E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia Science & Technology Asia
Vol.30 No.3 July - September 2025 Page: [243-253]
Original research article

Association Mapping of Early Mortality Syndrome - Acute Hepatopancreatic Necrosis Disease Tolerance in *Litopenaeus vannamei*

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Received 11 April 2025; Received in revised form 2 June 2025
Accepted 19 June 2025; Available online 30 September 2025

ABSTRACT

Early Mortality Syndrome (EMS)-Acute Hepatopancreatic Necrosis Disease (AHPND) is a severe bacterial disease that significantly impacts Pacific white shrimp (*Litopenaeus vannamei*) farming, leading to substantial declines in shrimp production. To mitigate losses caused by EMS-AHPND, molecular breeding presents a promising approach for sustainable disease prevention. This study focused on the identification of single nucleotide polymorphism (SNP) markers associated with EMS-AHPND phenotypes using Genotyping-by-sequencing (GBS). SNP markers were identified in fourth-generation selective breeding lines of *L. vannamei*. A total of 9,504 filtered SNPs were analyzed for their association with EMS-AHPND phenotypes using the Fixed and Random Model Circulating Probability Unification (FarmCPU), accounting for population stratification and cryptic relatedness. Seven SNPs were identified as significantly associated with EMS-AHPND phenotypes, with P-values passing the Bonferroni-adjusted threshold. This study provides a valuable genetic tool for the genetic improvement of EMS-AHPND tolerance in *L. vannamei*.

Keywords: Association mapping; EMS-AHPND; Genotyping-by sequencing; *Litopenaeus vannamei*

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1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is a commercially important aquaculture species and continues to dominate global production. The decade-long boom of the shrimp aquaculture industry has been halted by the outbreak of early mortality syndrome (EMS). Acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus* (*Vp*AHPND), has led to significant economic losses in the shrimp industry [1]. AHPND is a relatively recent bacterial disease affecting farmed penaeid shrimp. The shrimp production in AHPND affected regions has dropped to approximately 60% and the disease has caused a global loss of USD 43 billion to the shrimp farming industry [2]. It has caused significant economic losses in the shrimp industry, particularly in Asian countries like Thailand, Vietnam, and Malaysia, as well as in South America and the United States [3].

*Vp*AHPND contains a plasmid of approximately 69 kilobase pairs that carries genes encoding homologues of the Photorhabdus insect-related (Pir) binary toxins, PirA and PirB [4-6]. The toxins are produced in the shrimp's stomach but cause death by inducing massive sloughing of hepatopancreatic tubule epithelial cells (pathognomonic AHPND lesions) [7]. The binary toxins result in severe acute mortality in shrimp approximately within the first 35 days after stocking cultivation ponds [8].

To reduce the production losses caused by AHPND in shrimp farming, strategies such as improving shrimp culture conditions and enhancing farm management have been implemented. Additionally, focusing on breeding improvement offers a sustainable approach to reducing disease outbreaks of *L. vannamei* [9]. Recent advances in genomic technologies have facilitated the discovery and application of DNA markers, such as single nucleotide polymorphisms (SNPs), to enhance the genetics of various aquaculture species. By identifying specific genomic regions associated with economically significant traits through methods like genome-wide association studies (GWAS), researchers have uncovered markers linked to quantitative trait loci (QTL) and integrated them into aquaculture breeding programs using marker-assisted selection (MAS) [10]. In *L. vannamei*, markers associated with economically important traits have been identified in several studies using a genome-wide approach, such as growth traits [11, 12], ammonia nitrogen tolerance [13], sex [14], white spot syndrome virus (WSSV) tolerance [15], and tolerance to acute hepatopancreatic necrosis disease (AHPND) [9].

Association mapping (AM) is a method that utilizes thousands of polymorphisms to assess the impact of QTL. It serves as a crucial tool for identifying alleles, discovering new genes, and unraveling complex traits. Compared to linkage analysis, AM offers a higher resolution, largely due to its reliance on linkage disequilibrium (LD). Key factors to consider when applying AM include marker density, population type, sample size, and population structure [16]. Genotyping-by-sequencing (GBS) is a method used for discovering SNPs across the entire genome. It has quickly gained recognition as a versatile and cost-effective approach for generating comprehensive genome-wide marker data. GBS is both flexible and highly efficient, and it does not depend on pre-existing genomic resources, such as a reference genome assembly for the species of interest [17]. GBS necessitates extensive quality control (QC) measures to ensure reliable genotype data.

In this study, GBS was used to genotype 210 individuals of *L. vannamei* from fourth-generation selective breeding lines. The objectives of this study were to assess the association between SNPs and EMS-AHPND tolerance traits in *L. vannamei*. These findings can be utilized to develop DNA markers as tools for selecting *L. vannamei* with enhanced tolerance to EMS-AHPND disease.

2. Materials and Methods

All animal experiments were conducted and managed in strict compliance with the established guidelines for the ethical use of animals in scientific research.

2.1 Experimental shrimp challenge tests

Experimental shrimp and *V. parahaemolyticus* challenge tests were conducted to assess the shrimp's tolerance to bacterial infections and understand the pathogen's impact on shrimp health. First, 3150 post-larvae 20 (PL20) samples of *L. vannamei* (35 families, with 90 individuals per family), derived from 35 families of fourth-generation (F4) selective breeding lines were obtained from the Songkhla Aquatic Animal Health Research and Development Center. Each family was cultured separately in designated tanks for seedling rearing. This population was a part of the project titled "Founding of the SPF and high growth-performance broodstock of Pacific white shrimp, *L. vannamei*, for Thailand aquaculture and sustainable utilization". The experimental animals were inoculated with *Vp*AHPND by adding the pathogen, cultured in tryptic soy broth, directly into the water of each challenge tank. This achieved an estimated bacterial concentration at the LC50 level (1.12×10^4 CFU/mL) in the bioassay tank water. The challenge test continued for a duration of 14 days. Shrimp deaths were recorded three times per day. Surviving shrimps were collected at 14 days after challenge. Shrimp samples were preserved in 95% ethyl alcohol until DNA extraction. Phenotypes of susceptibility and tolerance were evaluated based on survival time following the challenge. The EMS-AHPND tolerance traits used for association mapping analysis were categorized into two phenotypes. The first phenotype, 'type' referred to survival outcome: individuals that died within 3 days after *V. parahaemolyticus* exposure were classified as the susceptible group, while those that survived for 14 days post-infection were classified as the resistant group. The second phenotype, 'score,' represented time to death for which individuals were categorized into one of three groups: within 22 hours, 28.5 hours, and 32 hours after *V. parahaemolyticus* exposure. Due to the high mortality rate observed in the shrimp after infection, individuals that died within the three intervals were selected for association mapping analysis to enhance the clarity and distinction of phenotypic differentiation. The cause of shrimp mortality was verified and confirmed to be due to *Vibrio parahaemolyticus* infection through PCR analysis.

2.2 Population structure

The population structure was analyzed using 10 microsatellite loci, namely TUMXLv9.178, TUMXLv10.455, TUMXLv10.411, TUMXLv8.224, TUMXLv7.121, TUMXLv5.45, TUMXLv7.148 [18], CNM-MG 357 [19], Lvan17 [20], and TUSWLvSU233 [21]. The PCR reactions were prepared to a total volume of 20 μL, comprising 20 ng of DNA, 10 pmol of each primer, 2.1 mM MgCl2, 0.2 mM of each dNTP, and 5U/ul of Taq polymerase. PCR amplification

was conducted using the T100 Thermal Cycler, involving an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at the locus-specific temperature for 30 seconds, and extension at 72°C for 1 minute. The process ended with a final extension at 72°C for 5 minutes. Subsequently, the PCR products were separated through 1% agarose gel electrophoresis. The DNA fragment lengths were analyzed using ZAG DNA Analyzer Systems. The determination of allelic size was carried out using ProSize data analysis software (Agilent Technologies). The population structure was analyzed using a model-based Bayesian approach as implemented in STRUCTURE software version 2.3.4 [22]. The analysis was performed ten times for each K value ranging from $K = 1 to K = 10$. For each run, both the burn-in period and the number of iterations were set to 100,000. Individuals with a membership probability (Q value) of less than 0.6 were classified as admixtures. The number of sub-populations (ΔK) was determined using the ad-hoc statistics proposed by Evanno et al. [23]. Pairwise kinship coefficients were calculated using SPAGeDi v.1.5a [24]. Negative values between individuals were adjusted to 0, indicating a relationship weaker than that of random individuals [25].

2.3 GBS-based SNP discovery

Total DNA was extracted from whole tissue samples of shrimp PL20 using Genomic DNA Mini Kit (Tissue) (Geneaid, New Taipei City, Taiwan) according to manufacturer's instructions. The quality and concentration of the extracted DNA were assessed through agarose gel electrophoresis, and absorbance measurements were taken at wavelengths of 260 nm and 280 nm using a spectrophotometer. The collected DNA samples were stored at -20°C until used. All individuals from 35 families were genome-wide genotyped using GBS, performed by Novogene Co., Ltd. DNA was digested by *Ms*eI and *Ms*pI and then ligated with barcoded adapters for sequencing. Following standard Illumina protocols, a pair-end library with a 350 bp insert size was constructed for each DNA sample. DNA sequencing of the libraries was performed with 150 bp paired-end mode. The raw sequencing reads were processed to remove adapter sequences and low-quality reads, resulting in a set of high-quality, filtered reads. SNPs with missing data ≥ 10%, minor allele frequency (MAF) < 0.05, and depth of coverage < 10 were discarded. The filtered reads were then aligned to the reference genome [26] using BWA with default parameters. Subsequent processing, including duplicate removal, was preformed using SAMtools and PICARD (http://picard.sourceforge.net). Screened SNPs were then mapped to the genome of *L. vannamei* (ASM378908v1) employed in this study.

2.4 Association analysis

The associations between SNP markers and EMS-AHPND tolerance traits were analyzed using Fixed and random model Circulating Probability Unification (FarmCPU) [27] integrated in Memory efficient, Visualization-enhanced, and Parallel-accelerated R package (rMVP) [28]. The correction for population stratification and cryptic relatedness was performed by employing the coefficient of co-ancestry kinship and the Q matrix as covariates by looking at the model fit using Quantile-Quantile (QQ) plots generated using the 'qqman' package in R. The significance threshold for declaring a significant association was set at

the Bonferroni-correction at 0.05 (p-value $\leq 5.26 \times 10^6$) [29]. A Manhattan plot visualizes the statistical significance of associations as $\log 10$ (p-value) on the y-axis, plotted against chromosome coordinates on the x-axis. Each dot represents a single variant, and a higher $\log 10$ (p-value) indicates a stronger association between the corresponding SNP and the phenotype.

3. Results and Discussion

3.1 Determining shrimp AHPND tolerance

The majority of shrimp died within 2–3 days following infection, with peak mortality observed on day 3. Approximately 15% of the shrimp survived until day 14 post-infection. Phenotypic classification based on the type phenotype was performed, with 105 dead shrimp (D) assigned a binary value of 0, and 105 surviving shrimp (S) assigned a value of 1. For the score phenotype, the 105 surviving individuals were assigned a score of 1. The deceased shrimp were further categorized based on time to mortality: 60 shrimp that died within 22 hours were assigned a score of 2, 33 shrimp that died within 28.5 hours received a score of 3, and 12 shrimp that died within 32 hours were assigned a score of 4.

The shrimp mortality rate observed in this study was consistent with the findings of Whankaew et al. [30], who reported high mortality rates in shrimp within 2–3 days after infection. In the present study, 15% of the shrimp survived until day 14 post-infection, which was notably higher than the 5% survival rate reported by Whankaew et al. [30] over an equivalent experimental period. This difference in survival outcomes may be attributed to the lower concentration of the pathogen used in this study (1.12×10^4 CFU/ml), in contrast to the higher concentration (1×10^5 CFU/ml) employed by Whankaew et al. [30].

3.2 Population structure

In the present study, population structure was inferred using an independent set of microsatellite markers to avoid dependency among terms in the model and to prevent the structure from absorbing QTL effects [22]. Admixture model-based simulations to population structure suggested $K = 2$ as the most probable number of clusters, calculated following the Evanno criteria (Fig. 1). This suggests that our panel could be split into two clusters, cluster 1 (red bars) and cluster 2 (green bars) (Fig. 2). Population genetic structure refers to the distribution of genetic variation both within and between populations. This structure is influenced by factors such as differing origins, geographical sources, genetic drift, and natural selection. The Bayesian structural analysis revealed two subpopulations, indicating that the base population likely originated from two private hatcheries in Krabi and Songkla provinces. These findings align with Huang et al. [31], who investigated the genetic diversity of seedling samples collected from seven cultured populations across three major shrimp production zones in Guangdong. Their study grouped the seven populations into three clusters, highlighting distinct genetic characteristics among the various cultured populations of *L. vannamei*.

3.3 SNP detection

Based on the GBS approach used in this study, a total of 87.072G of raw data from 210 samples was sequenced in this run. The mapping rate of each sample ranged from 56.29% to 89.98%. The average depth on the reference genome

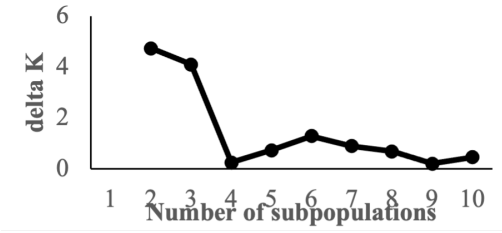

Fig. 1. Number of subpopulations calculated following the Evanno criteria. Delta K values show a clear peak at a cluster of two.

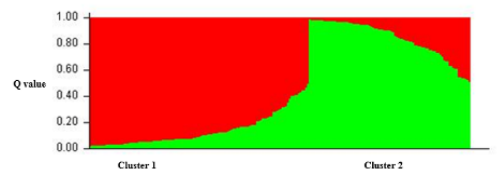

Fig. 2. A population genetic structure diagram, generated by the STRUCTURE program with $K = 2$.

ranged from 2.36× to 6.12×, while coverage exceeding 1× was more than 2.87%. This result falls within the qualified normal range. After filtering out low-quality data, 86.953G of clean data was generated. The raw data production for each sample ranged from 157.992M to 904.349M. With Q20 and Q30 values reaching 87.75% and 75.17%, respectively, the sequencing quality met the requirements for proper analysis. A total of 9,504 SNPs were retained after filtering out SNPs with a read depth greater than 10, a missing rate of less than 10%, and a minor allele frequency greater than 0.05. The GBS library construction in this study was successful, and the filtered SNPs were suitable for association mapping and related analyses.

Various techniques have been utilized for genome-wide SNP discovery in *L. vannamei* to identify loci associated with economically important traits. The number of SNPs retained after filtering in this study was higher than that reported by Whankaew et al. [30], who used the DArT sequencing technique to identify DNA markers associated with the AHPND-tolerant phenotype in *L. vannamei*. Their filtering process removed variants with PIC < 0.1, MAF < 0.05, and a call rate < 80%, resulting in the retention of 516 SNPs and 2,292 InDels. Additionally, the number of retained SNPs in this study exceeded that reported by Guppy et al. [32], who utilized RAD-Seq target-capture genotyping for breeding programs of black tiger shrimp. Different SNP development techniques, populations used in different studies, and filtering parameters can affect the number of SNPs discovered.

3.4 SNPs associated with EMS-AHPND tolerance

The effectiveness of AM in identifying true associations depends on its ability to separate marker-QTL LD from LD caused by confounding factors such as population structure, family relatedness, selection, genetic drift, inbreeding, and admixture [33-35]. In this study, the population structures derived from the Q matrix and kinship effectively controlled both false positives and false negatives, as indicated by the QQ plots for both type (Fig. 3A) and score (Fig. 3B), which showed a straight line with a sharply deviated tail. The FarmCPU+K+Q model efficiently detected LD linked to population structure and family relatedness.

In this study, a total of seven and one SNPs were significantly associated with the type and score phenotypes, respectively, as illustrated in the Manhattan plots (Figs. 3C-3D) and summarized in Table 1. The significance threshold for SNP markers, set at 5.26×10^{-6} after applying the Bonferroni correction, was more stringent than those used in several previous studies to en-

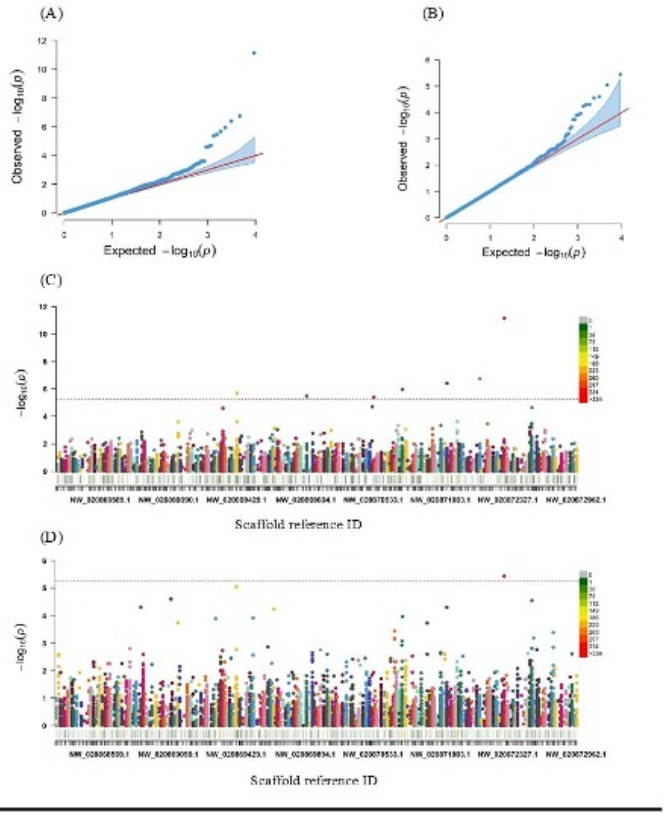

Fig. 3. QQ plots and Manhattan plots of EMS-AHPND tolerance in terms of type (survival and mortality) and score (survival and rate of time to death). (A) and (B) QQ plot of type and score, respectively. The X-axis represents the $\log_{10}$-transformed expected *p*-values, while the Y-axis represents the $\log_{10}$-transformed observed *p*-values. (C) and (D) Manhattan plots of type and score, respectively. The X-axis represents the scaffold reference ID and the Y-axis represents $\log_{10}$ *p*-values. The red dotted line represents the significance threshold adjusted using Bonferroni correction (P = 5.26×10^{-6}).

Table 1. Markers associated with EMS-AHPND tolerance in *L. vannamei*.

Trait	SNP	Scaffold reference ID	Position	*p*-value	MAF	Effect	PVE (%)	Annotation	AnnoPos
type	SNP8225 C/A	NW_020872260.1	440338	7.21E-12	0.26	-0.31	21.85	rna-XM_027380988.1,rna-XM_027380990.1	intergenic
type	SNP7640 A/G	NW_020871441.1	101150	1.78E-07	0.44	0.23	12.47	rna-Trnav-cac-62,rna-XM_027378499.1	intergenic
type	SNP6998 G/A	NW_020871044.1	362854	4.02E-07	0.29	0.16	11.53	rna-XM_027376640.1,rna-XM_027376642.1	intergenic
type	SNP5982 T/C	NW_020870781.1	939212	1.12E-06	0.07	0.19	11.27	rna-XM_027374118.1,rna-XR_003477203.1	intergenic
type	SNP3072 A/G	NW_020869429.1	1622620	2.07E-06	0.29	0.16	9.37	rna-XM_027361145.1,rna-XM_027361146.1	intergenic
type	SNP4253 T/C	NW_020869894.1	1212063	3.35E-06	0.27	0.13	10.48	rna-XM_027365628.1,rna-XM_027365654.1	intergenic
type	SNP5255 G/A	NW_020870533.1	108736	4.16E-06	0.16	0.13	9.58	rna-XM_027371776.1,rna-XM_027371789.1	intergenic
score	SNP8225 C/A	NW_020872260.1	440338	3.66E-06	0.26	0.39	10.02	rna-XM_027380988.1,rna-XM_027380990.1	intergenic

Allele are represented as major allele/minor allele; *p*-value, indicate statistically significant values from the Bonferroni corrected (5.26E-06); MAF, minor allele frequency; Effect, effect of the minor allele in allelic test; PVE (%), The phenotypic variation explained; Annotation, gene names, functions; AnnoPOS, SNP location.

hance the reliability of the identified associations. For instance, Whankaew et al. [30] reported no significant variants associated with AHPND tolerance in 93 *L. vannamei* individuals under the Bonferroni threshold. Similarly, Fu and Liu [13] found no SNPs exceeding the Bonferroni threshold (1.38×10^{-6}) in association with ammonia

nitrogen tolerance in *L. vannamei*.

The absence of significant SNP associations in some studies may be attributed to several factors. A small sample size can reduce the statistical power to detect true associations. Additionally, traits with a polygenic architecture controlled by multiple loci each with small effects are less likely to yield individually significant SNPs. Low heritability of the trait may also contribute, as environmental influences or gene–environment interactions can obscure genetic signals.

Among all SNPs associated with EMS-AHPND tolerance, SNP8225 consistently demonstrated a strong association with both the type and score phenotypes (Table 1). This SNP exhibited the highest levels of association and phenotypic variance explained (PVE), accounting for 21.85% of the variation in type and 10.02% in score. The PVE value for SNP8225 surpassed the 5–13% range reported by Whankaew et al. [30] for SNPs linked to AHPND tolerance in *L. vannamei*, and also exceeded the PVE values previously reported for other traits such as nitrite tolerance (8.42–10.31%) [36]. These findings suggest that SNP8225 could serve as a valuable genetic marker for marker-assisted selection in breeding programs aimed at enhancing EMS-AHPND tolerance. However, further validation in diverse genetic backgrounds is essential to confirm its robustness and applicability across populations.

All SNP markers associated with EMS-AHPND tolerance in this study were located in intergenic regions. These regions lie between genes and do not directly impact coding sequences. However, variants in intergenic regions can still play critical regulatory roles. Whankaew et al. [30] similarly reported that most candidate InDels associated with AHPND tolerance in *L. vannamei* were located in intergenic regions. Likewise, Fu and Liu [13] identified four SNPs associated with ammonia nitrogen tolerance in *L. vannamei* that were also located in intergenic regions. Comparable findings have been reported in cattle, where Fernandes Júnior et al. [37] observed that the majority of SNPs and InDels 59.9% and 58.7%, respectively were located in intergenic regions. Although intergenic SNPs do not affect protein-coding sequences directly, they may influence gene expression through their presence in regulatory elements such as promoters and enhancers, potentially exerting substantial effects on phenotypic traits [38–40].

4. Conclusion

In this study, we performed association mapping of EMS-AHPND tolerance in *L vannamei* using GBS. Several SNPs significantly associated with EMS-AHPND tolerance were identified, with SNP8225 demonstrating the strongest association for both phenotypic traits, type and score. This SNP holds promise as a reliable genetic marker for marker-assisted selection in breeding programs. All identified SNPs will undergo further validation in additional populations to confirm their applicability. Overall, this research provides a valuable genomic resource and introduces a novel strategy for the genetic improvement of *L. vannamei* in Thailand.

Acknowledgements

The authors express gratitude to the project "Founding of the SPF and high growth-performance broodstock of Pacific white shrimp, *Litopenaeus vannamei*, for Thailand aquaculture and sustainable utilization" for providing SPF *L. vannamei* used in this study. The study was sup-

ported by grants from the Agricultural Research Development Agency (public organization). We sincerely thank Ms.Patrawee Nilplub for valuable advice on utilizing various programs for data analysis.

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