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Identification and Molecular Analysis of Hemocyanin as a Body Resistance Gene in *Litopenaeus vannamei*

Talitha Amanda[1*], Yuni Kilawati[2], Maftuch[2]

[1] Master Degree Program in Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia.
[2] Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia.

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Corresponding Author:
Talitha Amanda
talithadirwanto@gmail.com

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Abstract: Production of white shrimp (*Litopenaeus vannamei*) tends to decrease every year caused by diseases, one of which is White Feces Disease (WFD). Genetically, monitoring has been carried out on the parent characters of SPF (Specific Pathogen Free) shrimp imported from Hawaii and Florida with low genetic variation. If there is a threat such as disease caused by bacteria and the gene that codes for disease control or the body's resistance gene is missing, it allows the individual to become susceptible to disease. Gene that codes for body resistance in white shrimp is hemocyanin. Hemocyanin gene studies are needed as a first step in controlling disease in white shrimp. Method used is a descriptive method. Procedures carried out are sample collection, detection of WFD, molecular analysis of hemocyanin and data analysis. Results showed that healthy shrimp had hemocyanin while shrimp with WFD had very little or no amount of hemocyanin. Hemocyanin gene profile in healthy shrimp was also analyzed and there were 4 nucleotide change made changes to 4 amino acid. Changes in amino acids affect the stability of hemocyanin and cause polymorphism of hemocyanin. Phylogenetic analysis showed dendogram split into two clades between *L. vannamei* and *P. monodon*.

Keywords: Body resistance; Gene; Hemocyanin; *Litopenaeus vannamei*; White feces disease

Introduction

White shrimp (*Litopenaeus vannamei*) according to Wei et al. (2014) and Inayah et al. (2023) is one of the most important fishery species economically and market needs because it is widespread throughout the world. Kumar et al. (2022) stated that the most important problem in decreasing the production of white shrimp (*Litopenaeus vannamei*) farming is disease attacks. Examples of diseases that most commonly attack white shrimp include White Feces Disease (WFD) caused by *Vibrio parahaemolyticus* (Sugiharta et al., 2023). Environmental conditions also play an important role in the growth of white shrimp (Pratiwi et al., 2023; Rahmi et al., 2023).

Genetically, monitoring has been carried out on the parent characters of SPF (Specific Pathogen Free) shrimp imported from Hawaii and Florida have homozygous genetic characters or low genetic variation. In Hawaii and Florida, this brood is the 8th filial (F8) which was later imported into Indonesia and then known as F0. Breeding can caused low genetic variation (Yamin et al., 2022).

Low genetic variation is caused because the mother white shrimp is the result of selective breeding from Hawaii and Florida. This is in accordance with the statements of Wyban (2019) and Aramburu et al. (2020) that breeding with selective breeding has a risk of decreasing the value of genetic diversity because the expected traits inherited from the parent are few and uniform so that the offspring do not have diverse genetic traits (Kang et al., 2024). In the process of cultivation by selective breeding there may be genes coding for a trait that are missing. If there is a threat such as disease

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caused by bacteria, viruses or parasites and the gene that codes for disease control or the body's resistance gene is missing, it allows the individual to become susceptible to disease.

In white shrimp (*L. vannamei*) there is a gene that codes for body resistance namely the hemocyanin gene. The hemocyanin gene is found in the hepatopancreas organ of shrimp which helps maintain shrimp immunity and the process of oxygen transport (Yudiati et al., 2024). The hemocyanin gene is also antiviral and antimicrobial (Aweya et al., 2021). However, in reality, molecular hemocyanin gene information in GenBank is still not widely found. Polymorphism of hemocyanin in white shrimp has also not been studied much (Hassan et al., 2022).

Polymorphism analysis in hemocyanin genes is needed to determine the diversity of hemocyanin genes and the specification of nucleotide base sequences in shrimp bodies. This research is needed to explore the hemocyanin gene as the first step in disease control in *L. vannamei*.

Method

Location and Time

This research was conducted from June 2023 to September 2023 at Central Laboratory of Life Sciences, University of Brawijaya.

Sample Collection and Preparation

Samples were taken as many as 10 individuals of shrimp with hepatopancreatic characteristics not soft and light in color (indication the shrimp is healthy) and shrimp with morphological diagnosis of White Stool Disease (WFD) with flaccid and pale hepatopancreas (Kumar et al., 2021).

White Feces Disease (WFD) Detection using Polymerase Chain Reaction (PCR)

WFD detection using the PCR refers to Anissa et al. (2024) research that detecting shrimp disease with PCR method. PCR method begins with DNA extraction or isolation according to the procedure from the Promega kit, followed by amplification using PCR, after which a 50-volt electrophoresis process is carried out within 40 minutes and visualization using the UV Transilluminator Gel Documentation.

Isolation of RNA

RNA isolation was carried out based on the handbook guide of Qiagen's QIAzol® Lysis Reagent kit (Lederer et al., 2024). Hepatopancreas weighed 0.1 grams then given Qiazol as much as 1000 µl and centrifuged at a rate of 12000 x g for 15 minutes at 4°C. The sample was then given chloroform as much as 200 µl, incubated at room temperature for 3 minutes and centrifuged again at a speed of 12000 x g for 10 minutes at 4 °C. The sample was then given 500 µl isopropanol and 1000 µl 75% ethanol and recentrifuged at 7500 x g for 5 minutes at 4°C. The sample was dried and given 50 µl of nuclease free water. The isolation results were then tested for the quantity and quality of RNA with a nanodrop spectrophotometer on the A260/280 absorbance wave to see the concentration and purity.

Reverse Transcription

The reverse transcription process is carried out according to the kit to be used, namely ReverTra Ace™ qPCR RT Master Mix with gDNA Remover from Toyobo (Mo et al., 2012). Samples were incubated 5 minutes at 65°C and given 4x DNA Master Mix and gDNA remover. The sample was again incubated at 37°C for 5 minutes and put into ice and given 5x DNA Master Mix and then incubated again at 27°C for 5 minutes, 50°C for 5 minutes and 98°C for 5 minutes. The cDNA is then tested for quality and quantity using a nanodrop spectrophotometer just like RNA.

Amplification of cDNA

Amplification process refers to Rajalakshmi (2017). Denaturation is carried out at 95°C. The second process is annealing or pasting. The annealing process is primary attachment with a cDNA template resulting from the denaturation process. The annealing process is carried out at a temperature range of 59°C within 30-45 seconds. The last process is the extension or elongation of DNA strands.with a temperature of 72°C. The primers used are forward Hemocyanin_115F24 primer (5'-CAT ATG GAA TTC CCM TTC TGG TGG-3') and reverse Hemocyanin_1673R21 primer (5'-GGG ATC ACG GGT GGC AGT TTC-3').

Electrophoresis

Electrophoresis for cDNA visualization refers to Kusumaningrum et al. (2014) was performed using 1.5% agarose media at 50 volts for 55 minutes and then visualized with Gel Documentation UV Transilluminator.

Sequencing

Sequencing process is carried out by the Sanger method (Lokapirnasari et al., 2017) by preparing samples and sending DNA samples to PT. Genetics Science Indonesia which will proceed to 1st BASE DNA Sequencing in Singapore.

Data Analysis

Analysis of electropherogram data from sequencing results and then processed in several applications The first step is to make improvements to

the eceltropherogram using Chromas v2.6.6. The forward and reverse eceltropherograms cut the noise section at the beginning and end of the eceltropherogram. Noise itself is a stacked peak on the eceltropherogram. The base is cut along about 18-20 bp, if exceeded, it is feared that DNA information data can be lost. Once the eceltropherogram is fixed using Chromas v2.6.6, the next step is to combine the forward and reverse electropherograms to form a consensus using the UGENE v1.31.1 application. Consensus results are saved in files with FASTA format. After the UGENE process, a dataset is created in the Microsoft Word application and combined with comparison data obtained from GenBank. Hemocyanin sequence profile analysis using BioEdit software for alignment and analyzed nucleotide base mutations and amino acid changes (Mahmoodi et al., 2018) from *L. vannamei* hemocyanin sequences. Data were compared with 3 *L. vannamei* hemocyanin sequences and 3 *Penaeus monodon* hemocyanin sequences from the National Center of Biotechnology Information (NCBI) Gen Bank. Phylogenetic analysis was performed using MEGA 11 software to visualize hemocyanin mutations with a dendogram refers to Tamura et al. (2021) with the neighbor joining analysis method which has the principle of determining phylogenetic tree topology, branch length and evolutionary models.

Result and Discussion

White Feces Disease (WFD) Detection using Polymerase Chain Reaction (PCR)

Molecular diagnosis of White Feces Disease (WFD) uses the Polymerase Chain Reaction (PCR) method to ensure that the sample is diagnosed WFD or not. Primers used is specific primer *Enterocytozoon hepatopenaei*. *Enterozytozoon hepatopenaei* primers can used as first detect of White Feces Disease (WFD) in *Litopenaeus vannamei* (Alfiansah et al., 2020). Primer forward spefication is EHP 510F (5′-GCCTGAGAGATGGCTCCCACGT-3′) and primer reverse specification is EHP 510R (5′-GCGTACTATCCCCAGAGCCCGA-3′). The primer used is a design primer with the addition of immunostimulants as a booster in white shrimp and for the detection of hemocyanins. PCR results are shown by Figure 1 (for health shrimp) and Figure 2 (for WFD-diagnosed shrimp).

Based on the Figure 2, there are two controls used, namely negative control (K-) and positive control (K+). Negative control is used as a reference that a sample is negative WFD or undiagnosed WFD. Conversely, a positive control describes a positive sample exposed to WFD (Kim et al., 2023). Samples with W code indicate samples with WFD diagnosis at the time of morphological analysis and based on PCR results, all W samples are proven to be WFD diagnosed because there is a band that is parallel to the positive control. Sample S indicates a healthy sample seen on when morphological analysis and PCR results show none. The band is the same as the negative control so it is concluded that all S samples are not diagnosed WFD. The length of the resulting base is estimated at ±100 bp. WFD is caused by *Vibrio parahaemolyticus* infecting the hepatopancreas.

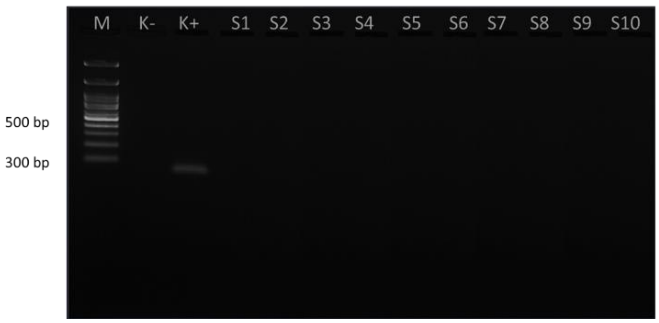

Figure 1. PCR result of health shrimp (S); M: marker, K-: negative control, K+: positive control

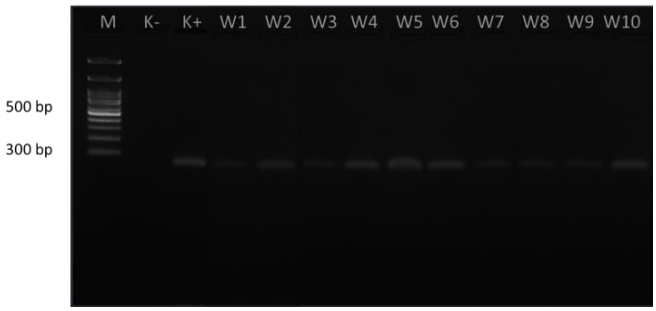

Figure 2. PCR result of WFD shrimp (W); M: marker, K-: negative control, K+: positive control

Quality and Quantity Test of RNA and cDNA

In this study, the quality and quantity test of RNA and cDNA isolation results was used to determine the concentration and purity of the isolate obtained. The quality test of RNA isolation results was carried out using a nanodrop spectrophotometer at wavelengths A260 nm and A280 nm. The absorbance value at the wavelength ratio A260/A280 indicates the purity value of the insulation result., The quantity test of RNA isolation results was also carried out with a nanodrop spectrophotometer where the RNA concentration was obtained from the multiplication between the absorbance value at a wavelength of 260 nm, constant and dilution factor (A260 x 50 x 1). Results are shown in Table 1 and 2.

Based on the results in the Table 1, the RNA purity value of healthy samples ranges from 1.71 – 1.99. The RNA concentration of healthy samples ranged from 636 ng/µl – 2310 ng/µl. RNA concentrations in shrimp with WFD diagnosis ranged from 552 – 2240 ng/µl and purity

values ranged from 1.71 – 1.98. Based on the data above, it can be concluded that the RNA concentration results have met the standard, which is above 100 ng / µl and all samples have good RNA purity (1.7 – 2.0) so that it can be said that the RNA obtained is pure RNA isolate (Liu et al., 2022). The low purity value of RNA is caused because in the RNA isolate obtained there are still lipids, proteins and polysaccharides. While the purity value of RNA that exceeds 2.0 indicates that there are contaminants in the form of DNA in it. Low RNA concentration values can be caused by imperfections in RNA isolation processes such as RNA precipitation so that there is no RNA in the supernatant (Maharana et al., 2018).

Table 1. Quality and Quantity Test of RNA

Sample	RNA	
	Concentration (ng/ul)	Purity(A260/280)
S1	1,536	1.93
S2	745	1.99
S3	636	1.91
S4	2,160	1.76
S5	1,299	1.97
S6	782	1.98
S7	2,261	1.73
S8	1,259	1.94
S9	636	1.91
S10	2,310	1.71
W1	2,186	1.73
W2	1,629	1.90
W3	882	1.79
W4	2,240	1.71
W5	2,160	1.82
W6	1,445	1.98
W7	2,094	1.85
W8	552	1.81
W9	597	1.92
W10	1,514	1.98

Based on the results in the Table 2, the cDNA concentration value of healthy samples ranges from 1.717 – 1.870 ng/µl. The cDNA purity of healthy samples ranges from 1.7 – 1.75. The concentration of cDNA in shrimp with WFD diagnosis ranged from 29 – 2.321 ng/µl. The purity of cDNA in shrimp samples with WFD diagnosis ranged from 1.7 – 1.76.

Based on the data above, it can be concluded that the cDNA of healthy samples and samples with WFD diagnosis synthesized is pure cDNA because it has a cDNA concentration above the minimum value of above 20 ng / µl and a purity value between the values of 1.7 – 2.0 (Sophian, 2021; Utaminingsih & Sophian, 2022). The value of cDNA concentration is influenced by the speed at the time of extraction. CDNA deposition may occur if the lysis and precipitation process is carried out for a long time so that when the supernatant is taken, cDNA is not taken. Purity values that are below the minimum threshold of 1.7 are due to RNA contamination (Dewanata & Mushlih, 2021).

Table 2. Quality and Quantity Test of cDNA

Sample	cDNA	
	Concentration (ng/ul)	Purity(A260/280)
S1	1,717	1.74
S2	1,734	1.74
S3	1,751	1.73
S4	1,768	1.75
S5	1,785	1.72
S6	1,802	1.74
S7	1,819	1.73
S8	1,836	1.74
S9	1,853	1.74
S10	1,870	1.70
W1	29	1.71
W2	1,588	1.76
W3	2,010	1.73
W4	1,393	1.77
W5	122	1.70
W6	1,860	1.73
W7	1,444	1.77
W8	2,321	1.70
W9	1,705	1.75
W10	1,705	1.75

PCR Result of cDNA

PCR amplification using hemocyanin-specific primers aims to double hemocyanin and see if hemocyanin is present in the sample. The difference in the presence of hemocyanin in healthy shrimp samples and WFD-infected shrimp samples can be seen based on the results of electrophoresis visualization from amplification. The primers used are forward Hemocyanin_115F24 primer (5'-CAT ATG GAA TTC CCM TTC TGG TGG-3') and reverse Hemocyanin_1673R21 primer (5'-GGG ATC ACG GGT GGC AGT TTC-3').

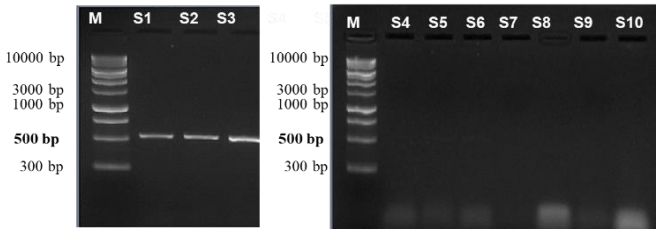

Figure 3. PCR result of health shrimp (S) cDNA; M: marker

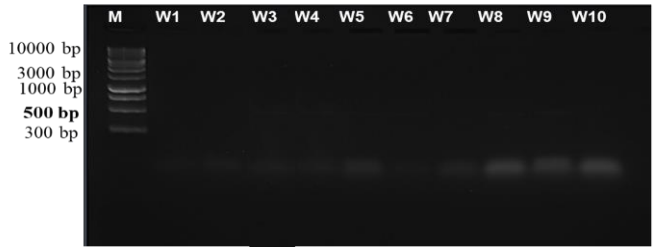

Figure 4. PCR result of WFD shrimp (W) cDNA; M: marker

The amplification results indicate that the primary successfully binds and amplifies the target gene, in this study, the hemocyanin gene in shrimp samples is characterized by the presence of bands in the visualization of PCR results. In healthy samples (Figure 3) it can be seen that there are bright and clear bands in samples with codes S1, S2 and S3. This indicates that the hemocyanin gene as the target gene was successfully obtained and amplified in the three healthy samples. Samples S4, S6, S7, S8, S9 and S10 do not show any bands. The results of PCR visualization on samples with WFD diagnosis (Figure 4) showed that there were very thin bands in W3, W4, W5, W6, W8 and W9 samples. However, the bands in all six WFD diagnosis samples were barely noticeable. While in the W1, W2 and W10 samples there are no bands at all.

There is a band-like luminescence in the visualization of PCR results of healthy samples with codes S4, S5, S6, S7, S8, S9 and 10 and all samples with WFD diagnosis. The band-like luminescence is not an amplified band because it is below the minimum line of marker magnitude, this is called a dimer primer. Primary dimer can occur because the bond between 2 nucleotide base sites is identical to the primer, or it can occur because the hot start process in the PCR program does not occur perfectly. Dimer primers have the potential to cause false-positive results because they are often considered amplified bands (Wang et al., 2020) so accuracy is needed to analyze the visualization of electrophoresis results to ascertain whether the bands obtained are DNA amplification or dimer primers.

Based on the data above, hemocyanin was successfully obtained and amplified in three healthy samples, namely S1, S2 and S3 amounting to ±500 bp so that these three samples were continued in the sequencing While in W3, W4, W5, W6, W8 and W9 samples, it is suspected that these samples still have hemocyanin in them with very small levels. This happens because hemocyanin as a gene coding for body resistance is still contained in samples infected with the disease but in small quantities (Zhao et al., 2013). It can also be influenced by the primary design in which there are immunostimulant contents. Samples with thin bands cannot proceed to the sequencing process.

Molecular Analysis of Hemocyanin

Molecular analysis of hemocyanin begins with a blast test looking for comparison data for hemocyanin sequences. The comparison data used were hemocyanin sequences of *L. vannamei* and *P. monodon* to see the polymorphism. The blast test results shown in Table 3.

Zhao et al., (2013) stated that the percent identity value shows the similarity in the percentage of sequences. The percentage identity value of 96-99% is a related sequence, while the percent identity value of 99-100% is categorized as an identical sequence. The percentage identity value that is categorized as related is most likely to occur in it mutations in several nucleotide bases. The E.v (E-value) indicates the homology of the sequence where the E-value of 0 indicates that the sequence is homologous. The 100% query cover shows the percentage of nucleotide base length aligned with nucleotide data on GenBank NCBI. The next step is to align the sequence of the research sample with the comparison data and then analyze the nucleotide base mutation as can be seen in Figures 5 and 6.

Table 3. Blast Result of Hemocyanin Sequence

Species	Query (%)	E.v	Per. Ident (%)	Acc	Code
L. vannamei	100	0.0	99.00	XM_027383260.1	P1
	100	0.0	98.59	KY695246.1	P2
	100	0.0	97.99	XR_003475559.1	P3
P. monodon	99	0.0	92.15	XM_037939491.1	O1
	99	0.0	91.95	XR_0052302388.1	O2
	99	0.0	91.15	XR_0052302389.1	O3

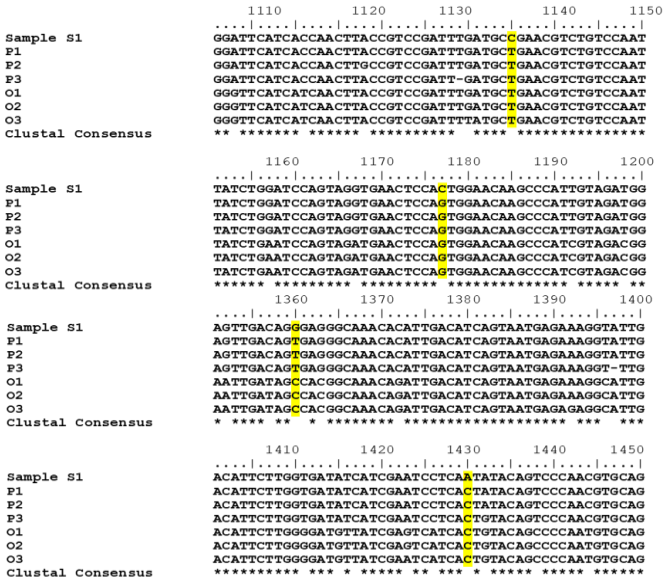

Figure 5. Nucleotide base mutation in hemocyanin of L. vannamei sequence

Based on figure 5, Sample S1 has 4 polymorphisms in its nucleotide base sequence. In the number of 1135 nucleotide base sequence, the hemocyanin sequence *L. vannamei* in the comparison data has a thymine nucleotide base (T) and changes to cytosine (C) in sample S1. At the sequence of nucleotide bases to 1177 there is a change in guanine base (G) to cytosine (C). At the number of 1365 nucleotide base sequence, there is a change in thymine base (T) to guanine (G). At the number of 1430 nucleotide base sequence, there is a change in the nucleotide base cytosine (C) to adenine (A). Species differences allow for many nucleotide base

changes and show different characterizations of each species. The change of adenine nucleotide bases (A) to guanine (G) and thymine (T) to cytosine (C) and vice versa is a type of transitional substitution mutation, which occurs between purine bases with purines and pyrimidines with pyrimidines. The change of purine nucleotide bases to pyrimidines e.g. adenine (A) to cytosine (C) or thymine (T), cytosine (C) to adenine (A) or guanine (G) and vice versa is also called transversion substitution (Badua et al., 2021; Shahzadi et al., 2019). Polymorphism in the genes of two different species according to Orfanoudaki et al. (2019) occurs because the characteristics of these species are different so that it is an effort to adapt each species to its environment. Changes in nucleotide bases cause changes in the amino acids formed as shown in Figure 6.

```
                    360       370       380       390       400
                    ....|....|....|....|....|....|....|....|....|
Sample S1           X*CLRPSSGSQRRKLLLDSSPTYRPI*CRTSVQLSGSSR*TPLEQAHCRW
P1                  E*CLRPSSGSQRRKLLLDSSPTYRPI*C*TSVQLSGSSR*TPVEQAHCRW
P2                  E*CLRPSSGSQRRKLLLDSSPTCRPI*C*TSVQLSGSSR*TPVEQAHCRW
P3                  E*CLRPSSGSQRRKLLLDSSPTYRPIXC*TSVQLSGSSR*TPVEQAHCRW
O1                  G*RVRPSSRSQGRELLLGSSSTYRPI*C*TSVQLSESSR*TPVEQAHRRR
O2                  G*RVRPSSRSQGRELLLGSSSTYRPI*C*TSVQLSESSR*TPVEQAHRRR
O3                  G*RVRPSSRSQGRELLLGSSSTYRPILC*TSVQLSESSR*TPVEQAHRRR
Clustal Consensus   XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
                    460       470       480       490       500
                    ....|....|....|....|....|....|....|....|....|....|
Sample S1           S*QGGQTH*HQ**ERY*HSW*YHRILNIQSQRAVLWSFT*HCPYCTRPSR
P1                  S*Q*GQTH*HQ**ERY*HSW*YHRILTIQSQRAVLWSFT*HCPYCTRPSR
P2                  S*Q*GQTH*HQ**ERY*HSW*YHRILTIQSQRAVLWSFT*HCPYCTRPSR
P3                  S*Q*GQTH*HQ**ERX*HSW*YHRILTVQSQRAVLWSFT*HCPYCTRPSR
O1                  N**PRQTD*HQ**ERH*HSWGCYRVITVQPQCAVLWSFT*HCPYCTRSSR
O2                  N**PRQTD*HQ**ERH*HSWGCYRVITVQPQCAVLWSFT*HCPYCTRSSR
O3                  N**PRQTD*HQ**ERH*HSWGCYRIITVQPQCAVLWSFT*HCPYCTRSSR
Clustal Consensus   XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
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Figure 6. Amino acid mutation in hemocyanin of *L. vannamei* sequence

The amino changes shown were amino acid changes from S1 samples that were different from the *L. vannamei* hemocyanin sequence in the comparison data and the *P. monodon* hemocyanin sequence. In the 351st amino acid sequence, there is a change in the amino acid glutamic acid (Glu/E) to amino acid X, where amino acid X is an unknown amino acid (Unk/X). This may happen because some sequences show different amino acids or if the quality of the original sequence is too poor to translate into definite amino acids (Martoni, 2019). Amino acid on base number 398 sequence also changes from valine (Val/V) to leucine (Leu/L). In amino acid number of 455, there is a change in the amino acid sequence hemocyanin *L. vannamei* sample S1 from the stop codon to glycine (Gly/G) while the amino acid sequence hemocyanin *P. monodon* is different from *L. vannamei*, namely proline (Pro/P). The last amino acid change is shown in the 477 order amino acid, namely from threonine (Thr/T) to asparagine (Asn/N). Mutations in nucleotide base sequences will often cause amino acids to change, affecting the proteins produced (Fan et al., 2019; Morgil et al., 2020; Robert & Pelletier, 2018). For amino acid changes in the hemocyanin gene (HMC) allows changes in the expression of the hemocyanin gene itself, causing diversity in the characteristics of hemocyanin in *Litopenaeus vannamei*.

Phylogenetic Analysis

Phylogenetic analysis is performed using the Neighbor Joining method which will select sequences that when combined will produce the best estimate of the length of the branches that most closely reflect the actual distance between the sequences (Dharyamanti, 2021). The results of the reconstruction of the phylogenetic tree compiled by the Neighbor Joining method. can be seen in Figure 7.

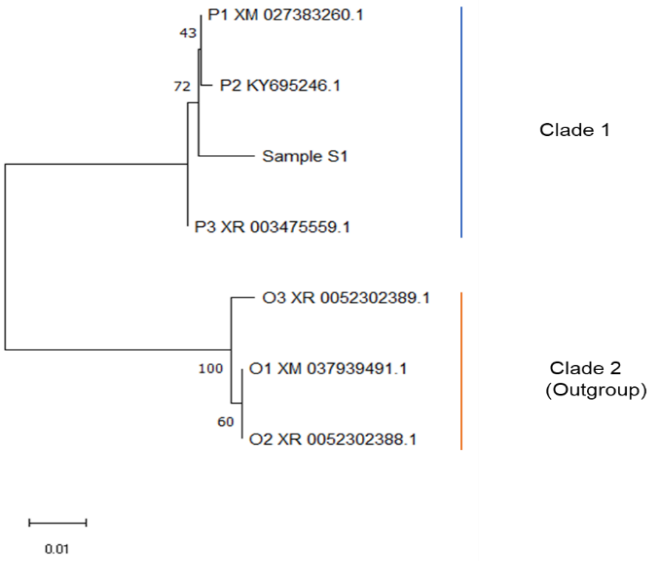

Figure 7. Dendogram of samples

All individuals are divided into 2 clades consisting of an ingroup clade and 1 outgroup clade. Clade 1 consists of S1 samples and three comparative data of *L. vannamei* hemocyanin sequences, namely P1, P2 and P3. Clade 2 consists of all outgroup samples of *P. monodon* hemocyanin sequences, namely O1, O2 and O3 samples. Group determination is based on genetic distance visualized by branch length. If the branches get shorter, the genetic distance is small, which means that the closer the kinship and enter into one clade (Suryani et al., 2022). Based on the dendogram above, it can be concluded that hemocyanin in *L. vannamei* and *P. monodon* has differences in molecular characters due to mutations in them by 1%.

Conclusion

Hemocyanin works optimally in healthy shrimp and does not work optimally in shrimp infected with White Feces Disease (WFD). Hemocyanin polymorphisms are possible as in this study.

Hemocyanin gene profile in healthy shrimp was also analyzed and there were 4 nucleotide change made changes to 4 amino acid. Changes in amino acids affect the stability of hemocyanin and cause polymorphism of hemocyanin. Phylogenetic analysis showed dendogram split into two clades between *L. vannamei* and *P. monodon*. Polymorphism occurs due to genetic mutations that can cause changes in amino acids. Changes in amino acids allow for differences in genes to be encoded so that changes in gene expression can occur. Knowing the profile and characteristics of hemocyanin molecularly can be the first step in disease control in white shrimp cultivation.

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Author Contributions
Conceptualization, Amanda, T and Kilawati, Y.; methodology, Amanda, T, Kilawati, Y and Maftuch.; software, Amanda, T.; validation, Kilawati, Y and Maftuch.; formal analysis, Kilawati, Y.; investigation, Amanda, T., Kilawati, Y and Maftuch; resources, Kilawati, Y and Maftuch.; data curation, Amanda, T and Kilawati, Y.; writing—original draft preparation, Amanda, T., Kilawati, Y and Maftuch; writing—review and editing, Amanda, T., Kilawati, Y and Maftuch; visualization, Amanda, T.; supervision, Kilawati, Y. and Maftuch; project administration, Kilawati, Y.; funding acquisition, Kilawati, Y.

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Conflicts of Interest
The authors declare no conflict of interest.

References

Alfiansah, Y. R., Peters, S., Harder, J., Hassenrück, C., & Gärdes, A. (2020). Structure and co-occurrence patterns of bacterial communities associated with white faeces disease outbreaks in Pacific white-leg shrimp Penaeus vannamei aquaculture. *Scientific Reports*, *10*(1), 11980. https://doi.org/10.1038/s41598-020-68891-6

Anissa, R. K., Lisdiana, L., & Widyayanti, A. T. (2024). Optimasi metode Nested PCR untuk deteksi Vibrio parahaemolyticus AHPND pada udang vaname (Litopenaeus vannamei). *LenteraBio: Berkala Ilmiah Biologi*, *13*(1), 1–13. https://doi.org/10.26740/lenterabio.v13n1.p1-13

Aramburu, O., Ceballos, F., Casanova, A., Le Moan, A., Hemmer-Hansen, J., Bekkevold, D., Bouza, C., & Martínez, P. (2020). Genomic signatures after five generations of intensive selective breeding: Runs of homozygosity and genetic diversity in representative domestic and wild populations of turbot (Scophthalmus maximus). *Frontiers in Genetics*, *11*, 296. https://doi.org/10.3389/fgene.2020.00296

Aweya, J. J., Zheng, Z., Zheng, X., Yao, D., & Zhang, Y. (2021). The expanding repertoire of immune-related molecules with antimicrobial activity in penaeid shrimps: a review. *Reviews in Aquaculture*, *13*(4), 1907–1937. https://doi.org/10.1111/raq.12551

Badua, C. L. D. C., Baldo, K. A. T., & Medina, P. M. B. (2021). Genomic and proteomic mutation landscapes of SARS-CoV-2. *Journal of Medical Virology*, *93*(3), 1702–1721. https://doi.org/10.1002/jmv.26548

Dewanata, P. A., & Mushlih, M. (2021). Differences in DNA purity test using UV-Vis Spectrophotometer and Nanodrop Spectrophotometer in type 2 diabetes mellitus patients. *Indonesian Journal of Innovation Studies*, *15*. https://doi.org/10.21070/ijins.v15i.553

Dharyamanti, I. (2021). *Filogenetika molekuler: metode taksonomi organisme berdasarkan sejarah evolusi*. Retrieved from https://www.scirp.org/reference/referencespapers?referenceid=755496

Fan, J., Li, X., Lu, H., Lin, R., Aweya, J. J., & Zhang, Y. (2019). N-terminal diversity of Litopenaeus vannamei hemocyanin and immunity. *Molecular Immunology*, *112*, 360–368. https://doi.org/10.1016/j.molimm.2019.06.019

Hassan, S. A. H., Sharawy, Z. Z., El Nahas, A. F., Hemeda, S. A., El-Haroun, E., & Abbas, E. M. (2022). Modulatory effects of various carbon sources on growth indices, digestive enzymes activity and expression of growth-related genes in whiteleg shrimp, Litopenaeus vannamei reared under an outdoor zero-exchange system. *Aquaculture Research*, *53*(16), 5594–5605. https://doi.org/10.1111/are.16041

Inayah, Z. N., Musa, M., & Arfiati, D. (2023). Growth of vannamei Shrimp (Litopenaeus vannamei) in Intensive Cultivation Systems. *Jurnal Penelitian Pendidikan IPA*, *9*(10), 8821–8829. https://doi.org/10.29303/jppipa.v9i10.4278

Kang, Z., Kong, J., Sui, J., Dai, P., Luo, K., Meng, X., Liu, J., Chen, B., Cao, J., & Tan, J. (2024). Optimal open nucleus breeding system for long-term genetic gain in the Pacific white shrimp using genomic selection. *Aquaculture*. https://doi.org/10.1016/j.aquaculture.2024.740760

Kim, H.-J., Shyam, K. U., Oh, M.-J., Lee, J., Rajendran, K. V, Kim, D.-H., Kim, H. J., & Kim, W.-S. (2023). A multiplex real-time polymerase chain reaction

(qPCR) kit targeting VP664 and VP28 genes of white spot syndrome virus (WSSV). *Aquaculture*, *577*, 739968. https://doi.org/10.1016/j.aquaculture.2023.739968

Kumar, R., Huang, J. Y., Ng, Y. S., Chen, C. Y., & Wang, H. C. (2022). The regulation of shrimp metabolism by the white spot syndrome virus (WSSV). *Reviews in Aquaculture*, *14*(ue 3), 1150–1169. https://doi.org/10.1111/raq.12643

Kumar, V., Roy, S., Behera, B. K., Bossier, P., & Das, B. K. (2021). Acute hepatopancreatic necrosis disease (AHPND): Virulence, pathogenesis and mitigation strategies in Shrimp aquaculture. *Toxins*, *13*(8). https://doi.org/10.3390/toxins13080524

Kusumaningrum, H. P., Budi, W. S., Azam, M., & Bawono, A. (2014). Design of electrophoresis device for Optimation of DNA visualization and DNA concentration using software. *Jurnal Pendidikan Fisika Indonesia*, *10*(2), 194–202. https://doi.org/10.15294/jpfi.v10i2.3456

Lederer, T., Hipler, N. M., Thon, C., Kupcinskas, J., & Link, A. (2024). Comparison of fecal MicroRNA isolation using various total RNA isolation kits. *Genes*, *15*(4), 498. https://doi.org/10.3390/genes15040498

Liu, Y., Bhagwate, A., Winham, S. J., Stephens, M. T., Harker, B. W., McDonough, S. J., Stallings-Mann, M. L., Heinzen, E. P., Vierkant, R. A., Hoskin, T. L., Frost, M. H., Carter, J. M., Pfrender, M. E., Littlepage, L., Radisky, D. C., Cunningham, J. M., Degnim, A. C., & Wang, C. (2022). Quality control recommendations for RNASeq using FFPE samples based on pre-sequencing lab metrics and post-sequencing bioinformatics metrics. *BMC Medical Genomics*, *15*(1). https://doi.org/10.1186/s12920-022-01355-0

Lokapirnasari, W., Sahidu, A., Nurhajati, T., Supranianondo, S., & Yulianto, A. (2017). Sekuensing 16S DNA bakteri selulolitik asal limbah cairan rumen sapi peranakan Ongole (Sequencing of 16s DNA of cellulolytic bacteria from bovine rumen fluid waste ongole crossbreed. *Jurnal Veteriner*, *18*(1), 76–82. https://doi.org/10.19087/jveteriner.2017.18.1.76

Maharana, S., Wang, J., Papadopoulos, D. K., Richter, D., Pozniakovsky, A., Poser, I., Bickle, M., Rizk, S., Guillén-Boixet, J., & Franzmann, T. M. (2018). RNA buffers the phase separation behavior of prion-like RNA binding proteins. *Science*, *360*(6391), 918–921. https://doi.org/10.1126/science.aar7366

Mahmoodi, M., Afshari, K. P., Seyedabadi, H. R., & Aboozari, M. (2018). Sequence analysis of 12S rRNA and 16S rRNA mitochondrial genes in Iranian Afshari sheep. *Banat's Journal of Biotechnology*, *9*(18). https://doi.org/10.7904/2068-4738-IX(18)-5

Martoni, F. (2019). *Meaning X in the amino acid sequence*. Retrieved from https://www.researchgate.net/post/Meaning-X-in-the-amino-acid-sequence

Mo, Y., Wan, R., & Zhang, Q. (2012). *Application of Reverse Transcription-PCR and Real-Time PCR in Nanotoxicity Research* (pp. 99–112). https://doi.org/10.1007/978-1-62703-002-1_7

Morgil, H., Gercek, Y. C., & Tulum, I. (2020). Single nucleotide polymorphisms (SNPs) in plant genetics and breeding. In *The Recent Topics in Genetic Polymorphisms* (pp. 400–825). https://doi.org/10.5772/intechopen.91886

Orfanoudaki, M., Hartmann, A., Karsten, U., & Ganzera, M. (2019). Chemical profiling of mycosporine-like amino acids in twenty-three red algal species. *Journal of Phycology*, *55*(2), 393–403. https://doi.org/10.1111%2Fjpy.12827

Pratiwi, R. K., Mahmudi, M., Faqih, A. R., & Arfiati, D. (2023). Dynamics of water quality for vannamei shrimp cultivation in intensive ponds in coastal areas. *Jurnal Penelitian Pendidikan IPA*, *9*(10), 8656–8664. https://doi.org/10.29303/jppipa.v9i10.4322

Rahmi, I., Arfiati, D., Musa, M., & Karimah, K. (2023). *Dynamics of Physics and Chemistry of Vanamei Shrimp (Litopenaeus vannamei*. https://doi.org/10.29303/jppipa.v9i1.2528

Rajalakshmi, S. (2017). Different types of PCR techniques and its applications. *International Journal of Pharmaceutical, Chemical & Biological Sciences*, *7*(3). Retrieved from https://www.scirp.org/reference/referencespapers?referenceid=2818692

Robert, F., & Pelletier, J. (2018). Exploring the impact of single-nucleotide polymorphisms on translation. *Frontiers in Genetics*, *9*, 507. https://doi.org/10.3389/fgene.2018.00507

Shahzadi, I., Mehmood, F., Ali, Z., Malik, M. S., Waseem, S., Mirza, B., Ahmed, I., & Waheed, M. T. (2019). Comparative analyses of chloroplast genomes among three Firmiana species: Identification of mutational hotspots and phylogenetic relationship with other species of Malvaceae. *Plant Gene*, *19*, 100199. https://doi.org/10.1016/j.plgene.2019.100199

Sophian, A. (2021). DNA isolation of chicken feathers from the base of the young feathers, the base of the old feathers, and the tip of the feathers. *Bioeduscience*, *5*(2), 104–108. https://doi.org/10.22236/j.bes/526211

Sugiharta, A., Fadjar, M., & Hardoko, H. (2023). The use of purik leaf extract (Mitragyna speciosa) on hematological and histopathological profile of

vannamei shrimp (Litopenaeus vannamei) on Vibrio parahaemolyticus infection. *Jurnal Penelitian Pendidikan IPA*, *9*(SpecialIssue), 1262–1270. https://doi.org/10.29303/jppipa.v9ispecialissue.4216

Suryani, C., Prasetya, E., Harsono, T., Amalia, L., Muammar, M., Musdary, F., Aulia, R. N., & Husna, A. (2022). Genetic and kinship analysis of Kenanga (Cananga odorata) from North Sumatra. *AIP Conference Proceedings*, *2659*(1). https://doi.org/10.1063/5.0116463

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, *38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Utaminingsih, S., & Sophian, A. (2022). Analysis of purity and concentration of DNA isolation results on chondroitin samples. *BiosciED: Journal of Biological Science and Education*, *3*(2), 56–61. Retrieved from https://e-journal.upr.ac.id/index.php/bed

Wang, Z., Zhou, J., Li, J., Zou, J., & Fan, L. (2020). The immune defense response of Pacific white shrimp (Litopenaeus vannamei) to temperature fluctuation. *Fish & Shellfish Immunology*, *103*, 103–110. https://doi.org/10.1016/j.fsi.2020.04.053

Wei, J., Zhang, X., Yu, Y., Huang, H., Li, F., & Xiang, J. (2014). Comparative transcriptomic characterization of the early development in Pacific white shrimp Litopenaeus vannamei. *PLoS ONE*, *9*(9). https://doi.org/10.1371/journal.pone.0106201

Wyban, J. (2019). Selective breeding of Penaeus vannamei: impact on world aquaculture and lessons for future. *Journal of Coastal Research*, *86*(SI), 1–5. https://doi.org/10.2112/SI86-001.1

Yamin, M., Khairuddin, K., & Padusung, P. (2022). Breeding of Philemon buceroides by Synchronization of Pregnant Mare Serum Gonadotropine (PMSG) Reproductive Hormone and Estrogen as Conservation for Preventing Its Extinction. *Jurnal Penelitian Pendidikan IPA*, *8*(2), 957–962. https://doi.org/10.29303/jppipa.v8i2.1482

Yudiati, E., Azhar, N., Ambariyanto, A., Alghazeer, R., & Trianto, A. (2024). Immunostimulatory effects of ulvan on trypsin-mediated protein digestion in the gut of pacific whiteleg shrimp (Litopenaeus vannamei. *Jurnal Riset Akuakultur*, *19*(1), 45–56. https://doi.org/10.15578/jra.19.1.2024.45-56

Zhao, S., Lu, X., Zhang, Y., Zhao, X., Zhong, M., Li, S., Guo, E., & Lun, J. (2013). Identification of a novel alternative splicing variant of hemocyanin from shrimp Litopenaeus vannamei. *Immunology Letters*, *154*(1–2), 1–6. https://doi.org/10.1016/j.imlet.2013.08.003