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Research Article

Identification of a Potential SNP Related to the Expression of Immune Genes and Its Possible Application to Selection of WSSV-Resistant Pacific White Shrimp (*Litopenaeus vannamei*)

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Abstract

The Pacific white shrimp (*Litopenaeus vannamei*) is Indonesia's main export commodity, but its production is constrained by the white spot syndrome virus (WSSV). Selective breeding of disease-resistant broodstock based on single nucleotide polymorphism (SNP) in the anti-lipopolysaccharide factor (ALF) gene is an alternative strategy for solving the disease problem. This study aimed to detect the SNP g.455 A>G in the anti-lipopolysaccharide factor (ALF) shrimp gene, evaluate the correlation of SNP with WSSV-resistance trait, analyze the expression level of immunity genes and genotype frequencies of the WSSV-resistance population shrimp and analyze the SNP inheritance in the first generation of selected shrimp. A total of 120 individuals from 4 families were used to detect the SNP marker using tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). The correlation of the SNP marker with survival rate (SR) was analyzed using a general linear model (GLM) between genotype frequencies and SR. Genotypic similarities between broodstock and pedigree were analyzed using Chi-square. SNP g.455 A>G was successfully detected using the ARMS-PCR method and had a strong correlation between the marker and SR (p-value of AA = 0.012; AG = 0.359, and GG = 0.001). The resistant population has significantly higher ALF and SOD gene expression levels and AA genotype frequency. The SNP marker was inherited, so the broodstock and pedigree have the same genotype frequencies according to chi-square analysis ($\chi 2$ = 0.46 and p-value = 0.497). These results suggested that the g.455 genotype AA could be selected to produce WSSV-resistant Pacific white shrimp.

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1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is the most economically valued and cultivated species, with a productivity reach of about a 5.8 million tons a year (FAO, 2022). Moreover, Pacific white shrimp is Indonesia's main export commodity in the international market, and Indonesia was ranked fourth in shrimp production in Asia (Anderson *et al.*, 2019; Ramadhani *et al.*, 2022). In recent years, viral diseases have significantly constrained Pacific white shrimp production (Walker and Mohan, 2009). The GOAL survey indicated that diseases are the main challenge in shrimp aquaculture (Anderson *et al.*, 2019). The primary viral pathogen in Pacific white shrimp aquaculture is the white spot syndrome virus (WSSV) (Bir *et al.*, 2017; Robinson *et al.*, 2022). White spots under the cuticle are a common clinical sign of WSSV infection and cause mass mortality of up to 100% in a short time (Bir *et al.*, 2017; OIE, 2019; Sabapathy *et al.*, 2019). The economic loss due to WSSV infection is estimated to reach US$ 6 billion per year worldwide (Sabapathy *et al.*, 2019). Furthermore, there is still no consistently effective prevention for WSSV infection (OIE, 2019).

The immune system in shrimp does not have memory cells, unlike vertebrates, which have specific antibodies and complements. The shrimp immune system does not have immunoglobulins that play important role in the immune mechanism, shrimp only have a natural immune system (Baladrat *et.al.*, 2022). The innate immune system dominated by hemocytes is the primary defense against pathogenic infections such as WSSV (Burnett and Burnett, 2015). The antimicrobial peptide (AMP) gene strongly influences hemocyte activity in fighting pathogenic infections (Rowley, 2016). The anti-lipopolysaccharide factor (ALF) is one of the AMP systems that play an essential role against WSSV infection (Li *et al.*, 2015; Zhan *et al.*, 2015). Based on studies of ALF gene expression and WSSV infection, it can be seen that the ALF gene has the highest activity when WSSV infection occurs (Li *et al.*, 2018). SOD and ProPO genes also have a significant role when there is an attack from WSSV through the reactive oxygen system mechanism (Ji *et al.*, 2011).

Single nucleotide polymorphisms (SNPs) in immune system-related genes are known to correlate with resistance to pathogen infection (Guo *et al.*, 2013a; Nasrullah *et al.*, 2020). SNPs that correlate with specific traits have great potential to be used as molecular markers (Liu *et al.*, 2014). Molecular markers are genetic variations between individuals at specific loci and affect the phenotype, so they can be used to select the desired trait (Saefuddin and Afendi, 2006; Vaseeharan *et al.*, 2013; Eze, 2019). SNPs as molecular markers have been developed in African catfish (*Clarias gariepinus*) resistant to *Aeromonas hydrophila* (Nasrullah *et al.*, 2020), fast-growing gourami (*Osphronemus goramy*) (Sandra *et al.*, 2021), and fast-growing giant freshwater prawns (*Macrobrachium rosenbergii*) (Sopian *et al.*, 2017). Molecular markers selection has advantages over phenotypic selection. Molecular markers can apply to a trait with low heritability and for selecting traits that are difficult to measure (e.g., disease resistance). Molecular markers are inherited to the next generation and can detect desired traits at the beginning phase (Rothschild and Ruvinsky, 2007; Zenger *et al.*, 2019; Garcia *et al.*, 2021).

Selection of superior traits using Genome high-density SNPs has been successfully carried out on many aquatic species. However, high-density markers will increase genotyping costs and require complex mathematical calculations. To reduce costs and simplify mathematical calculations, it is a reasonable step to select a few SNPs in the major genes that have a high correlation associated with specific traits (Wang *et al.*, 2022). Few SNPs on major genes as biomarkers of disease resistance selection have been successfully applied in many fields of agronomy. An example is the selection of salinity-resistant rice using SNP g-1975 A>G in OsRR22 (hst1) gene with genotype AA as salinity-resistant (Rana *et al.*, 2019) and citrus resistant to Alternaria brown spot (ABS) using SNP08 g-25862085 G>T in chromosome III with TT genotype as ABS-resistance (Cuenca *et al.*, 2016). In aquaculture breeding, selection has developed using a few SNPs on major genes related to specific traits. Marker development for the selection of *Sinonovacula constricta* resistant to *Vibrio parahaemolyticus* infection using two SNPs in the ABC transporter genes coding regions (CDS) that were filtered from 2 million SNPs and only Unigene0039666 A>G, which is on CDS with p-value of 0.012 (Zhao *et al.*, 2021). Another example of using SNP markers on major genes is the selection of mandarin fish *Siniperca chuatsi* resistant to ISKNV using the SNP g-1625 C>T in the IL-6 gene (Jin *et al.*, 2021).

In the preliminary study, the ALF gene of resistant and susceptible Pacific white shrimp to WSSV was sequenced in 2018. It concluded that the SNP g.455 A>G has the potential as a molecular marker for WSSV-resistant traits. However, further research must be conducted to evaluate SNP g.455 A>G as a molecular marker. This study aims to detect the variation of SNP g.455 A>G in the ALF gene of white shrimp with an applicable method, evaluate the correlation of SNP markers in the ALF gene with shrimp resistance to WSSV infection, analyze the level of expression of immunity genes and genotypes of the population of vannamei shrimp resistant to WSSV, and analyze inheritance of SNP markers in the first generation of selected Pacific white shrimp.

2. Materials and Methods

All animal experimental and rearing procedures were handled and complied with animal welfare under national accreditation no. SNI 7311:2009 & SNI 8037.1:2014 of Republic of Indonesia.

2.1 Shrimp Population

This research was conducted from October 2021 to July 2022 at the National Broodstock Centre for Shrimp and Mollusca (BPIU2K) Karangasem, Bali. The specific pathogen-free (SPF) shrimp population of ten families were obtained from BPIU2K. Ten shrimp families (FA−FJ) and one control family (FK) with 140 days of culture (DOC140) were reared separately and called G_0 or founder generation. A commercial feed with 36% protein was given five times a day at satiation until the G_0 population became a mature broodstock. Three families with the best growth and survival were selected and spawned to produce the first generation (G_1) shrimp.

2.2 Founder Generation (G_0) Genotype

SNP detection was carried out using the tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. ARMS-PCR was conducted on three families with the best growth and survival, and one with the lowest WSSV resistance (n=120; 30 shrimps/family). ARMS-PCR primers (Table 1) were designed using the Primer1 program, accessed online at http://primer1.soton.ac.uk/primer1.html (Collins and Ke, 2012).

The gDNA was extracted using the gSYNC™ DNA Extraction Kit (Geneaid, Taiwan) according to the kit instructions. Amplification was carried out using the MyTaq™ HS Red DNA Polymerase (Bioline, UK) enzyme and optimized on the SimpliAmp 96-Well Thermal Cycler (Applied Biosystems, USA) with a program: 95 °C for three minutes, then 35 cycles consisting of 95 °C for 15 seconds, 57°C for 15 seconds, and 72°C for 90 seconds. The 20 µL PCR components consisted of 4 µL MyTaq Red Reaction Buffer, 0.4 µL MyTaq HS Red DNA Polymerase, 2 µL of 6 µM Outer R’ primer, 2 µL of 4 µM other primers, and 1.8 µL of DNA template. The PCR products were evaluated using the electrophoresis method on the Owl Easy Cast B1 Mini Gel Electrophoresis System (Thermo Scientific, USA) using 1.5% agarose gel for 90 minutes at a voltage of 75 Volts.

2.3 The Correlation of SNP g.455 A>G and WSSV Resistance Trait

The correlation between SNP g.455 A>G in the ALF gene and the resistance of white shrimp to WSSV was obtained by comparing the frequency of the SNP (AA, AG, and GG) and the survival rate (SR) after the WSSV challenge test using the LD70.

The challenge test was carried out by taking shrimp from each G_0 family separated from the stock and reared in 50 L containers with ten fish per container. Shrimp were injected with 100 µL of LD70 WSSV filtrate (4×10^2 copies/µL) and 100 µL of 0.9% NaCl as a control. The challenge test was carried out with three replications. Shrimp were reared for 96 hours post-infection. During observation, commercial pellet feed (36% protein content) was given four times a day, as much as 5% of the total biomass. Shrimp mortality was observed every hour, and dead shrimp were removed from the rearing container.

2.4 Immune Gene Expression

The immune-related gene expression levels were analyzed to evaluate the differences between resistant and non-resistant populations. A total of 50 Pacific white shrimps were used for each family with the highest SR FI (96.67 ± 3.33%) and the lowest SR FC (30 ± 20%). Gill tissue from three individual surviving shrimp every hour was collected after LD70 WSSV injection at 0 hours (before WSSV injection), 20th, 40th, 60th, 80th, and 100th hours post-injection. Total RNA was extracted from shrimp gill according to the GENEzol Reagent (Geneaid, Taiwan) product manual. Total RNA was dissolved in 100 µL nuclease-free water (NFW). The purity and concentration of total RNA were measured using spectrophotometry at 260 nm and 280 nm wavelengths. Complementary DNA (cDNA) synthesis was carried out using the RevertraAce ® qPCR RT Mastermix kit with gDNA remover (Toyobo, Japan). The genes evaluated in this study were anti-lipopolysaccharide factor (ALF), superoxide dismutase (SOD), and prophenoloxidase (ProPO) genes (Table 2). The elongation factor 1 alpha (EF1α) gene was used as the internal control (Table 2). The genes expression was analyzed using the quantitative real-time polymerase chain reaction (qPCR) method in a 7,500 fast RealTime-PCR machine (Applied Biosystem, USA) using the Sensi FAST™ SYBR Lo-ROX Kit (Bioline, UK). qPCR was performed in 20 µL and consisted of 1 µL cDNA, 10 µL 2x Sensi FAST™ SYBR Lo-ROX mix, 0.8 µL of 10 uM forward primer, 0.8 µL of 10 uM reverse primer, and 7.4 µL of NFW. The qPCR temperature was optimized using the following program, pre-denaturation at 95°C for two minutes followed by 40 cycles, denaturation from 95°C for five seconds, annealing at 60°C for 10 seconds, extension at 72°C for 15 seconds, and final extension at 72°C for seven minutes. Relative gene expression levels were evaluated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

2.5 First Generation (G_1) Shrimp Production and Inheritance analysis of SNP marker

Three families of shrimp populations with the highest growth and survival rate of G_0 were used as broodstock candidates with family codes FF, FG, and FI, with average daily growth (ADG) of 0.185 g/day, 0.204 g/day and 0.185 g/day, respectively. The G_1 shrimp population was generated from a random reciprocal cross-mating between three G_0 families (Table 3).

The G_1 shrimp were kept in one rearing tank until they reached the post-larvae (PL15) stage. A total of 30 individuals were taken randomly from the G_1 population for SNP detection. Marker inheritance analysis was carried out by comparing the SNP frequencies in G_0 and G_1 based on the Hardy-Weinberg equilibrium.

2.6 Statistical Analysis

The research data were processed and analyzed using Microsoft Excel 2019 and the Minitab 18.1 program (Minitab, USA). The survival rate (SR) of G_0 shrimp populations after WSSV injection was compared using one-way ANOVA. The correlation between molecular markers genotype (AA, AG and GG) and resistance to WSSV traits were evaluated using the general linear model (GLM). The inheritance of SNPs was analyzed using chi-square ($\chi 2$) with 1 degree of freedom (binomial) referring to Hardy-Weinberg equilibrium. The results of the quantification of gene expression for each population G_0 at 0 hours (before WSSV injection), 20th hours, 40th hours, 60th hours, 80th hours, and 100th hours post-injection, were analyzed using one-way ANOVA ($p<0.05$) followed by Fisher's posthoc test.

3. Results and Discussion

3.1 Development of a simple method for detection of a g.455 A>G SNP

SNP g.455 A>G in the ALF gene was successfully detected at 57 °C annealing temperature.

Table 1. Primer sequences for the detection of SNP g.455 A>G in the ALF gene of white shrimp

Primer	Sequence (5' – 3')	Allele PCR band size
Set-1		
Inner_A F'	GCAGGACTTCGTCAGGAAAGCTTGCA	A/A: 248 bp
Inner_G R'	TCTGATTCGGTGATGAGACCCGCTCC	G/G: 120 bp
Outer F'	TATACTAACCCTTTCGCTCCCACCCACAGC	A/G: 248 and 120 bp
Outer R'	TGGATGAGGTATCAACATTCGCGGAAGAA	

Table 2. qPCR primer sequences of vannamei shrimp immunity genes

Primer	Gen	Sequence (5' – 3')	Reference
nLvALF2 F'	ALF	GCGAACAAACTCACTGGACTG	(Wang *et al*., 2021)
nLvALF2 R'		ACATGCGACCCTGGAATACAG	
cMnSOD F'	SOD	CGTAGAGGGTATTGTCGT	(Zhou *et al*., 2010)
cMnSOD R'		TTGAAATCATACTTGAGGG	
proPO F'	ProPO	TCTTCGCCTCACGCATCTC	(Wang *et al*., 2021)
proPO R'		TATCCTCACAGTCACCTCCTTC	
LvEf F'	EF1α	CTGTGGTCTGGTTGGTGTTG	(Rubio-Castro *et al*., 2016)
LvEf R'		TCAGATGGGTTCTTGGGTTC	

Table 3. Matrix of interfamily reciprocal crosses from three selected G0 shrimp families, each 100 individuals

G0 Families	♀FF	♀FG	♀FI
♂FF	♀FF×♂FF	♀FG×♂FF	♀FI×♂FF
♂FG	♀FF×♂FG	♀FG×♂FG	♀FI×♂FG
♂FI	♀FF×♂FI	♀FG×♂FI	♀FI×♂FI

PCR products of the G and A alleles are 120 bp and 248 bp, respectively (Figure 1). The key to detecting SNP using the ARMS-PCR method was influenced by the reagent concentration, annealing temperature, percentage of GC region, and SNP type (Collins and Ke, 2012; Medrano and De Oliveira, 2014).

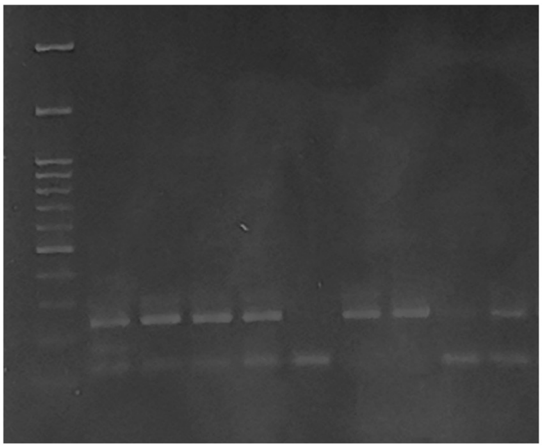

Figure 1. The SNP g.455 A>G in the ALF gene amplification results

The ARMS-PCR method is based on the principle of bi-directional PCR amplification using two pairs of primers that can amplify two different alleles in one operation (Rincón and Medrano, 2003). The primer used has a mismatch at the 3' terminal base, so the Taq DNA polymerase reaction will slow down or even stop. Specific inner and outer primers in the ARMS-PCR tetra-primer allow for obtaining different bands of each SNP when electrophoresis is performed (Medrano and De Oliveira, 2014; Zhang *et al*., 2015).

Several methods can detect SNP, including sequencing, colorimetric mutector assay, allele discrimination real-time PCR, SYBR-green high-resolution melting (HRM) analysis, COLD PCR, and restriction fragment length polymorphism (RFLP). These methods have several limitations, such as the high cost, because they require additional materials and tools such as restriction enzymes, fluorescent materials, probe primers, and real-time PCR. Another limitation is that it requires a reasonably complex procedure because it requires optimization after the PCR amplification reaction (Huang *et al*., 2013). The ARMS-PCR method can reduce the complexity of the work, is relatively low in cost, and has high sensitivity so that it can be used routinely to detect SNPs in marker-assisted selection programs (Huang *et al*., 2013; Ehnert *et al*., 2019).

3.2 Founder generation (G_0) genotype

The results of SNP observations in four G_0 families showed that the FC family, with the lowest SR, has the lowest frequency of AA genotype compared to other selected families (Table 4). In the analysis of the similarity of genotypes between families using chi-squared with a degree of freedom of 1, it was found that the genotype of the FC family was significantly different from the combination of other selected families at α= 0.05.

Table 4. The observed genotype and allele frequencies of G0 shrimp families

Family code	Observation genotype frequency			Allele frequency	
	AA	AG	GG	A = p	G = q
FC	0.18	0.41	0.41	0.39	0.61
FF	0.62	0.34	0.04	0.79	0.21
FG	0.27	0.53	0.20	0.53	0.47
FI	0.22	0.44	0.34	0.42	0.57

Description: Family code is an alphabetical code given to 10 families of G0 shrimp. FC is the C shrimp family, FF is the F shrimp family, FG is the G shrimp family, and FI is the I shrimp family.

3.3 The Correlation of SNP g.455 A>G and WSSV resistance trait

The correlation was carried out by comparing the genotype frequency (AA, AG, and GG) to the survival rate (SR) in three selected families and one family with the lowest SR (n= 120). Disease resistance markers were identified in population groups with significant phenotypic differences to identify the highly correlated markers. Such as the study by Zhao *et al*. (2021), which used 15 heads of razor clams for each group with different resistance to *V. parahaemolyticus* infection. However, the greater number of families used the more sensible results.

The survival rate (SR) after WSSV infection showed that the FC family shrimp had the lowest SR and was significantly different from other families (Figure 2). A correlation p-value of genotypes AA, AG and GG was 0.012, 0.359, and 0.001. The frequency of the GG genotype has the highest correlation significant level (r = -0.908; p-value = 0.001) and R-square = 82.52%, which indicated that 82.52% of SR change was influenced by GG genotype frequency (*q*2). A strong negative correlation coefficient of GG genotype frequency (*q*2) and SR indicated that the GG genotype carries susceptible WSSV traits; the higher the GG genotype in the population will lower the resistance to WSSV infection.

Therefore, the association between SNP genotypes and lifespan can be analyzed for each shrimp individually. However, the lifespan of surviving shrimp cannot be obtained at the end of the study. Association between SNP genotypes and lifespan may be made if higher doses of WSSV are used, and all shrimp die at the end of observation.

3.4 Immune Gene Expression

Gene expression analysis was performed in the FI and FC families to evaluate differences in gene expression levels in the two populations. The gene expression analysis showed that the ALF gene expression level in the FI shrimp family was higher than in the FC shrimp family at the 40th and 60th hours post-WSSV infection, which was suspected by the role of the g.455 A>G SNP in the ALF gene (Figure 3). Robert and Pelletier (2018) stated that SNPs could cause changes in expression levels, but the protein's function does not change if the tertiary structure of the protein does not change. The SNP g.455 A>G in the ALF gene occurs in the exon but does not change the tertiary structure based on the model predictions from swissmodel.expasy.org (Figure 4). Moreover, researchers speculate that a synonymous SNP in CDS may influence protein expression by slowing down the translation process (Jin *et al*., 2021). Further research is needed to verify an association analysis between genotype and gene expression levels.

SOD gene expression of the FI shrimp family was also higher than the FC shrimp family at the 40th, 60th, 80th, and 100th-hour post-WSSV infection (Figure 3). That was in line with (Ji *et al*., 2011) research which showed that SOD gene expression increases as a defense mechanism against WSSV infection. Reactive oxygen intermediate (ROI) and reactive oxygen species (ROS) increased in the presence of oxidative stress or pathogens such as WSSV. The effect of increasing ROI and ROS is minimized or eliminated by SOD, and this process can destroy pathogens effectively. SOD gene expression increased after WSSV infection, peaked at the 80th hour post-WSSV infection, and decreased the next hour. These results were similar to previous studies; the expression of the SOD gene will increase as a defense mechanism against WSSV acute infection and decrease after prolonged infection (Ji *et al*., 2011; Miranda-Cruz *et al*., 2018). Shrimp maintained a dynamic equilibrium

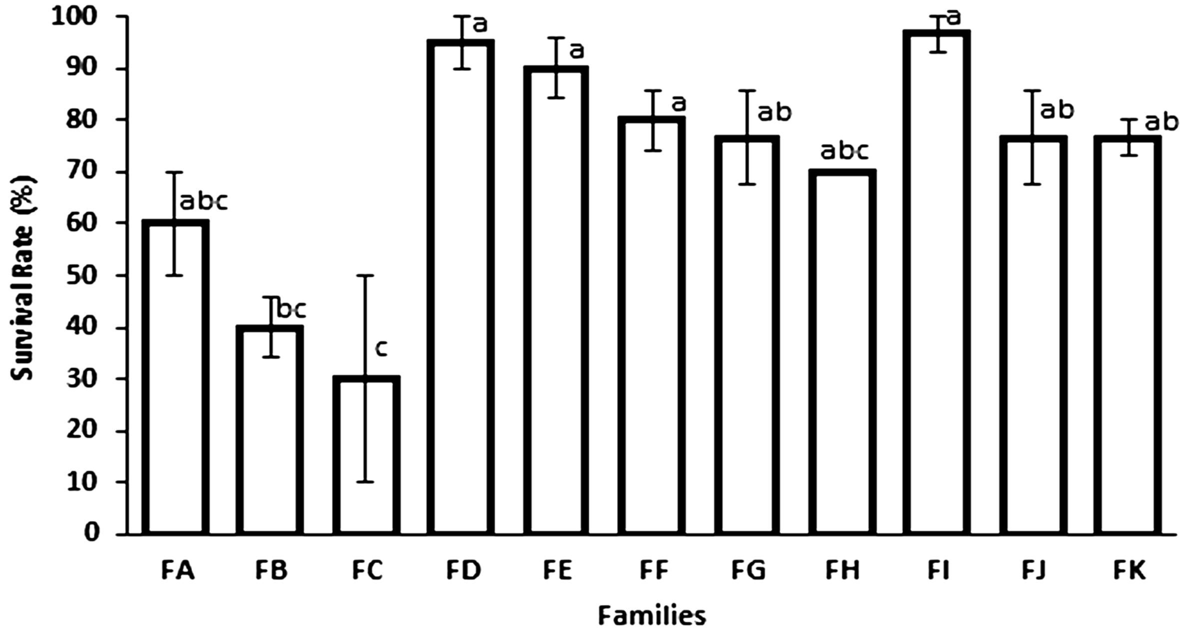

Figure 2. Survival rate (SR) of shrimp families after WSSV infection for 96 hours of observation. FA is the A shrimp family, FB is the B shrimp family, and so on until FJ is the J shrimp family, and FK is the control family. Different letters indicate a significant difference in ANOVA (α = 0.05) with Tukey's posthoc test. The data is presented as the mean of SR (n = 30), and the vertical line (bar) is the standard error of the mean (SEM).

between WSSV and SOD activities (Ning *et al*., 2016), and SOD gene expression decreased because WSSV is deploying effectively, so very few WSSVs could attack the SOD immune system (Chen *et al*., 2016).

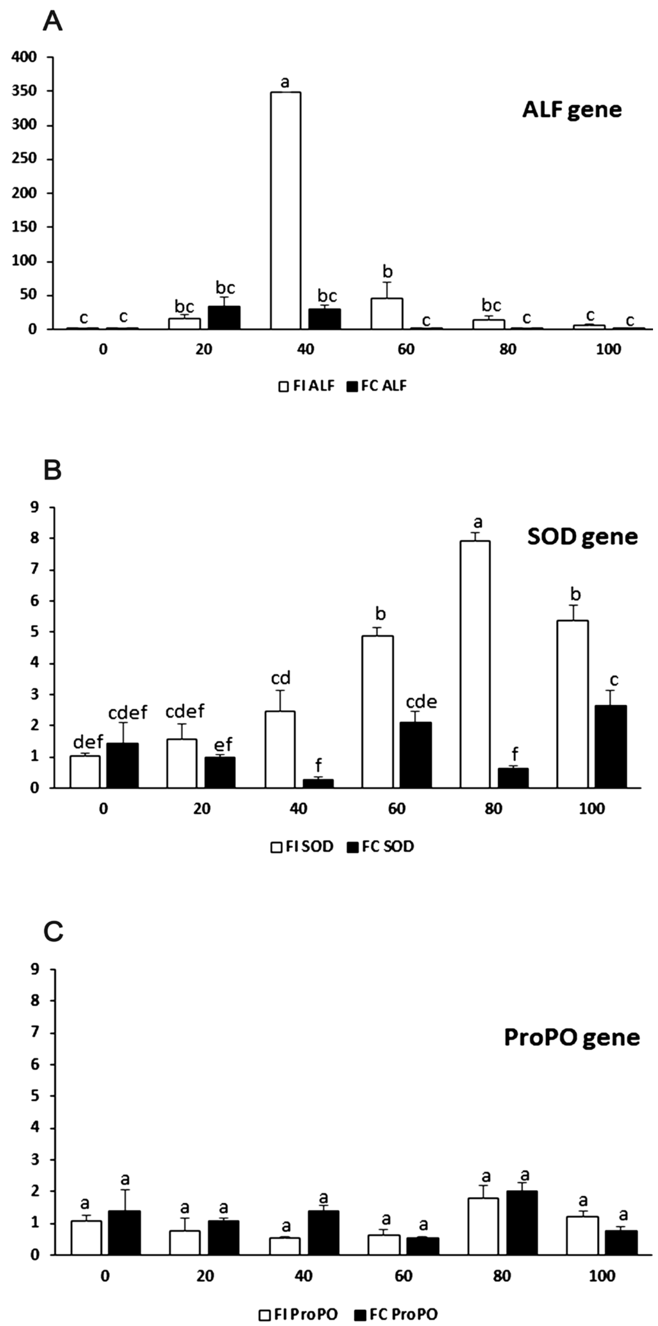

Figure 3. The expression level of immunity genes after WSSV infection in the FI and FC shrimp families. (A) ALF gene expression, (B) SOD gene expression, and (C) ProPO gene expression. The x-axis shows the 0th-hour, 20th-hour, 40th-hour, 60th-hour, 80th-hour, and 100th-hour post-infection (p.i), the relative expression levels of ALF, SOD, and ProPO genes present in the y-axis. Different letters indicate a significant difference in ANOVA (α = 0.05) with Fisher's posthoc test. The data is presented as the mean of the gene expression (n = 3), and the vertical line (bar) is the standard error of the mean (SEM).

ProPO gene expression was not significantly different (Figure 3). The ProPO system is essential in protecting shrimp from the pathogen, especially for melanin production, adhesion, encapsulation, and phagocytosis. The results of the ProPO gene expression after WSSV infection in this study differed from Ji *et al*. (2011) research, which mentioned an increase in the ProPO gene expression after WSSV and AHPND infection. Ji *et al*. (2011) study used two different types of pathogens, namely viruses and bacteria, so it should be suspected that the activity of the ProPO gene is for bacterial infection defense.

WSSV infection causes NF-kb to activate and initiate transcription factors such as dorsal, thereby increasing the expression of effector genes such as antimicrobial peptide or AMP, like the ALF gene (Li *et al*., 2019a, 2019b). ALF itself is critical to be used to defend against pathogens such as WSSV. Therefore, the expression of the ALF gene had a rapid increase earlier than the expression of the SOD gene.

The defense mechanism and activity of the ALF gene as a WSSV antiviral is through apoptosis. The increase in ALF gene expression was followed by an increase in apoptosis. According to Guo *et al*. (2013b, 2017) study, the increase in ROS is in line with the apoptosis that occurs. SOD eliminated the increases of ROS as a defense system against WSSV infection (Ji *et al*., 2011). It can be seen from this study of gene expression that the peak of SOD expression occurred after the peak of ALF expression. It is suspected that the performance of ALF triggers an increase in the SOD gene via apoptosis, as previously described.

WSSV is suspected of having a strategy to avoid the ProPO defense mechanism. In the process of WSSV replication, WSSV also secretes proteins that can increase Serine proteinase activity and inhibit ProPO activation (Flegel and Sritunyalucksana, 2011; Li *et al*., 2019b). It supports this research results of the ProPO gene expression, which showed no increase after WSSV infection, and also, there was no significant difference in the relative expression of the ProPO gene in resistant and susceptible shrimp.

3.5 Inheritance of SNP g.455 A>G in the First Generation (G_1)

SNP g.455 A>G in the ALF gene are detected in all G_1 samples (n=30). Chi-square analysis ($\chi 2$) with α = 0.05 and degree of freedom (df) = 1, shows that all broodstock and fry populations of white shrimp are in Hardy-Weinberg equilibrium (Table 5). The Combined

Table 5. Expected frequencies and Hardy-Weinberg equilibrium of broodstock and PL shrimp populations

Family code	Allele frequency		Hardy-Weinberg genotype frequency			Hardy-Weinberg equilibrium	
	A = *p*	G = *q*	*p2*	*2pq*	*q2*	χ2	*p-value*
FF Broodstock	0.79	0.21	0.63	0.34	0.04	0.07	0.785
FG Broodstock	0.53	0.47	0.28	0.5	0.22	0.15	0.696
FI Broodstock	0.42	0.57	0.20	0.49	0.31	0.27	0.603
Combined Broodstock	0.59	0.41	0.35	0.48	0.17	0.62	0.433
G1 PL	0.55	0.45	0.30	0.50	0.20	2.34	0.126

Description: Broodstock Family Code is an alphabetical code given to 10 shrimp families G0. The FF Broodstock is the FF shrimp family, the FG Broodstock is the FG shrimp family, and the FI Broodstock is the FI shrimp family. The Combined Broodstock is the genotype of the broodstock populations FF, FG, and FI. The G1 Fry is the shrimp fry produced from the FF, FG, and FI broodstock.

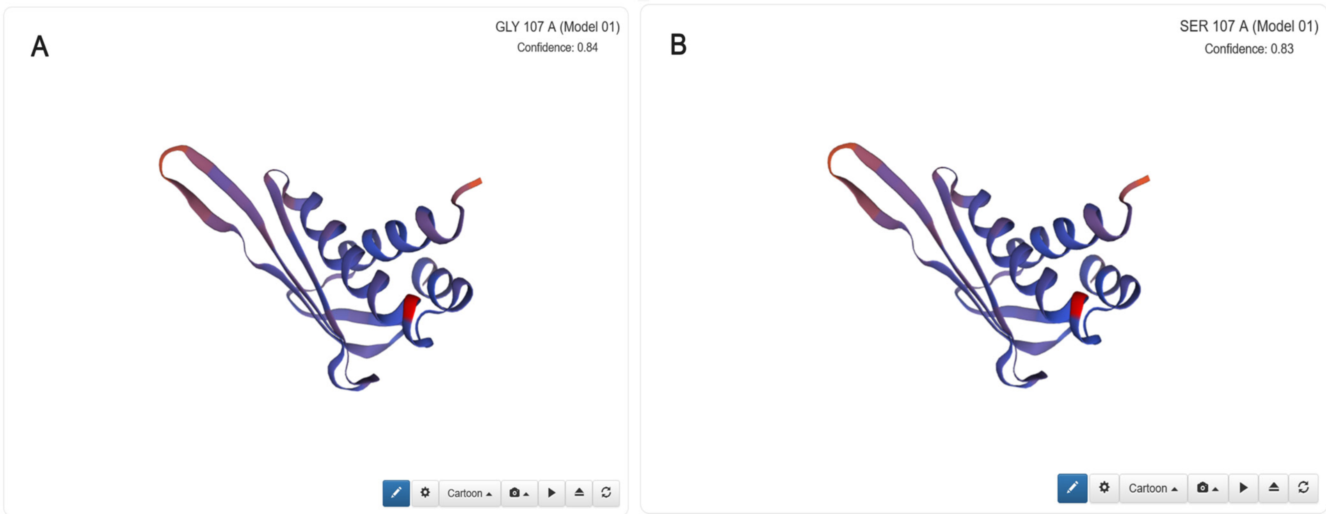

Figure 4. The tertiary protein structure prediction of the g.455 A>G SNP. The image on the left (A) for SNP g.455 G produces Glycine, and on the right (B) for SNP g.455 A produces Serine. There are no differences in tertiary structure prediction and no change in the function of the anti-lipopolysaccharide factor (ALF) protein produced.

Broodstock is the genotype of the broodstock population of the FF family, FG family, and FI family. Allele and genotype frequency are analyzed based on the Hardy-Weinberg equation.

According to the Hardy-Weinberg law, a population in Hardy-Weinberg equilibrium will have the same allele frequency and genotype frequency from generation to generation if there is random mating in a large population (Griffiths *et al*., 2015). The total of broodstock can be concluded based on the population's breeding number (Ne) for the allele frequency 0.01 in the 1st generation with p-value = 0.05, which is 150. With the ratio of males and females 1:1, it takes a minimum of 75 male and 75 female broodstock (Tave, 2016) to comply with the Ne value, and the Hardy-Weinberg law can be applied to predict the genotype frequency of the seeds produced. The genotype frequency of G_1 shrimp and the combined broodstock was similarly based on chi-square (χ2) analysis with α = 0.05 and df = 1. The χ2 = 0.46 and p-value = 0.497 was obtained. Further research is needed to determine the accuracy of the correlation between SNP g.455 A>G of G_1 shrimp by conducting a WSSV challenge test on G_1 shrimp and comparing it with genotype data. The accuracy of molecular markers for disease resistance across generations has also been reported in salmon (*Salmo salar*) (Fraslin *et al*., 2022). Furthermore, the accuracy of SR predictions can be verified by mating to generate all AA for a few families and all GG for a few families. These families are then tested for WSSV survival rates.

4. Conclusion

This study successfully detects single nucleotide polymorphisms (SNP) g.455 A>G in the ALF gene using the tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. SNP g.455 A>G in the ALF gene has a strong correlation with the survival rate (SR) after LD70 WSSV infection. The WSSV-resistant Pacific white shrimp population has significantly higher ALF and SOD gene expression levels and has a higher AA genotype than the WSSV-susceptible shrimp population. The SNP marker was stably inherited in the first generation following the Hardy-Weinberg law. These results indicate that the SNP g.455 A>G is a potential molecular marker for WSSV-resistance traits, and genotype AA is an SNP marker for selecting Pacific white shrimp resistant to the white spot syndrome virus. This study suggests selecting the WSSV-resistant shrimp population using the SNP marker g.455 A>G in the ALF gene to generate a homozygous AA population and challenge it with WSSV to confirm the marker accuracy.

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Authors' Contributions

All authors have contributed to the final manuscript. Each author's contribution is as follows, BRB; reared the shrimp, collected the data, drafted the manuscript, and designed the table & graph. AMD, DTS, and SN; devised the main conceptual ideas, analyzed, and evaluated the final data, and made critical revisions to the manuscript. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

All the authors declare that they have no competing interests upon the publication of this paper.

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