ASSOCIATION MAPPING OF EARLY MORTALITY SYNDROME (EMS)-ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND) TOLERANCE IN PACIFIC WHITE SHRIMP

BY

ANONG NIMLAMAI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (BIOTECHNOLOGY AND AGRICULTURE)

DEPARTMENT OF BIOTECHNOLOGY

FACULTY OF SCIENCE AND TECHNOLOGY

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Chairman _____
(Sithichoke Tangphatsornruang, Ph.D.)

Member and Advisor _____
(Associate Professor Kittipat Ukoskit, Ph.D.)

Member _____
(Associate Professor Theerachai Thanananta, Ph.D.)

Member _____
(Assistant Professor Chanitchote Piyapittayanun, Ph.D.)

Member _____
(Panom K. Sodsuk, Ph.D.)

Dean _____
(Associate Professor Supet Jirakajohnkool, Ph.D.)

Dissertation Title	ASSOCIATION MAPPING OF EARLY MORTALITY SYNDROME (EMS)-ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND) TOLERANCE IN PACIFIC WHITE SHRIMP
Author	Anong Nimlamai
Degree	Doctor of Philosophy (Biotechnology and Agriculture)
Department/Faculty/University	Biotechnology Faculty of Science and Technology Thammasat University
Dissertation Advisor	Associate Professor Kittipat Ukoskit, Ph.D.
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ABSTRACT

The Pacific white shrimp (*Litopenaeus vannamei* (Boone, 1931)) is a key aquaculture species in Thailand with substantial economic importance. Ensuring genetic diversity is critical for sustaining a robust gene pool in farmed populations, ensuring resilience to diseases, environmental changes, and enhancing overall productivity. In the present study, genetic variation and population structure across 35 families of fourth-generation selective breeding lines of *L. vannamei* were monitored using five microsatellite DNA markers. The polymorphism information content (PIC) all microsatellite loci showed high polymorphism , with PIC greater than 0.70, indicating that the DNA markers were highly informative and effective in detecting allele variations in this population. The number of alleles per locus and the number of effective alleles (N_e) ranged from 3 to 6 and 2.809 to 4.598, with averages of 4.4 and 3.734, respectively. These values indicate that the population possessed a moderate level of genetic diversity. Overall average of observed heterozygosity (H_o) ranged from 0.209 to 0.492, a value below the expected heterozygosity (H_e) (0.714-0.784). The mean inbreeding (F_{is}) coefficient across all loci was 0.336 suggesting a moderate degree of inbreeding in this population. The population structure analysis showed that the 35

families were classified into two subpopulations (K=2), related to the sources of the base population collected from two private hatcheries. This study will offer important insights into population genetics and help guide breeding strategies of *L. vannamei*.

Early Mortality Syndrome (EMS)-Acute Hepatopancreatic Necrosis Disease (AHPND) is a severe bacterial disease that significantly impacts *L. 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 was directed toward the identification of SNP markers associated with EMS-AHPND tolerances 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 tolerances 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 tolerances, 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 tolerance, Genotyping-by sequencing, Microsatellite, Pacific white shrimp, Single nucleotide polymorphisms

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Anong Nimlamai

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
AFLP	Amplified fragment length polymorphism
AHPND	Acute hepatopancreatic necrosis disease
AM	Association mapping
AMOVA	Analysis of molecular variance
DArT	Diversity Arrays Technology
DNA	Deoxy ribonucleic acid
DOF	Department of fisheries
EMS	Early mortality syndrome
FarmCPU	Fixed and Random Model Circulating Probability Unification
F_{is}	Inbreeding coefficient
GBS	Genotyping-by-Sequencing
GLM	Generalized linear model
GWAS	Genome-wide Association Study
H_e	Expected heterozygosity
H_o	Observed heterozygosity
InDel	Insertion-deletion
LD	Linkage Disequilibrium
LGs	linkage groups
MAF	Minor allele frequencies
MAS	Marker assisted selection
MLM	Mixed linear model
N_a	Number of alleles
N_e	Number of effective alleles
NGS	Next-generation sequencing
PCA	Principal component analysis
PCO	Principal coordinates analysis
PCR	Polymerase Chain Reaction

PIC	Polymorphism information content
PL	Post larva
Pir	Photorhabdus insect-related binary toxin
PVE	Phenotypic variance explained
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLPs	Restriction fragment length polymorphisms
SNP	Single nucleotide polymorphisms
SSR	Simple Sequence Repeats
STRs	Short tandem repeats
Vp	*Vibrio parahaemolyticus*
WSSV	White spot syndrome virus

CHAPTER 1
INTRODUCTION

1.1 Background

Shrimp aquaculture represents one of the most rapidly expanding sectors of global aquaculture and a major contributor to food security, livelihoods, and economic development in coastal regions. The industry has undergone tremendous expansion over the past four decades, driven by increasing consumer demand for shrimp as a premium seafood product in international markets. Globally, farmed shrimp production is valued at over USD 30 billion annually, with more than 5 million tons produced in 2022 (FAO, 2022). Among farmed crustaceans, penaeid shrimp dominate aquaculture production, with the *L. vannamei* now representing over 80% of the total farmed shrimp globally (Sampantamit et al., 2020).

After 2012, shrimp aquaculture production has decreased continually caused by the emergence of a bacterial disease. Shrimp diseases are recognized serving as one of the primary threats to the aquaculture sector. Outbreaks often result in mass mortalities, causing substantial financial losses and reduced productivity. Among the most devastating diseases affecting *L. vannamei* in the past decade is Early Mortality Syndrome (EMS), commonly known as Acute Hepatopancreatic Necrosis Disease (AHPND). The disease initial appeared in China in 2009 and has since extended to other major shrimp-producing countries in Southeast Asia, covering Vietnam, Malaysia, and Thailand, as well as Mexico (Lee et al., 2015; Tran et al., 2013).

EMS-AHPND is associated with a specific strain of *Vibrio parahaemolyticus* harboring the pVA plasmid that carries PirAB-like toxin genes (Han et al., 2015). The toxins produced by this plasmid cause extensive necrosis of the hepatopancreas in juvenile shrimp, leading to acute mortality often during the first month during pond stocking. Mortality may reach 70-100%, resulting in catastrophic production losses (FAO, 2013; Soto-Rodriguez et al., 2015). The global spread of EMS-AHPND has significantly impacted the economic viability of shrimp farming, reducing production outputs, increasing production costs, and undermining farmer confidence. The Food

and Agriculture Organization estimated that losses from EMS-AHPND exceeded USD 1 billion annually during the peak years of outbreaks (FAO, 2013). Conventional disease management strategies, such as improved pond management, biosecurity measures, probiotics, and restricted use of antimicrobials, have had limited success in preventing or controlling the disease. This underscores the urgent need for sustainable and long-term solutions to mitigate EMS-AHPND impacts on the shrimp industry.

Because of its high economic value, selective breeding for genetic improvement has been practiced for decades. Selective breeding for disease resistance or tolerance offers a promising, environmentally sustainable, and long-term strategy to mitigate the effects of infectious diseases in aquaculture species . In terrestrial livestock, genetic improvement programs have successfully enhanced resistance to pathogens, and similar strategies are now increasingly applied in aquaculture (Gjedrem & Baranski, 2009). In *L. vannamei*, substantial advances have been achieved in selective breeding for growth , survival, and viral resistance (Argue et al., 2002; Castillo-Juárez et al., 2015). However, the genetic factors underlying tolerance or resistance to bacterial diseases such as EMS-AHPND remains poorly understood. Determining genetic markers related to disease tolerance can accelerate the development of tolerance strains through marker-assisted selection (MAS). Unlike traditional selection methods, MAS allows for precise and efficient incorporation of favorable alleles into breeding populations, reducing reliance on time-consuming and costly challenge tests.

However, disease tolerance seems to have low heritability and is readily affected by environmental factors (Trang et al., 2019). Marker-assisted selection is regarded as an efficient approach for breeding complex traits, particularly disease tolerance. To perform MAS, markers associated with disease tolerance are required. Association analysis is considered a powerful tool for identifying trait-related markers. Two effective methods for association analysis are genome-wide association studies (GWAS) as well as candidate gene analyses, with GWAS frequently employed to map complex disease traits in humans, economically significant crops, and animals. Association mapping, occasionally termed linkage disequilibrium (LD) mapping, is a powerful approach for establishing associations between genetic markers and traits of interest by exploiting natural variation in populations (Zhu et al., 2008). Unlike classical linkage mapping, which requires controlled crosses, whereas association mapping

leverages the genetic diversity already present in populations, providing greater resolution for locating trait-associated loci.

With the rapid development of genomic resources for *L. vannamei*, including high-density genetic linkage maps, transcriptome assemblies, and whole-genome sequencing (Zhang et al., 2019), genome-wide association studies (GWAS) are becoming increasingly feasible. Molecular markers such as SNPs and SSRs have been extensively utilized for growth-related research, reproduction, and viral resistance traits in shrimp (Baranski et al., 2014; Lyu et al., 2021; Yu et al., 2015). However, studies employing association mapping to investigate EMS-AHPND tolerance are still limited.

Understanding the genetic basis of EMS-AHPND tolerance through association mapping will provide valuable insights into the host-pathogen interaction and identify genetic loci which can be applied in selective breeding programs.

1.2 Objectives

1.2.1 Analysis of genetic variation and population structure in *L. vannamei*.

1.2.2 Identification of SNPs marker using Genotyping-by-Sequencing.

1.2.3 Association mapping of EMS-AHPND tolerance in *L. vannamei* using SNPs marker.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Pacific white shrimp

The Pacific white shrimp, sometimes called whiteleg shrimp, is currently the highest important crustacean species in global aquaculture. Its dominance stems from several favorable traits, including fast growth and adaptability to various salinity levels, adaptability to intensive culture systems, and high reproductive efficiency in captivity (FAO, 2022). Since the early 2000s, *L. vannamei* has gradually replaced the black tiger shrimp (*Penaeus monodon*) in many Asian countries due to its superior performance under commercial farming conditions (Sampantamit et al., 2020). In Thailand, for instance, *L. vannamei* now accounts for nearly all shrimp production, contributing substantially to export revenues. Despite the benefits mentioned, the sustainability of shrimp aquaculture is continuously threatened by disease outbreaks, which constitute a major constraint on stable production (Lightner, 2011).

A high-quality reference genome is a critical resource for population genomics, marker discovery, GWAS/genomic selection, functional genomics, as well as understanding the molecular mechanisms underlying traits like growth, molting, vision, and disease tolerance. The first large-scale genome assembly and annotation for *L. vannamei* were published by a Chinese consortium, which provided the first comprehensive view of the species' genome architecture and gene repertoire. *L. vannamei* had a genome size of 1.66 Gb and had 44 linkage groups (LGs). There were up to 23.93% of simple sequence repeats (SSRs) (Figure 2.1) found for every 301 bp of SSRs, which is the highest compared to any other animal species which have posed a significant challenge for genome sequencing and assembly. Regarded as amomg of the most notable characteristics of the *L. vannamei* genome is the remarkable expansion of repetitive elements, particularly simple sequence repeats (SSRs). Both SSR density and average SSR length rank among the highest recorded in arthropod genomes. SSRs represent the predominant repetitive fraction and are considered the primary cause of fragmented assemblies (Zhang et al., 2019).

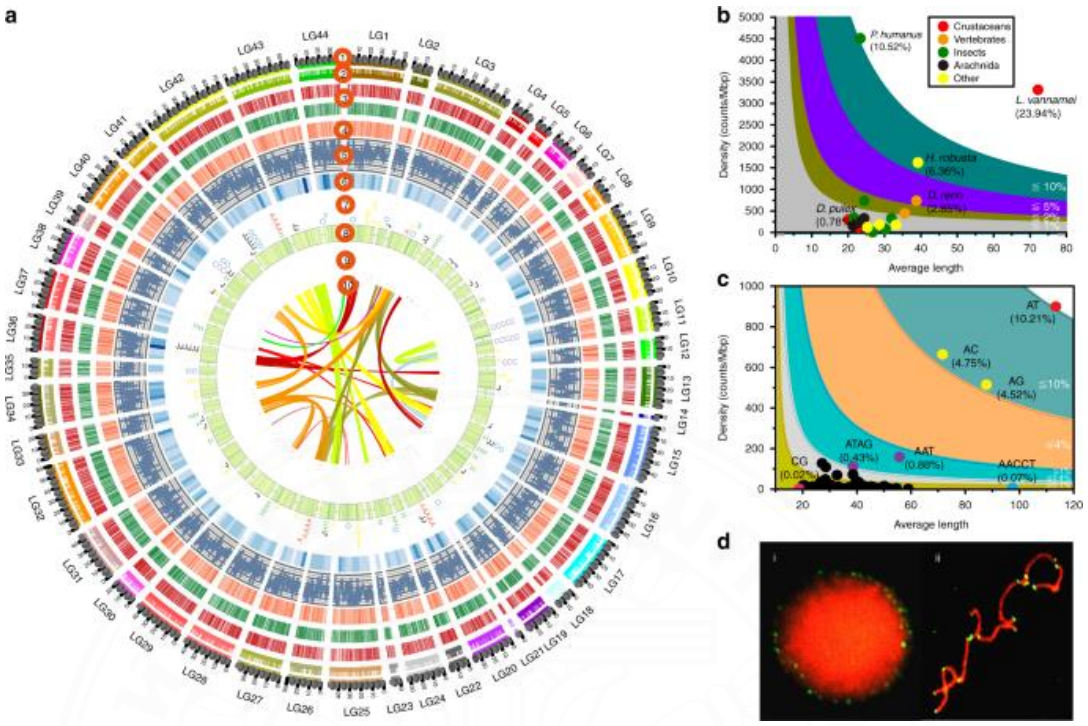

Figure 2.1 Genomic features of *L. vannamei*. (a) Schematic overview of *L. vannamei* genomic characteristics. (b) Comparative SSR content among different animal genomes. (c) SSR type distribution in the *L. vannamei* genome. (d) FISH analysis of the $(AACCT)_n$ SSR within the nucleus (Zhang et al., 2019).

Decoding *L. vannamei*'s genome serves to elucidate the genetic foundations of certain biological functions while supplying important information for selective breeding in shrimp aquaculture. Although a complete genome of the *L. vannamei* is not yet available in public databases, continuous studies are being conducted to generate increasingly complete genome data (Peng et al., 2023; Perez-Enriquez et al., 2024; Yuan et al., 2021; Zhang et al., 2019) as shown in Table 2.1.

Table 2.1 Overview of the *L. vannamei* de novo genome assembly statistics.

Parameter	Zhang et al. (2019)	Yuan et al. (2021)	Peng et al. (2023)	Perez-Enriquez et al. (2024)
Genome size	1.62 Gb	1.63 Gb	1.87 Gb	2.055 Gb
Scaffold number	4,682	28,409	10,682	15,726
Scaffold N50	605 kb	31.3 Mb	36.7 Mb	40.14 Mb
Scaffold L50	892	21	21	21
Largest scaffold	3.46 Mb	47.30 Mb	54.4 Mb	65.79 Mb
GC content	36.69%	35.68%	35.91%	36.71%
Number of gene models (<300 nt)	25,325	NA	21,548	21,816
Noncoding annotation				
Infernal/Rfam	5, 248	NA	NA	7, 427
Repetitive elements	39. 11%	NA	64.33%	39.41%

NA: not available.

2.2 Early mortality syndrome-acute hepatopancreatic necrosis disease (EMS-AHPND)

The emergence of Early Mortality Syndrome (EMS), or Acute Hepatopancreatic Necrosis Disease (AHPND), a bacterial disease mainly caused by *Vibrio parahaemolyticus*, has resulted in significant economic losses in aquaculture, a halophilic Gram-negative bacterium commonly present in seawater (Tran et al., 2013). It commonly causes food poisoning in those who eat raw or undercooked seafood. EMS-AHPND causes rapid, mortality can be as high as 100%, within approximately 30–35 days following stocking of post-larvae or juvenile shrimp. Clinical symptoms include a hepatopancreas that appears pale to white, soft shells, guts with discontinuous or the food was not full, the hepatopancreas may sometimes exhibit black spots or scratches. In addition, the hepatopancreas is firm and resists being compressed between the thumb and forefinger (Figure 2.2) (FAO, 2013; Hong et al., 2016). The pathogenic *V. parahaemolyticus* strains possess a 70.45 kbp plasmid (pVa), which carries genes encoding homologues of the Photorhabdus insect-related binary toxin, PirA and PirB it compose of 110 amino acid (332 bp) and 438 amino acid (1,313 bp), respectively. According to reports, the presence of both PirA and PirB is necessary to induce EMS-AHPND (Lee et al., 2015). The disease has been reported from China in 2009 (NACA et al., 2011), Vietnam in 2010, Malaysia in 2011, Thailand in 2012 (Lightner et al., 2012), Mexico in 2013 (Nunan et al, 2014), and Philippines in 2014 (Dabu et al., 2015)

Diagnostic approaches include PCR assays that detect pirA/pirB genes (or plasmid-specific markers), culture-based detection of Vibrio isolates followed by PCR, histopathology of hepatopancreas lesions, and newer protein-detection immunoassays targeting Pir proteins. Molecular detection of the pirAB genes is widely used for screening broodstock, post larvae and pond water/sediment. However, the presence of *pir* genes alone is not a reliable predictor of an imminent outbreak, as disease expression is influenced by factors such as bacterial abundance, plasmid copy number, environmental conditions, and host health. Therefore, a combined surveillance approach integrating molecular diagnostics, histopathology, and farm management data is recommended (Dangtip et al., 2015; Jeon et al., 2023).

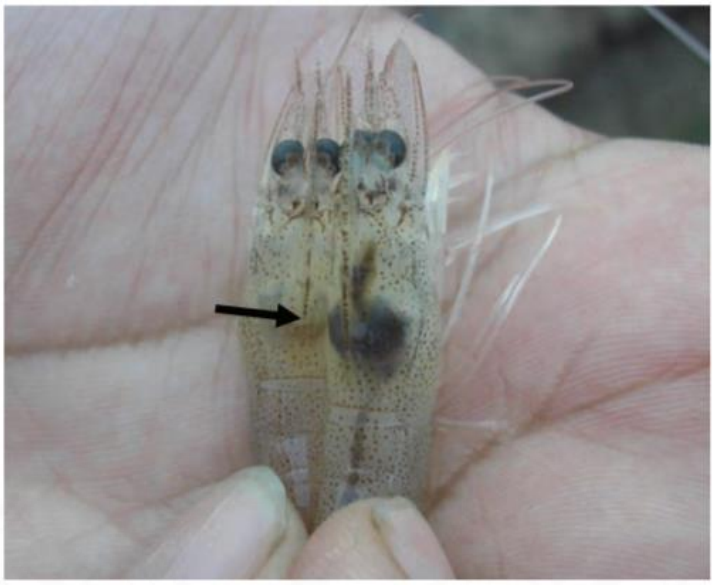

Figure 2.2 Juvenile *L. vannamei* showing gross signs of EMS-AHPND, specifically a pale, atrophied hepatopancreas and an empty stomach and midgut (FAO, 2013).

The receptor-binding mechanism of EMS-AHPND (PirA/PirB toxin) in *L. vannamei* begins when AHPND-causing strains of *V. parahaemolyticus* invade the shrimp host and simultaneously secrete the $PirAB^{VP}$ toxin. These toxins subsequently attach to the LvAPN1 receptor on shrimp hemocyte membranes, potentially inducing oligomerization of the $PirAB^{VP}$ complex. This interaction facilitates pore formation and insertion into the cell membrane, ultimately resulting in morphological alterations of hemocytes and hemocyte lysis. In addition, knockdown of *Lv*APN1 significantly decreased shrimp mortality, mitigated histopathological lesions in the hepatopancreas, and reduced the level of virulent VP_{AHPND} bacterial population in the stomach following challenge with VP_{AHPND} toxins. Furthermore, silencing *Lv*APN1 prevented severe damage to hemocytes and maintained both the total hemocyte count (THC) and the proportion of viable hemocytes. This result concluded that *Lv*APN1 is implicated in the pathogenesis of AHPND, serving as a receptor for VP_{AHPND} toxins and mediating their penetration into hemocytes (Luangtrakul et al., 2021).

2.3 Molecular markers

Molecular markers are genetic tools that enable the detection and analysis of DNA-level variation among individuals, populations, and species. In fisheries and aquaculture, they have revolutionized the understanding of genetic diversity, population structure, and evolutionary relationships, providing critical insights for resource management, stock assessment, and conservation programs (Liu & Cordes, 2004). Commonly used markers comprise microsatellites, single nucleotide polymorphisms (SNPs), and mitochondrial DNA sequences, each offering distinct advantages in terms of polymorphism detection, inheritance patterns, and resolution (Ovenden et al., 2015).

Molecular markers are commonly grouped into three categories depending on how they are detected and the extent of polymorphism revealed: (1) Hybridization-based markers, for example, restriction fragment length polymorphisms (RFLPs), which rely on DNA digestion and probe hybridization. These were the earliest DNA marker systems and depend on hybridization of labeled probes to DNA fragments separated by gel electrophoresis (Karakaş, 2024; Özbek, 2024); (2) Markers based on PCR techniques, such as AFLPs, RAPDs, and simple sequence repeats (SSRs or microsatellites), which exploit the amplification of target DNA sequences using specific or arbitrary primers. PCR-based markers are commonly employed due to their requirement for smaller quantities of DNA and no radioactivity, offering higher throughput and reproducibility (Karakaş, 2024); and (3) Sequence-based markers, for example single nucleotide polymorphisms (SNPs), which directly examine nucleotide variations within DNA sequences through sequencing technologies. Each marker type offers distinct advantages and limitations in terms of reproducibility, cost, throughput, and informativeness. For instance, SSRs are characterized by their high polymorphism and co-dominant inheritance, thus making them useful for population structure studies, Single nucleotide polymorphisms (SNPs), the most frequent genetic variants, are ideal for large-scale genotyping and GWAS (Agarwal et al., 2008; Davey et al., 2011). The choice of molecular markers is guided by research objectives, resource availability, and the genomic features of the target species.

2.3.1 microsatellite markers

Microsatellites, alternatively termed short tandem repeats (STRs) or simple sequence repeats (SSRs), are composed of tandemly repeated DNA motifs ranging from 2 to 6 base pairs in length (Figure 2.3) (Thitaram et al., 2008). Microsatellites are generally considered among the most powerful and popular genetic markers for evaluating genetic diversity, commonly used in studies of population genetics and conservation biology, and evolutionary biology. Owing to their co-dominant inheritance, high polymorphism, ease of genotyping, and Mendelian transmission, microsatellites are particularly well suited for analyzing population structure, conducting pedigree assessments, and distinguishing closely related species. In fisheries and aquaculture, microsatellite-based analyses provide critical insights into genetic diversity and population dynamics, thereby supplying essential information for developing effective conservation strategies and sustainable management practices (Abdul-Muneer, 2014).

In aquaculture, microsatellite markers have been widely employed for diverse applications. For example, they have been used to evaluate the genetic diversity of wild populations of Kuruma prawn (Zhang et al., 2023), selected strains of whiteleg shrimp (Zhang et al., 2023), wild and domesticated black tiger shrimp (Wong et al., 2021), and giant freshwater prawn (Ibrahim et al., 2025). Applied to assess for parentage and pedigree analysis in whiteleg shrimp (Ren et al., 2022), black tiger shrimp (Zhu et al., 2017), Chinese shrimp (Dong et al., 2006). Despite the emergence of high-throughput sequencing technologies, microsatellites remain highly relevant in fisheries genetics due to their cost-effectiveness, reproducibility, and ability to detect fine-scale population differentiation.

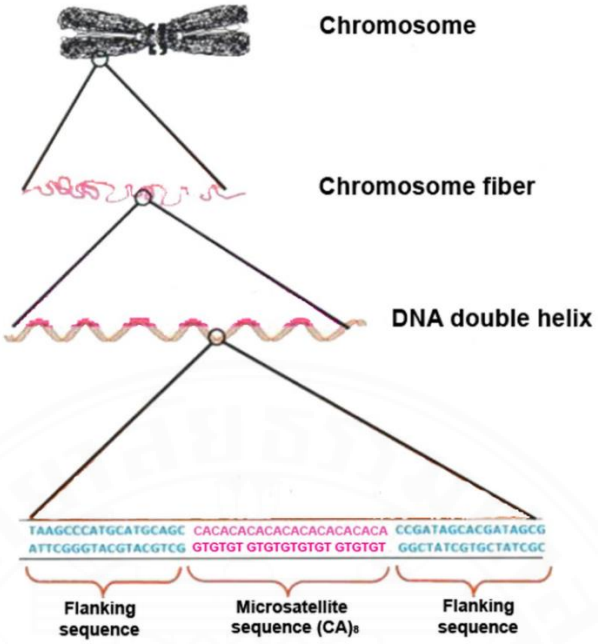

Figure 2.3 Diagram illustrating microsatellites, or variable number of tandem repeats (VNTRs), located at a specific locus within the chromosomal nuclear DNA (Thitaram et al., 2008).

2.3.2 Single nucleotide polymorphism (SNP) markers

SNPs represent one of the most frequent forms of genetic variation in the genome, defined by the criterion that the minor allele occurs at a frequency of at least 1%, distinguishing them from rare variants. Therefore, indels, which involve single-base insertions or deletions, are not formally considered SNPs (Brookes, 1999).

Most SNPs are located in the noncoding DNA between genes, but they can also are located within genes and regulatory regions, where they may directly influence gene function and lead to detectable phenotypic variation. Additionally, SNPs may assist in uncovering novel genes and their functions through alterations in gene expression and promoter activity at both the transcriptional and translational levels (Rafalski, 2002). In some cases, the positions of the substituted amino acids are critical, because certain residues govern the activity of the expressed regions (Morgil et al., 2020). SNP occurrence rates (density) reported in various aquaculture species. For example, the estimated SNP density is approximately one SNP per 500-600 base pairs

in Atlantic salmon (Lien et al., 2016), one SNP per 463 bp in Nile tilapia (Hong Xia et al., 2015), and one SNP per 300 bp in oyster (Zhang et al., 2012).

The SNP density in the *L. vannamei* genome is substantially higher than that observed in chickens (Rubin et al., 2010) and pigs (Groenen et al., 2012) and is comparable to that of the oyster (Zhang et al., 2012), a species characterized by exceptionally high heterozygosity. The majority of SNPs (86.68%) are located within intergenic regions, whereas coding regions exhibit markedly lower genetic diversity than intronic regions. Within the coding sequences, a total of 261,056 synonymous and 206,026 nonsynonymous SNPs were detected. In addition, about 1,400 SNPs were found at splice acceptor sites and 1,452 at splice donor sites. This collection is currently the most comprehensive set of high-quality SNPs for *L. vannamei*, serving as a valuable resource for future genetic research and breeding programs (Zhang et al., 2019).

In shrimp farming, SNP markers are widely applied to evaluate genetic diversity and population structure (Kijas et al., 2025; Li et al., 2021; Medrano-Mendoza et al., 2023; Vu et al., 2020; Vu et al., 2021), parentage assignment (Phuthaworn et al., 2023; Sellars et al., 2012; Silva et al., 2022; Yang et al., 2025), detect quantitative trait loci (QTLs) (Chen et al., 2022; Chen et al., 2024; Guo et al., 2019; Li et al., 2025; Zeng et al., 2020), and conduct genome-wide association studies (GWAS) (Fu & Liu, 2022; Garcia et al., 2021; Lyu et al., 2021).

2.4 Genotyping-by-sequencing (GBS)

Recent developments in next-generation sequencing (NGS) have enabled the development of high-throughput genotyping-by-sequencing (GBS) approaches, which simultaneously detect and genotype single nucleotide polymorphisms (SNPs) through whole-genome or reduced-representation sequencing. These methods have greatly lowered the cost of SNP discovery and genotyping in non-model species (Andrewset al., 2016; Elshire et al., 2011). As a result, GBS has become increasingly popular in aquaculture genetics (Robledo et al., 2018).

The most widely used GBS methods include steps for library preparation that generate deep sequencing data from a consistent subset of genomic regions, typically by employing a single or pair of restriction enzymes (RE). More recently,

new GBS approaches utilizing targeted sequencing have also been developed. The rationale for reducing genome complexity is that performing high-coverage genomic sequencing in a standard aquaculture species at sufficient depth to accurately call genotypes remains prohibitively costly given the large numbers of animals needed for high-resolution genetic studies and breeding program applications. Using restriction enzymes (RE) to reduce genome complexity offers a rapid and cost-effective alternative (Robledo et al., 2018).

GBS demands stricter quality control (QC) measures to ensure the data produced are reliable. This need arises mainly from the molecular characteristics of GBS, which can lead to issues such as missing genotype calls or the occurrence of spurious (false) genotypes, especially when sequencing depth is inconsistent or coverage across loci is incomplete. Without applying stringent filtering procedures and comprehensive quality control (QC) measures, these issues can undermine the accuracy of the dataset. If left unaddressed, such errors may lead to misleading biological interpretations, such as biased or inaccurate estimates of critical population genetic parameters, including genetic diversity, population structure, and relatedness among individuals (Andrews et al., 2016; Gorjanc et al., 2015).

Common sources of genotyping error in GBS include sequencing base-call errors, misalignment or clustering issues, and null alleles caused by low sequencing depth or mutations at restriction enzyme recognition sites during library preparation. These challenges are particularly pronounced in aquaculture species, many of which have highly polymorphic and repetitive genomes. Such genomic features increase the risk of erroneous genotypes, a problem that has been especially noted in crustaceans and oysters (Zenger et al., 2018).

The GBS procedure begins by measuring genomic DNA (gDNA) with a fluorescence-based method to determine its quantity, followed by adjusting the samples in a new plate so that all have equal concentrations of DNA and adapters. The DNA is then cut with restriction enzymes and buffer, and barcoded adapters, along with ligase and ligation buffer, are added to attach unique barcode sequences to each sample. After this, the samples are pooled together and cleaned to remove excess reagents, and the combined DNA library is amplified using PCR. The amplified library is cleaned again and analyzed with a capillary sizing system to confirm the correct

fragment sizes before sequencing. After sequencing, the raw data in FASTQ files are processed by sorting the reads according to their barcodes to assign them to individual samples. These sorted reads are aligned to a reference genome or, if no reference is available, compared with each other to identify single nucleotide polymorphisms (SNPs) based on small (1-2 base pair) differences. Finally, filtering steps are applied to distinguish true SNPs from sequencing errors (figure 2.4) (Poland & Rife, 2012).

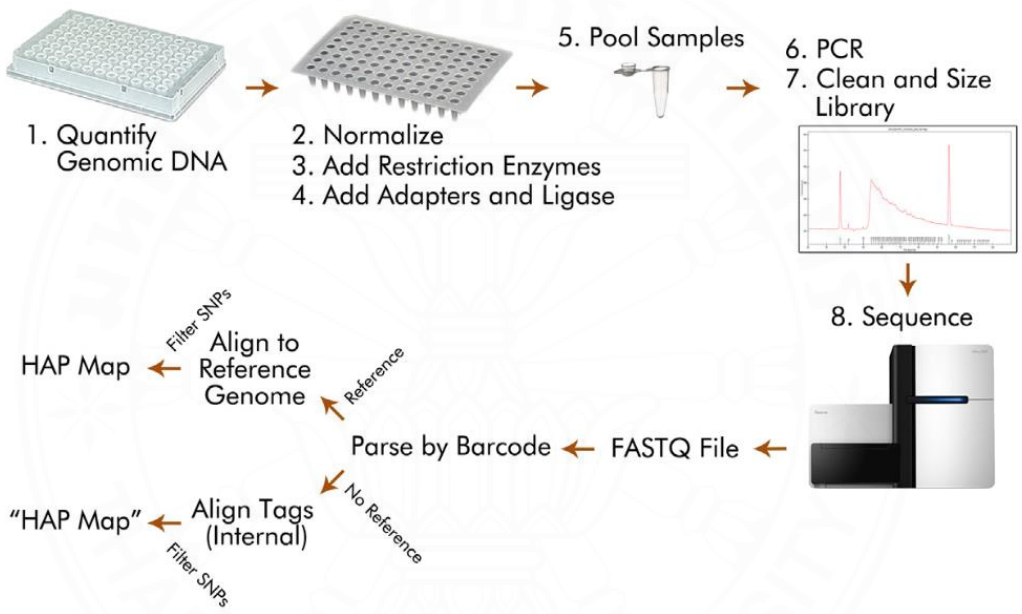

Figure 2.4 A schematic overview of the steps involved in GBS (Poland & Rife, 2012).

2.5 Association mapping

Association mapping, which is also referred to as linkage disequilibrium (LD) mapping, has emerged as a recent method and promising genetic approach for dissecting complex traits. By utilizing historical recombination events across diverse natural populations, this method offers the potential for higher mapping resolution, It facilitates gene-level mapping in non-model species where traditional linkage approaches may not be feasible. Association mapping utilizes ancestral recombination events and the existing genetic variation within a population to study quantitative traits,

based on the concept of linkage disequilibrium (Ersoz et al., 2007). Association mapping in aquaculture primarily uses single nucleotide polymorphisms (SNPs) as genetic markers due to their abundance, genome-wide coverage, and ease of genotyping. Genome-wide association studies (GWAS) are the most widely employed method, scanning the entire genome for associations between SNPs and phenotypic traits (Mastrochirico-Filho et al., 2020; Tsai et al., 2015; Zhao et al., 2023).

The advantage of association mapping is that there is no need to create a population, can use natural populations cultured population or population from breeding. The process of association mapping study consisted of 5 steps including of selecting a sample from the population, determining the level and influence of the population structure in the sample studied, identifying the phenotype data of the traits, genotyping the genes associated with the trait of interest or performing genotyping across the entire genome, and to assess the relationship between genotype and phenotype (Gomez et al., 2011).

However, challenges remain, such as population structure confounding (Xu & Shete, 2005). When performing association mapping, it is important to consider the effects of population structure and kinship (family relationships) within the studied population. If these factors are ignored, they can cause errors in estimating how genes affect traits and may create false associations between DNA markers and the traits of interest. To address this, a mixed model is often used. In this approach, population structure (Q-matrix) and kinship (Kinship) are included as variables in the analysis to reduce false positives. Understanding the relationship between genetic variation and important traits is crucial, and the information gained can be valuable for applying genomics research in food production industries (Abdelrahman et al., 2017). For other challenges, including small sample sizes and low frequency alleles difficult to detect, functional validation needed (Myles et al., 2009), and the need for functional validation of candidate loci (Xia et al., 2013). For polygenic traits influenced by many small-effect variants (Sinclair-Waters et al., 2020), machine learning (Luo et al., 2024) and multi-locus models can improve detection power and predictive accuracy (Getahun et al., 2025).

Association mapping can be performed using two main approaches. The first, genome-wide association mapping (GWAS), analyzes genetic variation across the entire genome to identify relationships between DNA markers and phenotypic traits, utilizing markers distributed throughout the genome. The second approach, candidate gene association mapping, focuses on polymorphisms within specific genes that are involved in biological pathways relevant to the trait of interest (Kumar et al., 2017).

Association mapping has been successfully applied to a variety of aquaculture species, demonstrating its value for genetic improvement programs. As for crustaceans, GWAS reports identifying SNPs or genes linked to key traits are limited. Using RAD sequencing of a biparental family, GWAS for growth in *L. vannamei* identified two candidate genes: protein kinase C delta type and ras-related protein Rap-2a (Yu et al., 2019). Another GWAS using 2b-RAD sequencing of 200 *L. vannamei* identified 23,049 SNPs and class C scavenger receptor as a growth-related candidate gene (Wang et al., 2019). With the genome assembled, Wang et al. (2019) identified and validated MMD2 as another growth-related candidate gene (Wang et al., 2020). A GWAS of 94,113 SNPs in 200 breeding shrimp identified four SNPs on LG19 and LG39 associated with body weight, with further analysis linking deoxycytidylate deaminase and non-receptor protein tyrosine kinase to this trait (Lyu et al., 2021). In addition to using GWAS to study growth traits, research has also been conducted on white spot syndrome virus (WSSV) resistance (Lyu et al., 2021; Sun, 2021), and ammonia nitrogen tolerance (Fu & Liu, 2022).

CHAPTER 3
EVALUATION OF GENETIC DIVERSITY IN FOURTH-GENERATION SELECTIVE BREEDING LINES OF PACIFIC WHITE SHRIMP USING MICROSATELLITES

3.1 Introduction

Pacific white shrimp is a key species in aquaculture worldwide. In Thailand, the shrimp industry plays a pivotal role in contributing to the country's economy by generating employment opportunities and income for millions of people. Approximately 40% of the overall aquaculture production in Thailand is attributed to shrimp farming (Sampantamit et al., 2020). Continued investment in selective breeding programs along with the implementation of advanced breeding technologies are essential for further advancing the genetic potential of shrimp stocks and to maintain the long-term sustainability and profitability of Thailand’s shrimp farming industry and globally. These efforts contribute to improving shrimp traits such as growth rate, resistance to disease and tolerance to environmental stressors, thereby enhancing productivity and profitability in the aquaculture industry. The prolonged practice of selective breeding, especially if carried out without proper management strategies, can lead to a decrease in genetic diversity within farmed shrimp populations. Diminished genetic variability can have several negative consequences, including increased risk of disease and decreased ability to adapt to shifting environmental conditions, and decreased overall resilience of the population. Moreover, limited genetic diversity may hinder future breeding efforts aimed at introducing new desirable traits or addressing emerging challenges in shrimp farming (Vaseeharan et al., 2013). In a population with high genetic variability, there is a greater likelihood of individuals possessing alleles that confer advantageous traits, such as disease resistance, tolerance to environmental stress, or enhanced growth rates. Regular monitoring of genetic variation is essential for shrimp breeders to detect any reductions in variability that may arise due to factors such as genetic drift, inbreeding, or selection pressures. Assessing genetic variation enables breeders to make well-informed decisions regarding breeding strategies , such

as selecting appropriate breeding pairs to maximize genetic diversity in offspring, introducing new genetic material from wild populations or other sources to increase variability, and implementing managed breeding programs to minimize the accumulation of deleterious genetic traits.

Recently, molecular markers are valuable tools in breeding programs for monitoring genetic variation over generations. Microsatellite markers also referred to as Simple Sequence Repeats (SSRs), are invaluable tools in genetic research and breeding programs across various species. These markers are characterized by short, tandemly repeated DNA sequences, typically consisting of 1-6 base pairs in length. Microsatellites are co-dominantly inherited in Mendelian patterns, meaning that both alleles at a given locus are expressed in heterozygotes (Zane et al., 2002). These markers are extensively employed for tasks such as estimating genetic distances, building phylogenetic trees, and assessing genetic diversity among species. The microsatellite markers have been employed for tracking genetic variation in marine species, including the Pacific oyster (Chen et al., 2022), summer flounder (Wirgin et al., 2022). In addition, the microsatellite markers also can be used to evaluate genetic diversity in captive populations across various generations and farmed populations of *Oreochromis niloticus* (Diyie et al., 2021). Multiple studies have provided explicit evidence of the diminishing genetic variation in cultured stocks across successive generations, as observed in *Penaeus stylirostris* (Bierne et al., 2000), *Penaeus monodon* (Xu et al., 2001) and *L. vannamei* (Cruz et al., 2004). So far, numerous microsatellite markers have been discovered and employed to assess the present condition of genetic resources in wild or hatchery populations of shrimp species like *L. vannamei* (Ren et al., 2018), and *P. monodon* (Wong et al., 2021).

In the present study, I employed five microsatellite markers to examine the genetic variation structure of 35 families of fourth-generation (F_4) selective breeding lines of *L. vannamei*. This study aimed to evaluate genetic diversity of the pacific white shrimp breeding lines obtained from the fourth-generation (F_4) selection. The findings from this research will provide breeder with valuable insights into the genetic makeup of selected strains, thus enhancing management approaches for shrimp farming initiatives.

3.2 Materials and methods

3.2.1 Samples and DNA extraction

One hundred eighty nine post larvae (PL20) samples, derived from 35 families of F_4 selective breeding lines of *L. vannamei*, were obtained from the Nakhon Si Thammarat Aquatic Animal Genetic Research and Development Center. The base population originated from two private hatcheries in Krabi province and Songkhla province. This population is 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". Shrimp samples were preserved in 95% ethyl alcohol. Total DNA was extracted from whole tissue samples of shrimp PL20 using the phenol-chloroform method (Sambrook & Russell, 2001). Both the quality and concentration of the extracted DNA were determined through agarose gel electrophoresis and absorbance readings were taken at wavelengths of 260 nm and 280 nm using a spectrophotometer. The collected DNA samples were stored at -20 °C until used.

3.2.2 Microsatellite amplification

The polymerase chain reaction (PCR) was employed to amplify microsatellite loci in the shrimp samples, using 5 specific primers, namely TUMXLv9.178, TUMXLv10.455, TUMXLv10.411, CNM-MG 357 (Pérez et al., 2005) and Lvan17 (Marques et al., 2013) (Table 3.1). PCR reactions were prepared in a total volume of 20 μL, containing 20 ng of genomic DNA, 10 pmol of each primer, 2.1 mM $MgCl_2$, 0.2 mM of each dNTP, and 5 U/μL of Taq polymerase. Amplification was performed using a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with 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. A final extension was carried out at 72°C for 5 minutes. The resulting PCR products were resolved on a 1% agarose gel, and fragment sizes were determined using the ZAG DNA Analyzer System (Agilent Technologies, Santa Clara, CA, USA). The determination of allelic size was carried out utilizing ProSize data analysis software (Agilent Technologies).

Table 3.1 Attributes of the microsatellite markers used in the present study.

Locus ID	Forward and Reverse Primer (5' > 3')	Tm (°C)	Size (bp)
TUMXLv9.178	F: CATTGAAAACGGAATCCTCG R: GATATTCCCATCAACACAGCG	55	188 - 199
TUMXLv10.455	F: AGAGTAGAAGAGGCAGGGCG R: GTCAAGAAGCAGGAAGGGTG	60	237 - 287
CNM-MG 357	F: GCTTGAATCGCTACTGC R: GTTGCTGCCACTCATT	60	283 - 291
Lvan17	F: GTAACATGCCCTCACTCACT R: GTCAAAAGCGCCTTAGTTTA	60	220 - 235
TUMXLv10.411	F: AGCACCTAGCACTTGCTGAAC R: AGAGACTCACATCCTCATCCTC	60	154 - 204

3.2.3 Data analysis

Genetic variation was assessed through the calculation of the number of alleles (N_a), the number of effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and the inbreeding coefficient (F_{is}). These computations were carried out using Popgen32 software (Yeh et al., 1999). The analysis of polymorphic information content (PIC) was conducted using PowerMarker (Liu & Muse, 2005). Principal component analysis (PCA) was applied to illustrate the non-hierarchical relationships among the samples. Eigenvalues and eigenvectors were computed using the EIGEN module, and 2D plot was generated to create the two-dimensional PCO plot. All calculations, modules, and procedures were performed using NTSYS-pc version 2.0 software (Rohlf, 1998). The population structure was assessed using a model-based Bayesian method utilized through the software package STRUCTURE v. 2.3.4 (Pritchard et al., 2000). The analysis was repeated ten times for each K value, ranging from K = 1 to K = 10, using burn-in period of 100,000 iterations. Individuals having a membership probability (Q value) less than 0.6 were considered admixed. The number of sub-populations (ΔK) was inferred using the ad hoc statistic proposed by Evanno et al, (2005). The analysis of molecular variance (AMOVA) and calculation of F_{st} among groups were performed with ARLEQUIN ver 3.1 (Excoffier & Lischer, 2010) with

100,000 permutations, considering both families and clusters suggested by Bayesian analysis.

3.3 Results and discussion

3.3.1 Genetic variation

All microsatellite markers were polymorphic across samples, The degree of polymorphism differed across loci. The assessment of genetic diversity based on the five microsatellite markers for the 189 samples were summarized in Table 3.2. The average PIC was 0.825 ranging from 0.770 to 0.897. Five microsatellite markers displayed a PIC greater than 0.7, signifying high levels of polymorphism.

Identifying genetic diversity is key in germplasm identification. It is widely accepted that the level of genetic diversity detected correlates positively with the viability and evolutionary potential of a population (Botstein et al., 1980). Detecting genetic diversity is especially crucial during selective breeding. Microsatellites serve as dependable molecular tools for this purpose and have been extensively utilized to evaluate genetic diversity levels and population structures. This is of significant importance for conserving germplasm resources and formulating shrimp breeding strategies. In this study, all microsatellite loci analyzed showed high levels of polymorphism with PIC above 0.70. According to Botstein et al. (1980), markers with PIC greater than 0.50 are deemed highly informative. Our results suggest that these microsatellite markers offer strong resolution for individual discrimination and were suitable for population structure analyses.

Genetic diversity is fundamental to shrimp breeding, as high levels of genetic variation are crucial for preserving a rich gene pool that confers advantageous traits, such as enhanced growth rates, disease resistance and, adaptation to changing climates (Zhang et al., 2023). The average number of alleles per locus, or allelic diversity, provides an important measure of genetic variability within a population, provided that sample sizes are similar. In this present study, the average number of alleles over the five loci was 4.400 ranging from 3 to 6, which was lower than reported by Valles-Jimenez et al. (2004). The TUMXLv9.178 locus had the most alleles (6 alleles), which is higher than the number reported by Meehan et al. (2003). Similarly,

TUMXLv10.455 had 5 alleles, which is higher than 4 alleles reported by Meehan et al. (2003). In contrast, TUMXLv10.411 had 4 alleles, which is lower than 7 alleles reported by Meehan et al. (2003). CNM-MG 357 had 3 alleles, which is lower than 4 alleles reported by Pérez et al. (2005). Lvan17 had 4 alleles, which is lower than 11 alleles reported by Marques et al. (2013). According to the number of alleles as a key indicator of genetic diversity, our results were similar to those of Klongklaew and Songsangjinda (2016), who studied the genetic diversity of *L. vannamei* broodstock from six populations: four from department of fisheries hatcheries (DOF) and two from private hatcheries in Thailand. Notably, the broodstock from the DOF Hatchery, Nakhon Si Thammarat province (WH06), which was included in their study, is the same population analyzed in this study. The WH06 broodstock had average number of alleles per locus (*N*a) of 4.71±1.38, while the *N*a value in this study is 4.400±1.140. These results indicated that the population possessed a moderate genetic diversity. Differences in the number of alleles may result from the different populations used in the studies.

The number of effective alleles per locus found in this work varied from 2.809 to 4.598 and was lower than the observed number of alleles in all locus (3-6 alleles). After seven generations of selection, similar patterns emerged, with a total of four alleles but an effective number of only 1.6. (Wolfus et al., 1997). This suggests that while many different alleles may be present, they are not equally frequent. Some alleles are much more common than others, probably due to selective breeding during each generation's selection process. As breeding programs focus on selecting individuals with enhanced performance across key traits, these results reflect a decline in genetic diversity. In order to broaden genetic diversity, to enhance genetic variability, the breeding stock should be supplemented by crossing with genetically distinct lineages from external sources.

In all cases, H_o values were lower than the values for H_e. The average inbreeding coefficient (F_{is}) was 0.336 ranging from 0.119 to 0.617. Differences in H_o, and H_e values reflect variations among the populations. Huang et al. (2019) reported similar levels for these parameters for the F_1 generation of seven introduced populations in China. Zhang et al. (2023) also reported comparable values. All loci show a heterozygote deficit caused by inbreeding, explaining most of the positive F_{is} values found in this study. This is commonly found in hatchery populations due to selection or inbreeding (Chareontawee et al., 2007). The average value of F_{is} was 0.336, indicating a moderate

level of inbreeding within this population, and suggesting that there is some degree of relatedness among individuals but it is not excessively high. Similar to the studied by Lima et al. (2010), which reported average F_{is} values of 0.38 for hatchery A and 0.25 for hatchery B, both of which were *L. vannamei* hatcheries located in Pernambuco, Brazil. This level of inbreeding may lead to a reduction in genetic diversity and could have potential implications for the health and viability of the population.

Table 3.2 Summary statistics of genetic variabilities detected in five microsatellite loci among 189 samples, derived from 35 families of fourth-generation (F_4) selective breeding lines.

NO.	Locus ID	Genetic variabilities				PIC	F_{is}
		(N_a)	(N_e)	(H_o)	(H_e)		
1	TUMXLv9.178	6	4.598	0.492	0.784	0.897	0.247
2	TUMXLv10.455	5	4.595	0.492	0.784	0.897	0.265
3	CNM-MG 357	3	2.809	0.450	0.646	0.778	0.136
4	Lvan17	4	3.467	0.209	0.714	0.770	0.610
5	TUMXLv10.411	4	3.282	0.287	0.697	0.782	0.449
	Mean	4.400	3.734	0.385	0.724	0.825	0.336
	±SD	1.140	0.804	0.135	0.060	-	-

N_a: number of alleles; N_e: number of effective alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; PIC: polymorphism information content; F_{is}: inbreeding coefficient.

3.3.2 Population structure

The results of AMOVA among families showed that the total genetic variation primarily occurred within individuals (54.78%), and among individuals within families (37.48%), while there was low diversity among families (7.74%). The F_{st} value of 0.077 there was no significant differentiation among the 35 families ($p > 0.05$) (Table 3.3). Population structure analyses using an admixture model identified K = 2 as the most plausible number of clusters, based on the Evanno criteria. (Figure 3.1). This suggests that our panel could be split into two clusters, cluster 1 (red bars) and cluster 2 (green bars) (Figure 3.2). When performing AMOVA by grouping

populations based on the results from the model-based Bayesian method (STRUCTURE software program), it was found that the total genetic variation primarily occurred within individuals (51.44%) and among individuals within populations (35.73%), while there was low diversity among populations (12.83%). The F_{st} value of 0.128 was significant (p<0.05) differentiation among populations (Table 3.3). The PCA yielded comparable results to the STRUCTURE analysis (Figure 3.3). Subpopulation 1 (red) comprised of 94 samples. Subpopulation 2 (green) comprised of 85 samples. Ten samples (orange), displayed a membership probability (Q value) ranging from 0.4 to 0.6 and were characterized as admixtures.

Table 3.3 Analysis of molecular variance (AMOVA).

Source of variation	**d.f.**	**Percentage of variation (%)**	**Fixation Index**	***P*-value**
Among families				
Among populations	34	7.74		
Among individuals within	154	37.48	*F*st = 0.077	0.130
Within individuals	189	54.78		
Classify populations according to the results from STRUCTURE				
Among populations	1	12.83		
Among individuals within	187	35.73	*F*st = 0.128	0.000*
Within individuals	189	51.44		

*P < 0.00000, after 1023 permutations.

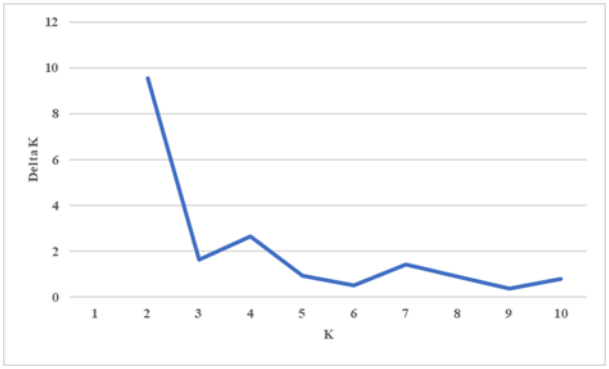

Figure 3.1 Number of subpopulations calculated following the Evanno criteria.

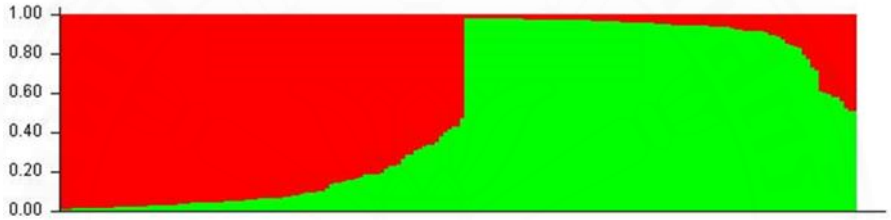

Figure 3.2 Neighbor-joining tree based on Nei's genetic distances was colored according to membership probability (Q value) from STRUCTURE.

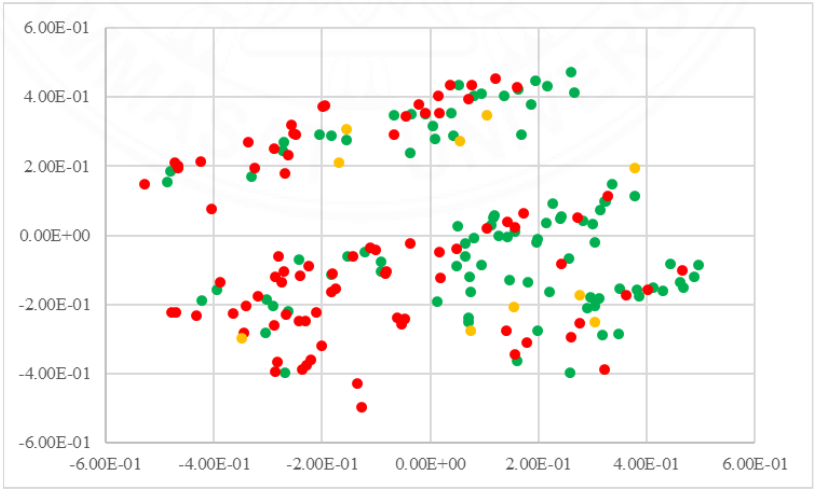

Figure 3.3 Principal Component Analysis (PCA). Subpopulation 1 (red) comprised of 94 samples. Subpopulation 2 (green) comprised of 85 samples. Admixtures (orange) comprised of 10 samples.

3.4 Conclusion

The microsatellites markers used in this study were informative and demonstrated substantial genetic diversity, making them suitable for assessing the genetic differentiation of *L. vannamei* within the fourth-generation (F_4) selective breeding lines. The heterozygosity observed indicates that the studied population possesses moderate genetic diversity. Signs of inbreeding were also obsered, contributing to a reduction in genetic variation within the population and potentially slowing down the response to selection. These results have important implications for managing *L. vannamei* in genetic improvement programs.

CHAPTER 4

ASSOCIATION MAPPING OF EARLY MORTALITY SYNDROME (EMS) - ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND) TOLERANCE IN *LITOPENAEUS VANNAMEI*

4.1 Introduction

The Pacific white shrimp is a commercially important aquaculture species and continues to dominate global production. The rapid growth of the shrimp aquaculture industry over the past decade has been disrupted by the emergence of early mortality syndrome (EMS). Acute hepatopancreatic necrosis disease (AHPND), caused by *Vibrio parahaemolyticus* (*Vp*AHPND), has caused major economic losses in the shrimp industry (Lyu et al., 2020). AHPND is a relatively recent bacterial disease affecting farmed penaeid shrimp. Shrimp production in regions impacted by AHPND has dropped to nearly 60%, and the disease has imposed an estimated global economic burden of USD 43 billion on the shrimp aquaculture sector (Kumar et al., 2021). The disease has imposed significant economic impacts on the shrimp industry, especially in Thailand, Vietnam, and Malaysia, and across regions of South America and the United States (Vandeputte et al., 2024).

*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 (Han et al., 2015; Lee et al., 2015; Yang et al., 2014). The toxins originate in the shrimp's stomach but lead to death by inducing the characteristic AHPND lesion: sloughing of hepatopancreatic tubule epithelial cells (Phiwsaiya et al., 2017). These binary toxins induce severe, rapid mortality in shrimp, generally occurring within the first 35 days following pond stocking (Sanguanrut et al., 2018).

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* (Wang et al., 2019). Progress in genomic technologies has greatly facilitated the identification and utilization of DNA markers, particularly 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) (Yáñez et al., 2023). In *L. vannamei*, markers associated with economically important traits have been identified in several studies using a genome-wide approach, such as growth traits (Lyu et al., 2021; Wang et al., 2017), ammonia nitrogen tolerance (Fu & Liu, 2022), sex (Jones et al., 2020), white spot syndrome virus (WSSV) tolerance (Medrano-Mendoza et al., 2023), and tolerance to acute hepatopancreatic necrosis disease (AHPND) (Wang et al., 2019).

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 (Ibrahim et al., 2020). 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, for example, the availability of a reference genome assembly for the species of interest. (Robledo et al., 2018). GBS necessitates extensive quality control (QC) measures to ensure reliable genotype data.

In this study, GBS was used to genotype 210 individuals of fourth-generation selective breeding lines of *L. vannamei*. The primary objective of this study was the development of SNP markers using GBS and 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.

4.2 Materials and methods

All experimental procedures involving animals were performed in full compliance with the established ethical guidelines for scientific research.

4.2.1 Experimental shrimp challenge tests

Experimental shrimp and *V. parahaemolyticus* challenge tests are conducted to assess the shrimp's tolerance to bacterial infections and understand the pathogen's impact on shrimp health. Three thousand one hundred fifty post-larvae 20 (PL20) samples (35 families, with 90 individuals per family) , derived from 35 families of fourth-generation (F_4) selective breeding lines of *L. vannamei* were obtained from the Nakhon Si Thammarat Aquatic Animal Genetic Research and Development Center and sent it to the Songkhla Aquatic Animal Health Research and Development Center for challenge testing. Each family was cultured separately in designated tanks for seedling rearing. This population is 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 LC_{50} level (1.12×10^4 CFU/mL) in the bioassay tank water. The challenge test continued for a duration of 14 days. The deaths of shrimp were recorded three time/day. Survived 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, 'type,' referred to survival outcome: individuals that died within 3 days after immersion in *V. parahaemolyticus* 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 and was categorized into three intervals: within 22 hours, 28.5 hours, and 32 hours after immersion in *V. parahaemolyticus*. Due to the high mortality rate observed in 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 *V. parahaemolyticus* infection through PCR analysis.

4.2.2 Population structure

The population structure were analyzed using 10 microsatellite loci (Table 4.1), namely TUMXLv9.178, TUMXLv10.455, TUMXLv10.411, TUMXLv8.224, TUMXLv7.121, TUMXLv5.45, TUMXLv7.148 (Meehan et al., 2003), CNM-MG 357 (Pérez et al., 2005), Lvan17 (Marques et al., 2013), and TUSWLvSU233 (Alcivar-Warren et al., 2007). 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 $MgCl_2$, 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 final extension at 72°C for 5 minutes. Subsequently, 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 utilizing 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 (Pritchard et al., 2000). 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. (2005). Pairwise kinship coefficients were estimated with SPAGeDi version 1.5a (Hardy & Vekemans, 2002). Negative values between individuals were adjusted to 0, indicating that their relationship is weaker than that of random individuals (Matthies et al., 2012).

Table 4.1 Attributes of ten microsatellite markers.

Locus ID	Forward and Reverse Primer (5' > 3')	Tm (°C)	Size (bp)
TUMXLv9.178	F: CATTGAAAACGGAATCCTCG	55	182 - 210
	R: GATATTCCCATCAACACAGCG		
TUMXLv10.455	F: AGAGTAGAAGAGGCAGGGCG	60	217 - 367
	R: GTCAAGAAGCAGGAAGGGTG		
CNM-MG 357	F: GCTTGAATCGCTACTGC	60	278 - 331
	R: GTTGCTGCCACTCATT		
Lvan17	F: GTAACATGCCCTCACTCACT	60	193 - 299
	R: GTCAAAAGCGCCTTAGTTTA		
TUMXLv8.224	F: TCGTGCGGTGAAATATAGGC	52	230 - 352
	R: TGAATGTCCCGTTGATTGAC		
TUSWLvSU233	F: CCCGACTTGGCTTTTAGTTG	55	292 - 310
	R: GAGATTGCTATCCTCGGCTG		
TUMXLv7.121	F: GGCACACTGTTTAGTCCTCG	60	149 - 244
	R: CGAACAGAATGGCAGAGGAG		
TUMXLv5.45	F: TTTGTCGTTTGTCTTTCTCC	60	151 - 198
	R: AGTAACTTACGTGAATGCTTGG		
TUMXLv10.411	F: AGCACCTAGCACTTGCTGAAC	60	151 - 215
	R: AGAGACTCACATCCTCATCCTC		
TUMXLv7.148	F: CATCGCTAAAATTCCGAAGC	55	179 - 312
	R: TAAAAATGAGGGGGTTGGAG		

4.2.3 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 the manual instruction. 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 method, performed by Novogene Co., Ltd. DNA was digested by *Mse*I 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 (Zhang et al., 2019) with BWA under default parameters, followed by downstream processing such as duplicate removal, was preformed using SAMtools and PICARD (http://picard.sourceforge.net). Screened SNPs were then mapped to the genome of *L. vannamei* (ASM378908v1) (Zhang et al., 2019) employed in this study.

4.2.4 Association analysis

The associations between SNPs markers and EMS-AHPND tolerance traits were analyzed using Fixed and random model Circulating Probability Unification (FarmCPU) (Liu et al., 2016) integrated in Memory efficient, Visualization-enhanced, and Parallel-accelerated R package (rMVP) (Yin et al., 2021). To account for population stratification and cryptic relatedness, the kinship coefficient (K) and Q matrix were incorporated as covariates. Model fit was examined through QQ plots generated in R using the 'qqman' package. The significance threshold for declaring a significant association was set at the Bonferroni-correction at 0.05 (*P*-value ≤ 5.26×10^{-6}) (Bonferroni, 1936). A Manhattan plot visualizes the statistical significance of associations as −log10 (*P*-value) on the y-axis, plotted against scaffold reference ID coordinates on the x-axis. Each dot represents a single variant, and a higher -log10 (*P*-value) indicates a stronger association between the corresponding SNP and the phenotype.

4.3 Results and discussion

4.3.1 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. (2024), who reported high mortality rates in shrimp within 2-3 days after infection. In the present study, 15% of shrimp survived until day 14 post-infection, which was notably higher than the 5% survival rate reported by Whankaew et al. (2024) 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. (2024).

4.3.2 Population structure

To avoid dependencies among model terms and prevent population structure estimates from absorbing QTL effects, an independent set of microsatellite markers was used to infer population structure in the present study (Pritchard et al., 2000). Population structure simulations under an admixture model identified K = 2 as the most plausible cluster number according to the Evanno criteria (Figure 4.1). This suggests that our panel could be split into two clusters, cluster 1 (red bars) and cluster 2 (green bars) (Figure 4.2). 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. (2019), 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*.

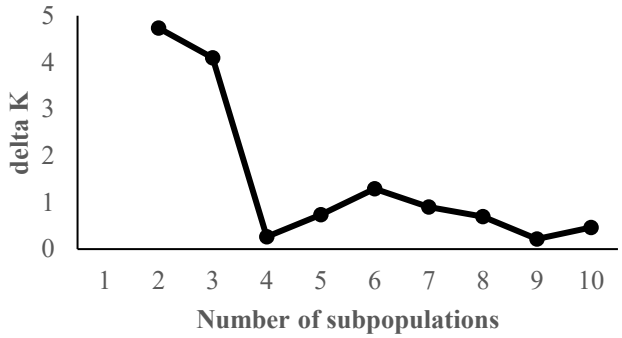

Figure 4.1 The number of subpopulations was determined using the Evanno method, with Delta K values indicating a distinct peak at two clusters.

Figure 4.2 A population genetic structure diagram, generated by the STRUCTURE program with K = 2.

4.3.3 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 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. (2024), 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. (2020), 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.

4.3.4 SNPs associated with EMS-AHPND tolerance

The effectiveness of AM in identifying true associations depends on its ability to separate marker-QTL linkage disequilibrium (LD) from LD caused by confounding factors such as population structure, family relatedness, selection, genetic drift, inbreeding, and admixture (Sun et al., 2013; Wang et al., 2019; Zhang et al., 2019). 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 (Figure 4.3A) and score (Figure 4.3B), which showed a straight line with a sharply deviated tail. The FarmCPU+K+Q model efficiently controlled 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 (Figure 4.3C and 4.3D) and summarized in Table 4.2. 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 enhance the reliability of the identified associations. For instance, Whankaew et al. (2024) reported no significant variants associated with AHPND tolerance in 93 *L. vannamei* individuals under the Bonferroni threshold. Similarly,

Fu and Liu (2022) 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 4.2). 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. (2024) 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%) (Peng et al., 2020) and growth traits (5.51-9.65%) (Ma et al., 2025). 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. (2024) similarly reported that most candidate InDels associated with AHPND tolerance in *L. vannamei* were located in intergenic regions. Likewise, Fu and Liu (2022) 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. (2020) 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 (Hoogendoorn et al., 2004; Mishiro et al., 2009; Wagschal et al., 2015).

Among the seven SNPs associated with tolerance to EMS-AHPND, only five showed a significant distinction between the genotypes of dead and servied based on chi-square analysis. SNP7640 and SNP5982 exhibited genotype patterns that did not clearly differentiate between dead and survied shrimp (Table 4.3). Several factors may explain why SNPs identified as statistically significant in association mapping do not clearly differentiate genotypic classes between phenotypic groups. First, significant SNPs may not represent causal variants but instead tag nearby functional mutations through linkage disequilibrium (LD) (Flint-Garcia et al., 2003; Slatkin, 2008), which can vary across populations and reduce genotype-phenotype resolution. For complex, polygenic traits such as disease resistance in shrimp, individual SNPs typically explain only a small proportion of phenotypic variance, further contributing to weak genotype clustering (Hill, 2010; Houston et al., 2020).

Table 4.2 Markers associated with EMS-AHPND tolerance in *L. vannamei* identified by FarmCPU+K+Q analysis.

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.

Table 4.3 Chi-square statistical analysis of SNPs with genotype patterns associated with surviving and dead shrimp samples.

SNP	Genotype/Allele		Dead		Survied		Total		Chi-Square	*P*-value
			number	%	number	%	number	%		
SNP8225	Genotype	CC	20	9.52	92	43.81	210	100	99.18	2.30E-23*
		CA	85	40.48	13	6.19				
	Allele	C	125	29.76	197	46.90	420	100	69.00	9.86E-17*
		A	85	20.24	13	3.10				
SNP6998	Genotype	GG	98	46.67	51	24.29	210	100	51.04	9.06E-13*
		GA	7	3.33	54	25.71				
	Allele	G	203	48.33	156	37.14	420	100	42.37	7.57E-11*
		A	7	1.67	54	12.86				
SNP3072	Genotype	GG	20	9.52	2	0.95	210	100	75.569	3.89E-13*
		GA	30	14.29	92	43.81				
		AA	55	26.19	11	5.24				
	Allele	G	70	16.67	96	22.86	420	100	6.73	0.009*
		A	140	33.33	114	27.14				
SNP4253	Genotype	TT	75	35.71	44	20.95	210	100	18.64	1.60E-05*
		TC	30	14.29	61	29.05				
	Allele	T	180	42.86	149	35.48	420	100	13.48	2.41E-04*
		C	30	7.14	61	14.52				
SNP5255	Genotype	GG	92	43.81	72	34.29	210	100	11.14	0.001*
		GA	13	6.19	33	15.71				
	Allele	G	197	46.90	177	42.14	420	100	9.77	0.002*
		A	13	3.10	33	7.86				

* Indicates that the number of surviving and dead shrimp is significantly associated with the observed genotype/allele patterns ($P < 0.01$).

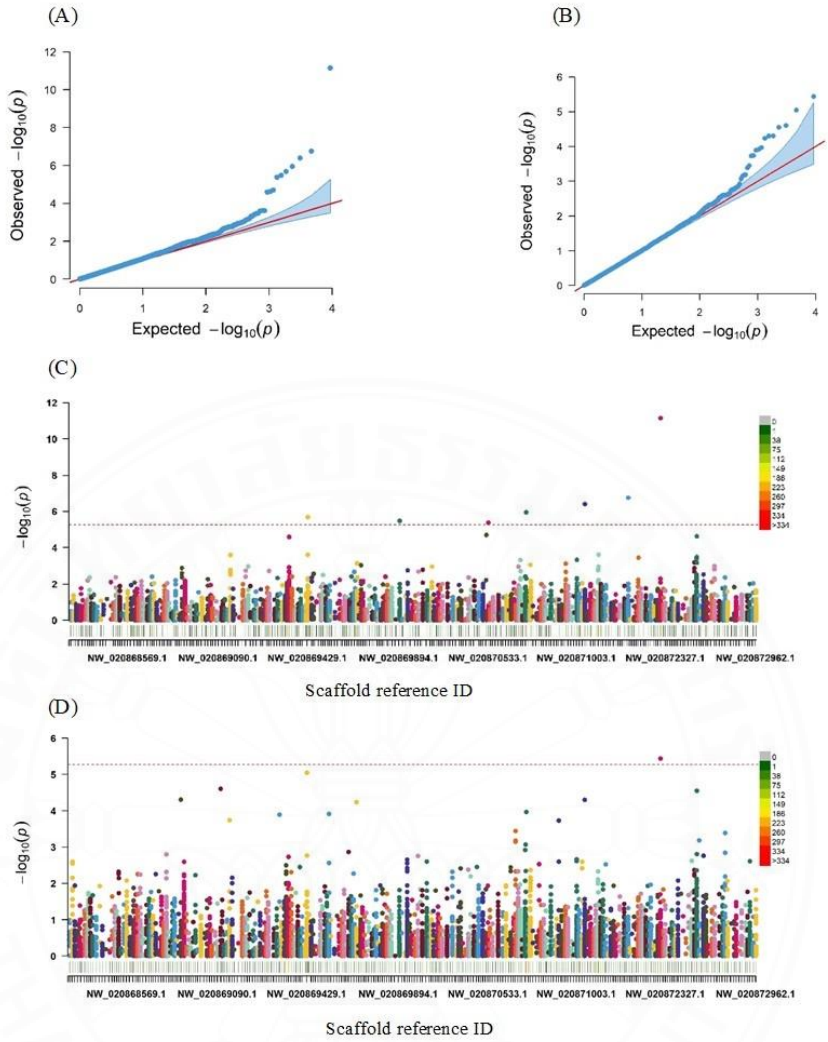

Figure 4.3 Quantile-Quantile (QQ) plots and Manhattan plots of EMS-AHPND tolerance in terms of type (survival and mortality) and score (survival and rate of time to death). (A) and (B) QQ plot of type and score, respectively. The X-axis represents the -log₁₀-transformed expected *p*-values, while the Y-axis represents the -log₁₀-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 -log10 *p*-values. The red dotted line represents the significance threshold adjusted using Bonferroni correction ($P = 5.26\times10^{-6}$).

4.4 Conclusion

In this study, I 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.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

In this study, microsatellite markers were used to assess the genetic variation and population structure of 35 families of fourth-generation (F_4) selective breeding lines of *L. vannamei*. This population was found to have a moderate level of genetic diversity. Additionally, signs of inbreeding were also observed, which contributed to a reduction in genetic variation within the population and may slow the response to selection. Recommendations for enhancing genetic diversity in the breeding process include the introduction of strains from diverse sources or distinct base populations for crossbreeding, as well as the maintenance of an adequate effective population size, since a small population size may result in elevated levels of inbreeding. The population structure analysis revealed that the 35 families were grouped into two subpopulations (K=2), corresponding to the origins of the base population obtained from two private hatcheries. The findings of this study will offer breeders valuable insights into the genetic composition of selected strains, thereby improving management strategies for Pacific white shrimp farming. Association mapping analysis of SNPs related to EMS-AHPND tolerance in *L. vannamei* using GBS. The FarmCPU+K+Q model provides a reliable and robust framework for association mapping in this study. Seven SNPs were identified as significantly associated with EMS-AHPND phenotypes, with their *P*-values exceeding the Bonferroni-corrected significance threshold. SNP8225 exhibited the strongest association with both phenotypic traits, namely type and score. This study offers a valuable genetic resource for enhancing EMS-AHPND tolerance in *L. vannamei*. However, all seven SNPs should be validated and further examined in additional populations to confirm their suitability for future applications. Overall, this study provides a valuable genomic resource and a new strategy for improving *L. vannamei* in Thailand.

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APPENDICES

APPENDIX A
THE TOP 50 SNP MARKERS FROM THE GLM MODEL

Table A The top 50 SNP markers obtained from the GLM model in term of type (survival and mortality) and score (survival and rate of time to death)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
1	SNP8850	NW_020872599.1	370124	T	A	0.195	0.72	0.051	1.361E-32	
2	SNP8225	NW_020872260.1	440338	C	A	0.262	-0.668	0.052	1.927E-28	
3	SNP8698	NW_020872599.1	310456	C	T	0.193	0.673	0.054	3.673E-27	
4	SNP3072	NW_020869429.1	1622620	A	G	0.29	0.64	0.053	5.684E-26	
5	SNP8763	NW_020872599.1	366931	A	T	0.157	0.677	0.056	7.767E-26	
6	SNP7232	NW_020871090.1	495393	A	G	0.252	0.629	0.054	1.711E-24	
7	SNP6998	NW_020871044.1	362854	G	A	0.293	0.638	0.055	1.807E-24	
8	SNP8703	NW_020872599.1	310572	G	C	0.226	0.625	0.055	7.268E-24	
9	SNP3661	NW_020869684.1	350398	C	A	0.269	-0.623	0.055	9.027E-24	
10	SNP8697	NW_020872599.1	310415	C	T	0.152	0.652	0.06	6.341E-22	
11	SNP6917	NW_020871044.1	361742	A	T	0.307	0.613	0.057	1.20033E-21	
12	SNP7775	NW_020871686.1	422755	G	T	0.381	0.6058	0.058	6.00328E-21	
13	SNP8577	NW_020872599.1	240548	T	C	0.169	0.6214	0.06	9.53358E-21	Type
14	SNP8699	NW_020872599.1	310509	G	A	0.219	0.5803	0.057	6.0374E-20	
15	SNP2734	NW_020869357.1	201355	T	A	0.281	0.5803	0.057	6.0374E-20	
16	SNP4030	NW_020869768.1	99447	A	G	0.276	0.5778	0.057	7.59136E-20	
17	SNP8227	NW_020872260.1	440351	A	G	0.131	-0.653	0.065	8.40854E-20	
18	SNP8753	NW_020872599.1	366769	C	T	0.164	0.63	0.063	1.98315E-19	
19	SNP2761	NW_020869357.1	201456	T	A	0.279	0.5693	0.058	3.73353E-19	
20	SNP8813	NW_020872599.1	367376	C	G	0.279	0.5693	0.058	3.73353E-19	
21	SNP5539	NW_020870762.1	460362	A	G	0.202	-0.575	0.058	5.14325E-19	
22	SNP5560	NW_020870762.1	466543	C	A	0.236	0.5637	0.057	6.06086E-19	
23	SNP8561	NW_020872599.1	240441	C	T	0.152	0.6068	0.062	1.24534E-18	
24	SNP5540	NW_020870762.1	460365	T	C	0.195	-0.573	0.059	1.48043E-18	
25	SNP3662	NW_020869684.1	350400	T	C	0.219	-0.561	0.058	1.74104E-18	

Table A The top 50 SNP markers obtained from the GLM model in term of type (survival and mortality) and score (survival and rate of time to death) (continue)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
26	SNP4021	NW_020869768.1	99400	A	C	0.302	0.5678	0.059	2.23754E-18	Type
27	SNP8747	NW_020872599.1	366700	T	C	0.200	0.5774	0.06	2.33866E-18	
28	SNP8803	NW_020872599.1	367304	T	A	0.271	0.5565	0.058	2.54266E-18	
29	SNP8855	NW_020872599.1	370180	T	A	0.117	0.6522	0.068	4.02604E-18	
30	SNP5583	NW_020870762.1	467337	T	C	0.293	-0.559	0.059	4.49619E-18	
31	SNP8737	NW_020872599.1	366613	G	T	0.369	0.5774	0.061	5.3451E-18	
32	SNP4949	NW_020870254.1	208627	T	C	0.236	-0.556	0.059	6.00278E-18	
33	SNP9102	NW_020872641.1	338842	A	C	0.283	0.5527	0.059	7.71819E-18	
34	SNP7808	NW_020871686.1	433023	G	A	0.321	0.5704	0.061	9.32781E-18	
35	SNP8859	NW_020872599.1	370188	T	C	0.121	0.6345	0.068	1.39194E-17	
36	SNP9188	NW_020872726.1	378060	C	T	0.264	-0.545	0.058	1.48458E-17	
37	SNP6978	NW_020871044.1	362224	G	C	0.260	0.5436	0.058	1.60849E-17	
38	SNP8679	NW_020872599.1	276894	A	G	0.207	-0.551	0.059	1.88067E-17	
39	SNP7000	NW_020871044.1	362869	A	T	0.329	-0.571	0.061	2.05498E-17	
40	SNP619	NW_020868581.1	844987	G	A	0.212	0.5477	0.059	2.52195E-17	
41	SNP8676	NW_020872599.1	276875	C	G	0.217	-0.544	0.059	3.26058E-17	
42	SNP8746	NW_020872599.1	366684	C	T	0.152	0.6088	0.066	3.50181E-17	
43	SNP8733	NW_020872599.1	366602	C	G	0.245	0.5416	0.059	4.07023E-17	
44	SNP8802	NW_020872599.1	367303	A	T	0.276	0.5393	0.059	4.9118E-17	
45	SNP4968	NW_020870254.1	208787	T	A	0.302	0.5479	0.06	5.38746E-17	
46	SNP5584	NW_020870762.1	467342	A	C	0.298	-0.544	0.06	7.52561E-17	
47	SNP8553	NW_020872599.1	240387	C	T	0.257	0.5338	0.059	7.55784E-17	
48	SNP8748	NW_020872599.1	366712	G	A	0.157	0.5955	0.066	8.88609E-17	
49	SNP8736	NW_020872599.1	366612	G	T	0.286	0.6035	0.067	1.17993E-16	
50	SNP424	NW_020868549.1	103210	C	T	0.288	0.5363	0.059	1.3156E-16	

Table A The top 50 SNP markers obtained from the GLM model in term of type (survival and mortality) and score (survival and rate of time to death) (continue)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
1	SNP8225	NW_020872260.1	440338	C	A	0.262	1.186	0.096	1.696E-26	Score
2	SNP3072	NW_020869429.1	1622620	A	G	0.290	-1.150	0.097	5.470E-25	
3	SNP2734	NW_020869357.1	201355	T	A	0.281	-1.161	0.099	9.965E-25	
4	SNP4030	NW_020869768.1	99447	A	G	0.276	-1.146	0.099	4.632E-24	
5	SNP8850	NW_020872599.1	370124	T	A	0.195	-1.166	0.102	5.889E-24	
6	SNP3661	NW_020869684.1	350398	C	A	0.269	1.127	0.100	2.781E-23	
7	SNP4021	NW_020869768.1	99400	A	C	0.302	-1.135	0.103	1.339E-22	
8	SNP6917	NW_020871044.1	361742	A	T	0.307	-1.136	0.103	2.097E-22	
9	SNP2761	NW_020869357.1	201456	T	A	0.279	-1.105	0.102	4.977E-22	
10	SNP3662	NW_020869684.1	350400	T	C	0.219	1.084	0.103	5.288E-21	
11	SNP5539	NW_020870762.1	460362	A	G	0.202	1.095	0.105	6.761E-21	
12	SNP4949	NW_020870254.1	208627	T	C	0.236	1.079	0.104	1.317E-20	
13	SNP8698	NW_020872599.1	310456	C	T	0.193	-1.095	0.106	1.395E-20	
14	SNP4015	NW_020869768.1	99375	G	T	0.417	-1.075	0.106	7.581E-20	
15	SNP8813	NW_020872599.1	367376	C	G	0.279	-1.028	0.106	1.123E-18	
16	SNP8583	NW_020872599.1	240680	T	C	0.374	-1.164	0.121	2.853E-18	
17	SNP5540	NW_020870762.1	460365	T	C	0.195	1.035	0.108	3.810E-18	
18	SNP8763	NW_020872599.1	366931	A	T	0.157	-1.056	0.112	6.459E-18	
19	SNP619	NW_020868581.1	844987	G	A	0.212	-1.015	0.107	6.811E-18	
20	SNP8703	NW_020872599.1	310572	G	C	0.226	-1.005	0.106	7.486E-18	
21	SNP9188	NW_020872726.1	378060	C	T	0.264	1.001	0.106	8.403E-18	
22	SNP4968	NW_020870254.1	208787	T	A	0.302	-1.015	0.109	1.471E-17	
23	SNP5583	NW_020870762.1	467337	T	C	0.293	1.003	0.108	2.228E-17	
24	SNP4044	NW_020869768.1	99524	A	G	0.343	-1.063	0.115	2.598E-17	
25	SNP7353	NW_020871124.1	308736	T	G	0.467	0.987	0.107	2.949E-17	

Table A The top 50 SNP markers obtained from the GLM model in term of type (survival and mortality) and score (survival and rate of time to death) (continue)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
26	SNP1699	NW_020869014.1	156141	A	G	0.35714	-1.09	0.118	3.007E-17	Score
27	SNP7354	NW_020871124.1	308741	T	A	0.27143	0.9797	0.107	6.415E-17	
28	SNP8579	NW_020872599.1	240635	G	T	0.22857	-1.16	0.127	7.299E-17	
29	SNP7232	NW_020871090.1	495393	A	G	0.25238	-0.967	0.107	1.385E-16	
30	SNP4022	NW_020869768.1	99406	A	T	0.29286	-0.979	0.109	1.731E-16	
31	SNP9200	NW_020872726.1	383667	T	C	0.18333	1.0843	0.121	1.750E-16	
32	SNP5584	NW_020870762.1	467342	A	C	0.29762	0.9798	0.11	2.165E-16	
33	SNP8697	NW_020872599.1	310415	C	T	0.15238	-1.042	0.117	2.647E-16	
34	SNP6983	NW_020871044.1	362800	A	T	0.24286	0.9622	0.109	5.345E-16	
35	SNP3715	NW_020869684.1	833683	T	G	0.32619	-0.744	0.085	6.157E-16	
36	SNP8289	NW_020872327.1	74626	T	C	0.22857	0.975	0.111	6.178E-16	
37	SNP8802	NW_020872599.1	367303	A	T	0.30714	-0.953	0.109	6.935E-16	
38	SNP8227	NW_020872260.1	440351	A	G	0.13095	1.0733	0.123	9.607E-16	
39	SNP4031	NW_020869768.1	99452	C	T	0.28095	-0.948	0.109	1.195E-15	
40	SNP9102	NW_020872641.1	338842	A	C	0.28333	-0.946	0.11	1.550E-15	
41	SNP1903	NW_020869103.1	604972	T	G	0.36667	-0.972	0.113	1.696E-15	
42	SNP7775	NW_020871686.1	422755	G	T	0.38095	-0.963	0.112	1.842E-15	
43	SNP8803	NW_020872599.1	367304	T	A	0.27143	-0.939	0.109	1.852E-15	
44	SNP6978	NW_020871044.1	362224	G	C	0.25952	-0.936	0.109	1.858E-15	
45	SNP929	NW_020868719.1	302908	G	A	0.22381	-0.957	0.111	1.865E-15	
46	SNP8292	NW_020872327.1	74654	G	T	0.45238	0.9427	0.11	2.547E-15	
47	SNP4043	NW_020869768.1	99518	C	T	0.33095	-0.984	0.115	2.760E-15	
48	SNP8577	NW_020872599.1	240548	T	C	0.16905	-0.986	0.116	3.07192E-15	
49	SNP6984	NW_020871044.1	362803	A	C	0.24048	0.9411	0.11	3.22782E-15	
50	SNP5427	NW_020870690.1	716901	A	T	0.26429	-0.929	0.109	3.47742E-15	

APPENDIX B

THE SNP MARKERS FROM THE FarmCPU+PCA MODEL

Table B The SNP markers obtained from the FarmCPU+PCA in term of type (survival and mortality) and score (survival and rate of time to death)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
1	SNP7640	NW_020871441.1	101150	C	T	0.438	0.2567	0.044	1.215E-09	Type
2	SNP7007	NW_020871044.1	364154	A	G	0.248	0.1735	0.031	3.625E-09	
3	SNP4411	NW_020869946.1	94362	T	C	0.193	0.128	0.028	1.363E-07	
4	SNP1022	NW_020868747.1	73519	G	A	0.405	-0.159	0.034	5.011E-07	
5	SNP8625	NW_020872599.1	242781	T	C	0.274	0.1065	0.026	1.802E-06	
6	SNP7597	NW_020871395.1	41093	G	A	0.21	0.119	0.028	2.296E-06	
7	SNP588	NW_020868573.1	803853	A	G	0.274	0.1158	0.028	2.871E-06	
8	SNP4253	NW_020869894.1	1212063	C	T	0.271	0.113	0.027	3.623E-06	
No.	**SNP**	**CHROM**	**POS**	**REF**	**ALT**	**MAF**	**Effect**	**SE**	***P*-value**	**Trait**
1	SNP3402	NW_020869525.1	157234	C	T	0.055	0.496	0.092	1.382E-08	Score
2	SNP2868	NW_020869357.1	385811	T	C	0.481	0.646	0.139	7.275E-08	
3	SNP6395	NW_020870918.1	243871	T	A	0.452	-0.422	0.091	4.389E-07	
4	SNP6449	NW_020870952.1	204341	A	T	0.100	0.314	0.071	8.651E-07	
5	SNP739	NW_020868623.1	4133	G	C	0.071	-0.376	0.090	2.912E-06	

APPENDIX C

THE SNP MARKERS FROM THE FarmCPU+Q MODEL

Table C The SNP markers obtained from the FarmCPU+Q in term of type (survival and mortality) and score (survival and rate of time to death)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
1	SNP1022	NW_020868747.1	73519	G	T	0.405	-0.161	0.031	3.050E-08	Type
2	SNP9287	NW_020872751.1	487685	A	G	0.452	0.2008	0.042	2.437E-07	
3	SNP5982	NW_020870781.1	939212	T	C	0.071	0.1621	0.036	6.679E-07	
4	SNP6140	NW_020870790.1	146066	T	C	0.06	0.1801	0.039	7.109E-07	
5	SNP296	NW_020868467.1	234072	G	T	0.324	0.1181	0.026	8.930E-07	
No.	**SNP**	**CHROM**	**POS**	**REF**	**ALT**	**MAF**	**Effect**	**SE**	***P*-value**	**Trait**
1	SNP3430	NW_020869525.1	157383	G	C	0.064	0.412	0.084	5.900E-08	Score
2	SNP3832	NW_020869707.1	108036	T	A	0.498	-1.797	0.398	2.242E-07	
3	SNP739	NW_020868623.1	4133	T	C	0.071	-0.421	0.087	3.199E-07	
4	SNP6152	NW_020870790.1	146148	T	C	0.057	-0.416	0.091	1.042E-06	

APPENDIX D

THE SNP MARKERS FROM THE FarmCPU+K MODEL

Table D The SNP markers obtained from the FarmCPU+K in term of type (survival and mortality) and score (survival and rate of time to death)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
1	SNP196	NW_020868383.1	19498	A	G	0.105	0.1354	0.034	3.377E-06	Type
2	SNP2755	NW_020869357.1	201411	G	A	0.048	0.2169	0.049	1.162E-06	
3	SNP3072	NW_020869429.1	1622620	A	G	0.29	0.1608	0.039	6.669E-07	
4	SNP4253	NW_020869894.1	1212063	T	C	0.271	0.1355	0.03	7.544E-07	
5	SNP6998	NW_020871044.1	362854	G	A	0.293	0.1718	0.036	1.319E-07	
6	SNP7640	NW_020871441.1	101150	A	G	0.438	0.2452	0.048	3.985E-08	
7	SNP8225	NW_020872260.1	440338	C	A	0.262	-0.293	0.045	2.561E-11	
8	SNP8850	NW_020872599.1	370124	T	A	0.195	0.1619	0.043	1.025E-06	
No.	**SNP**	**CHROM**	**POS**	**REF**	**ALT**	**MAF**	**Effect**	**SE**	***P*-value**	**Trait**
1	SNP9192	NW_020872726.1	378132	C	T	0.093	0.356	0.083	2.581E-06	Score

APPENDIX E

THE SNP MARKERS FROM THE FarmCPU+K+PCA MODEL

Table E The SNP markers obtained from the FarmCPU+K+PCA in term of type (survival and mortality) and score (survival and rate of time to death)

No.	SNP	CHROM	POS	REF	ALT	MAF	Effect	SE	*P*-value	Trait
1	SNP8225	NW_020872260.1	440338	C	A	0.262	-0.235	0.045	7.480E-09	Type
2	SNP6998	NW_020871044.1	362854	G	A	0.293	0.1835	0.036	8.190E-08	
3	SNP4253	NW_020869894.1	1212063	T	C	0.271	0.1426	0.03	3.080E-07	
4	SNP3072	NW_020869429.1	1622620	A	G	0.29	0.1572	0.039	1.950E-06	
5	SNP5982	NW_020870781.1	939212	T	C	0.069	0.1733	0.043	4.720E-06	
6	SNP1022	NW_020868747.1	73519	A	G	0.405	-0.154	0.037	4.770E-06	
No.	**SNP**	**CHROM**	**POS**	**REF**	**ALT**	**MAF**	**Effect**	**SE**	***P*-value**	**Trait**
1	SNP9192	NW_020872726.1	378132	C	T	0.093	0.387	0.081	1.370E-07	Score
2	SNP5601	NW_020870762.1	474494	A	T	0.333	-0.310	0.064	2.400E-07	
3	SNP8776	NW_020872599.1	367257	G	T	0.417	-0.361	0.078	1.000E-06	
4	SNP8225	NW_020872260.1	440338	C	A	0.262	0.380	0.091	2.030E-06	
5	SNP1435	NW_020868913.1	257200	T	A	0.050	-0.413	0.102	2.860E-06	

BIOGRAPHY

Name	Anong Nimlamai
Educational Attainment	2014: Master of Science (Agricultural Science Fisheries Science), Walailak University, Thailand 2001: Bachelor of Science (Fisheries), Kasetsart University, Thailand
Scholarship	2020: The Royal Golden Jubilee Ph.D. Scholarship Program with grant No. HRD6401004

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