The hemolymph (100 µl) sample was immediately centrifuged at 5,000 x g for 5 minutes at 4°C to separate the hemocyte (pellet) from the hemolymph (Vatanavicharn *et al.,* 2014). The supernatant was discarded whereas the pellet (the hemocyte) was used in RNA isolation. The remaining 100 µl of the hemolymph sample was stored at -20°C to be used in WSSV detection.

RNA extraction and cDNA synthesis

Total RNA was extracted from isolated hemocytes with TRIzol reagent (Invitrogen, USA), quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA), and treated with RNase free DNase I (Invitrogen, USA) to remove the contaminating DNA. Thermal cycling of cDNA synthesis was performed at 75°C for 5 minutes, chilled on ice for 5 minutes, 60°C for 15 minutes, and 85°C for 5 minutes with 1–160 ng RNA, 1 µM cocktails of antisense gene-specific primers, 1 µM dNTP, 0.2 U/µl RNasin (Promega, USA), 5 mM $MgCl_2$ (Promega, USA), 0.05 U/µl AMV RT-ase, and 5 µl of 5x AMV RT-buffer (Promega, USA).

Real-time PCR

The *Pm*VRP15 transcript levels from isolated RNA were assayed with 10 µl of 1x KAPA SYBR Fast qPCR Master Mix (Kapa Biosystems, USA), 0.1 µM of *Pm*VRP15-RTF/R and β-actin-F/R primers, and 10 x diluted cDNA in 7500 Fast Real-time PCR System (Applied Biosystems, USA). The sequences of primer set for β-actin (5' GAACCTCTCGTTGCCGATGGTG 3' [forward] and 5' GAAGCTGTGCTACGTG GCTCTG 3' [reverse]) and *Pm*VRP15 (5' CGTCCTTCAGTGCGCTTCCATA 3' [forward] and 5' ACAGCGACTCCAAGGTCT ACGA 3' [reverse]) used in this study were based on those described in Vatanavicharn *et al.* (2014). Thermal cycling was performed at 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. A melt curve stage was performed at 95°C for 15 seconds, 60°C for a minute, and 95°C for 30 seconds, and 60°C for 15 seconds. The results were presented as average relative expression ratio of *Pm*VRP15 transcript levels of the WSSV-infected shrimps compared with the non-infected shrimps after normalization to the reference gene β-actin. The relative expression ratios were calculated as $2^{-\Delta C_T}$ (Schmittgen and Livak, 2008).

Rapid DNA extraction and confirmation of WSSV infection

DNA extraction was conducted by homogenizing 50–100 µl of hemolymph or 0.2 g of tissue (gills/pleopods) with 500 µl of lysis buffer (10% SDS pH 7.2, 5M NaCl, 0.5M EDTA pH 8, and 1M Tris-HCl pH 8) and 1 µl of 10 µg/ml proteinase K until the tissues were finely dispersed. The mixture was then incubated at 95°C for 10 minutes using a heating block before centrifugation at 12,000 x g for 10 minutes. Supernatant (approximately 100 µl) was transferred to a new tube containing 400 µl of absolute ethanol. The tube was vortexed briefly and centrifuged at 12,000 x g for 5 minutes. Supernatant was removed and the DNA pellet was air-dried on the bench. The DNA pellet was then dissolved by adding 100 µl of dH_2O to the tube (Wang and Chang 2000). Before being used in PCR, the DNA was diluted 100 times by combining 1 µl of DNA isolate with 99 µl dH_2O. Detection of WSSV using nested PCR was conducted according to the Manual of Diagnostic Tests for Aquatic Animals (http://www.oie.int/international-standard-setting/aquatic-manual/accesss-online/) . Lightly WSSV-infected shrimps showed a positive result only in the second step of PCR, yielding a 941 bp PCR fragment, while severely WSSV-infected shrimps showed 1447 bp and 941 bp PCR fragments both in first step and second step of PCR (Lo *et al.* 1997).

Data Analysis

Cumulative mortality data were used to estimate median lethal time (LT_{50}) using 3-Parameter-Weibull survival distribution analysis in Minitab 16 software at the $P < 0.05$ level. Threshold cycle (C_T) data from ABI 7500 software was copied into Microsoft Excel to measure the relative expression change of *Pm*VRP15 transcript levels of the WSSV-infected shrimps compared with the non-infected shrimps after normalization to the reference gene β-actin. The statistical significance of differences among $2^{-\Delta C_T}$ values

of each population was calculated by a two-sample t-test in SigmaPlot 13.0 where the significance was accepted at the P < 0.05 level.

Results and Discussion

The first mortality in G_8 was recorded at 18 hpi whereas the first mortality in G_{8iA} was recorded at 30 hpi, indicating that the inbred population (G_8) died sooner than the outbred population (G_{8iA}) in responding to the WSSV infection. However, 100% mortality of both G_8 and G_{8iA} occurred at 60 hpi (Table 1). This demonstrates that all populations were susceptible to white spot disease. Nevertheless, the large standard error bar showed that G_{8iA} had more variable mortality time than G_8, suggesting that G_{8iA} showed a highly variable degree of susceptibility to WSSV within individuals in the population (Fig. 2 left). Based on confirmation of WSSV-infection using nested PCR, *P. monodon* from G_8 that were dead at 18 hpi (first mortality) showed a positive WSSV result only after the second step of amplification, which yielded a 941 bp PCR fragment (Fig. 2 right), indicating that the shrimps from G_8 died with a light infection of WSSV. Compared

with the light infection at early death in G_8, G_{8iA} individuals that died at 30 hpi (first mortality) gave WSSV-positive results in both steps of PCR and yielded 1447 bp and 941 bp PCR fragments (Fig. 2 right); thereby indicating that G_{8iA} shrimps were able to bear more severe infections in considerably longer periods than G_8. All control *P. monodon* from G_8 and G_{8iA} that were alive and terminated at 66 hpi were WSSV-negative.

Table 1. Median lethal time (LT_{50}) of WSSV-infected shrimp in the population of *P. monodon* Fabricius estimated with 3-Parameter-Weibull distribution analysis. Data were derived from three replicates. Supercripted letters indicate statistical signifance.

Population	Time of first mortality (hpi)	Time of 100% mortality (hpi)	LT_{50} (hpi)
G_8	18	60	45.17 ± 1.28^a
G_{8iA}	30	60	50.09 ± 1.04^b

Cumulative mortality G_8 and G_{8iA} was analyzed using 3-Parameter-Weibull survival distribution analysis and the LT_{50} of the two populations were compared to examine the degree of susceptibility. As Table 1 shows,
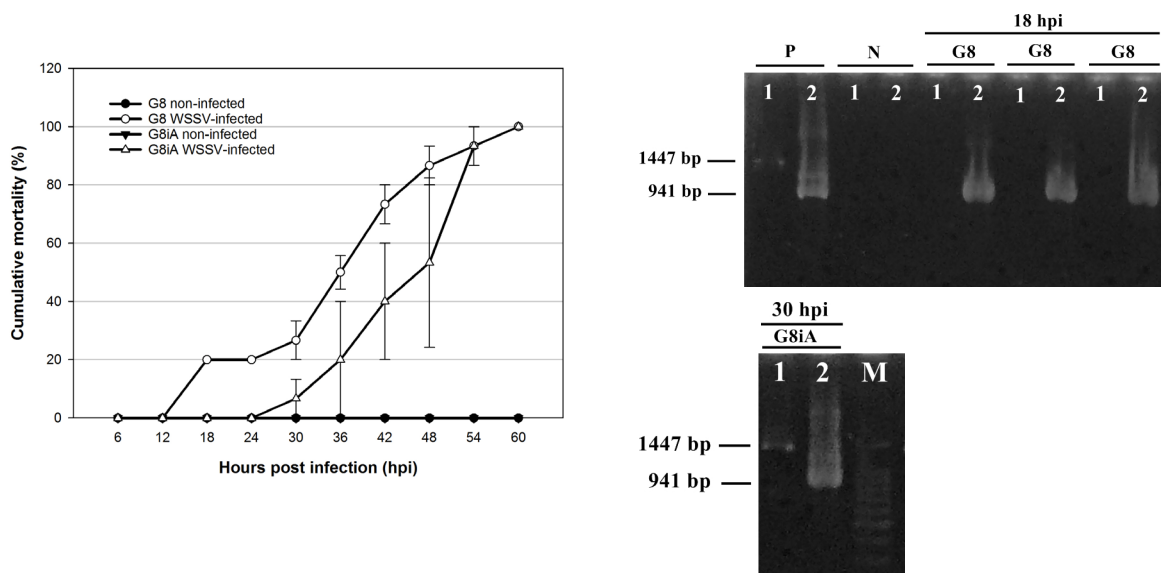

Fig. 2 Cumulative mortality of *P. monodon* Fabricius in G_8 and G_{8iA} populations in response to WSSV infection (left). The data represent the mean ± SEM derived from three replicates. Confirmation of WSSV-infection in the experimental *P. monodon* using nested PCR (right). Lanes: M: 100 bp DNA ladder, P: positive control (1447 bp and 941 bp), N: negative control; 1: first step PCR, 2: second step PCR.

the median lethal time (LT_{50}) of WSSV-infected G_{8iA} (50.09 ± 1.04 hpi; 95% confidence interval: 48.04–52.13) was significantly higher than that of G_8 (45.17 ± 1.28 hpi; 95% confidence interval: 42.67–47.68) (P < 0.05), indicating that G_{8iA} had a higher survival time than G_8. These results would suggest that the outbred population (G_{8iA}) was less susceptible to the WSSV infection than the inbred population (G_8). This result was consistent with previous studies reporting that wild type penaeid shrimps and outbred penaeid shrimps showed higher survival times than inbred populations in response to WSSC infection (Gopikrishna *et al.*, 2012; Hayes *et al.*, 2010). There is also evidence that genetic polymorphism between two different populations of *L. vannamei* affects their WSSV resistance/susceptibility level (Liu *et al.*, 2014).

The significant difference in median lethal time and distinct capability to bear infection suggested there was little genetic variation between G_8 and G_{8iA} involving in WSSV susceptibility. The genetic variation may promote different immune responses to WSSV infection. According to the MCBAF Jepara breeding history, G_8 was produced from a limited broodstock that had undergone decreased genetic variation since G_1 (Prastowo *et al.*, 2009). G_{8iA} also revealed higher genetic variation compared with G_8 (Purnamaningrum 2015). Moreover, wild type male *P. monodon* from Aceh, the parental population of G_{8iA}, showed higher heterozygosity compared with the other populations of male *P. monodon* in Indonesia (Prastowo *et al.*, 2010). Thus, the less WSSV susceptible G_{8iA} likely emerged from genetic variation resulting from breeding with the wild type Aceh population. A limited broodstock, which is often caused by harvest activities, and domestication by inbreeding could reduce genetic variation in apopulation, accumulate recessive alleles, and eliminate resistant alleles (Brieuc *et al.*, 2009; Cock *et al.*, 2009), thereby affecting the G_8 and G_{8iA} populations' ability to adapt to

the viral infection and contributing to their susceptibility.

*Pm*VRP15 has been found as a nuclear membrane-like protein acting to mediate replication of WSSV in hemocytes in early infection (Vatanavicharn *et al.*, 2014). A linear form of ΔC_T ($2^{-\Delta C_T}$) showed that baseline expression (non-infected) of *Pm*VRP15 in G_{8iA} was higher than G_8. WSSV infection induced an increased expression of *Pm*VRP15 in G_8 (Fig. 3). Contrarily, *Pm*VRP15 inducible expression (WSSV-infected) of G_{8iA} decreased (Fig. 3). The relative expression ratio showed that the *Pm*VRP15 transcripts level in G_8 was up-regulated to 2.12 fold, while G_{8iA} showed a down-regulation of *Pm*VRP15 transcripts level to 0.42 fold (Fig. 4). The melt curve which showed a single peak at 83–84°C (data not shown) indicated that amplification of *Pm*VRP15 and β-actin was specific. However, both the up-regulation of *Pm*VRP15 in G_8 (P = 0.399) and down-regulation in G_{8iA} (P = 0.393) were not statistically significant (Fig. 3), contradicting a previous study that showed that *Pm*VRP15 expression in the Thai population was significantly up-regulated in hemocytes at 48 hpi (Vatanavicharn *et al.*, 2014).
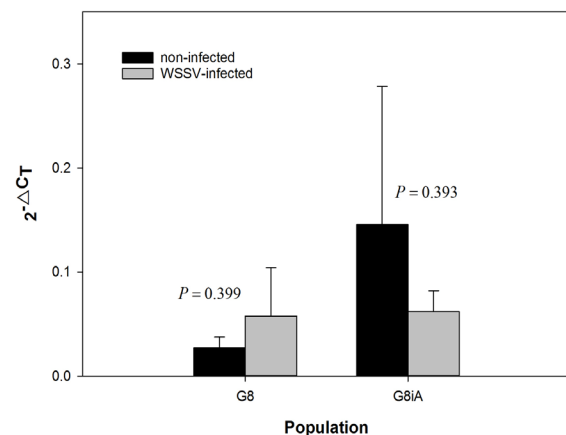
Fig. 3. Baseline expression (non-infected) of *Pm*VRP15 of each population compared with the inducible expression (WSSV-infected). The data represent the mean of $2^{-\Delta C_T}$ ± SEM of each population derived from nine different biological replicates. The P value resulted from a two-tailed t-test between the non-infected and WSSV-infected groups of each population.
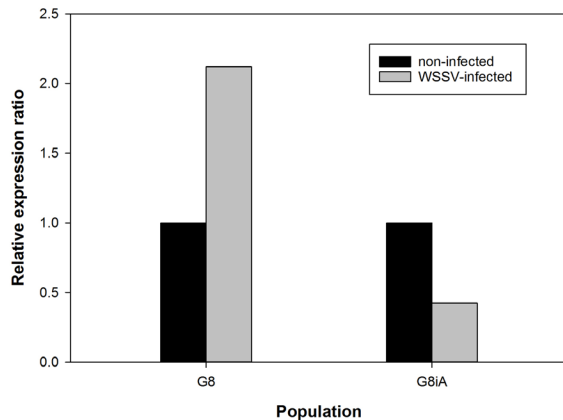
Fig. 4. *Pm*VRP15 relative expression ratios in G$_8$ and G$_{8iA}$ in response to WSSV infection. The data represent the fold change of each population calculated from nine different biological replicates. Relative expression ratio >1, 1, and <1 indicate that the gene expression level was up-regulated, remained the same, or was down-regulated compared with the non-infected *P. monodon*.

The disparity between this present study and Vatanavicharn *et al.* (2014) can be explained in two ways. Firstly, as Vatanavicharn *et al.* (2014) hypothesized, severity of WSSV infection may affect stimulation of *Pm*VRP15 expression in hemocytes. WSSV propagation of experimental *P. monodon* among these two local populations and the Thai population in the prior study were not monitored. Therefore, at present, we cannot determine how severe WSSV infection may regulate the expression of *Pm*VRP15. Refinement in the infection protocol by prior quantification of viral loads in the challenge test may also help to observe the impact of WSSV severity on *Pm*VRP15 expression. Secondly, *Pm*VRP15 expression in this present study was measured from individual challenged shrimps, while *Pm*VRP15 expression in Vatanavicharn *et al.* (2014) was measured from pooled challenged shrimps. As shown in Fig. 3, the large SEM bar of the *Pm*VRP15 transcripts level shows a high variability of *Pm*VRP15 expression within individuals. This indicates that the significant up-regulation of *Pm*VRP15 in Vatanavicharn *et al.* (2014) tends to represent the up-regulated

gene expression within the Thai population. Glass *et al.* (2005) argue that even though the gene expression increased by a treatment, the quality of representation of individuals does not improve the representation of the pool, implying that the gene expression information obtained by the pooled sample and individuals are not comparable. However, since the inferences of this study are needed for estimation in population, we assume that pooled sampling of hemocytes would be more representative to compare baseline expression and inducible expression within population. Number of individuals in pooling strategy should be examined carefully to consider population characteristics in expressing genes (Muniesa *et al.*, 2014; Peng *et al.*, 2003; Taylor *et al.*, 2010).

As the authors discussed before, the different median lethal time and severity of infection at time of death between G$_8$ and G$_{8iA}$ suggest that there is little variation for WSSV susceptibility within the local population of *P. monodon* in MCBAF Jepara. This implies that there may be a potential genetic marker for WSSV susceptibility in G$_8$ and G$_{8iA}$. In line with this result, Dutta *et al.* (2013) reported that WSSV susceptibility and WSSV resistance of *P. monodon* in an Indian population linked to a microsatellite 71 bp DNA marker. Hence, we speculate that *Pm*VRP15 is not universally associated with WSSV susceptibility in other populations besides the Thai population. Furthermore, there are extensive studies indicating that WSSV resistance and WSSV susceptibility in penaeid shrimps is synergic immune response regulated by many genes (Ghosh *et al.*, 2011), such as anti-lipopolysaccharide factor (ALF), which plays a role in defense against WSSV (Liu *et al.*, 2014; Wang *et al.* ,2013), *Penaeus monodon* antiviral (*Pm*AV), which presumably acts as an intermediate factor during antiviral reaction (Luo *et al.*, 2003), and other antimicrobial peptides like penaeidin class 5 that protect shrimps at early infection of WSSV (Jeswin *et al.*, 2013; Wang *et al.*, 2013). Perhaps WSSV susceptibility

in G_8 and G_{8iA} can be marked with other considerable immune-related gene expression rather than *Pm*VRP15. As suggested by Cock *et al.* (2009), it will be helpful to screen a large number of individuals from the G_8 and G_{8iA} populations that demonstrate either resistance or susceptibility to natural WSSV infection, thus exposing a representative WSSV susceptibility genetic marker to be used in MCBAF's Jepara selective breeding program. Despite this possibility, future research will still be needed to study the association of *Pm*VRP15 expression and WSSV susceptibility in another independent population of *P. monodon* to reveal the possibility of *Pm*VRP15 as a universal genetic marker for WSSV susceptibility.

Acknowledgements

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