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Original research article

Evaluation of Genetic Diversity in Fourth-Generation Selective Breeding Lines of Pacific White Shrimp Using Microsatellites

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ABSTRACT

The Pacific white shrimp (*Litopenaeus vannamei*) is a vital 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 this study, genetic diversity and structure of 35 families of fourth-generation selective breeding lines of *L. vannamei* were monitored using five microsatellite DNA markers. The polymorphism information content (PIC) of all microsatellite loci exhibited high levels of polymorphism, with PICs 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. The overall mean observed heterozygosity (H_o) ranged from 0.209 to 0.492, which was lower than the expected heterozygosity (H_e) (0.714-0.784). The mean inbreeding (F_{is}) coefficient across all loci was 0.336 indicating a moderate level of inbreeding within this population. The population structure analysis showed that the 35 families were divided into two subpopulations (K=2), related to the sources of the base population collected from two private hatcheries. This study provides valuable insights into population genetics and will help guide breeding strategies of Pacific white shrimp.

Keywords: Genetic variation; Microsatellite; Pacific white shrimp; Population structure

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1. Introduction

Litopenaeus vannamei, commonly known as Pacific white shrimp is an important species for aquaculture in the world. 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 [1]. Continued investment in selective breeding programs and the adoption of advanced breeding technologies are essential for further advancing the genetic potential of shrimp stocks and ensuring the long-term sustainability and profitability of the shrimp farming sector in Thailand and globally. These efforts contribute to improving shrimp traits such as growth rate, disease resistance, and tolerance to environmental stresses, 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 susceptibility to diseases, reduced adaptability to changing 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 [2]. 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. Monitoring genetic variation allows breeders to make informed decisions about 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 have been used 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 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 [3]. 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 [4] and summer flounder [5]. In addition, the microsatellite markers can also be used to evaluate genetic diversity in captive populations across various generations and farmed populations of *Oreochromis niloticus* [6]. Multiple studies have provided explicit evidence of

the diminishing genetic variation in cultured stocks across successive generations, as observed in *Penaeus stylirostris*. [7], *Penaeus monodon* [8], and *L. vannamei* [9]. 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* [10] and *P. monodon* [11].

In the present study, we 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 F_4 selection. The findings from this research will provide breeders with valuable insights into the genetic makeup of selected strains, thus enhancing management approaches for shrimp farming initiatives.

2. Materials and Methods

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 Songkhla aquatic animal health 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 [12]. 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.

Table 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

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 [13], CNM-MG 357 [14], and Lvan17 [15] (Table 1). The PCR reactions were prepared to a total volume of 20 µL, comprising 20 ng of DNA, 10 pmol of each primer, 2.1 mM MgCl2, 0.2 mM of each dNTP and 5U/ul of Taq polymerase. PCR amplification was conducted using the PCR thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories, Hercules, CA, USA), involving an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at the locus-specific temperature for 30 seconds, and extension at 72°C for 1 minute. The process ended with a final extension at 72°C for 5

minutes. Subsequently, PCR products were separated through 1% agarose gel electrophoresis. The DNA fragment lengths were analyzed using ZAG DNA Analyzer Systems (Agilent Technologies, Santa Clara, CA). The determination of allelic size was carried out utilizing ProSize data analysis software (Agilent Technologies).

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. [16]. The analysis of polymorphic information content (PIC) was conducted using PowerMarker [17]. 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 [18]. The population structure was assessed using a model-based Bayesian method utilized through the software package STRUCTURE v. 2.3.4 [19]. The analysis was repeated ten times for each K value, ranging from K = 1 to K = 10, using a burn-in period of 100,000 iterations. Individuals showing a membership probability (Q value) below 0.6 were defined as an admixture. The number of sub-populations (ΔK) was determined using the ad-hoc statistics of Evanno et al. [20]. The analysis of molecular variance (AMOVA) and calculation of F_{st} among groups were performed with ARLEQUIN v. 3.1 [21] with 100,000 permutations, considering both families and clusters suggested by Bayesian analysis.

3. Results and Discussion

3.1 Genetic variation

All microsatellite markers were polymorphic across samples. The degree of polymorphism differed across loci. The genetic diversity based on the five microsatellite markers for the 189 samples are summarized in Table 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.

Table 2. Summary statistics of genetic variabilities detected in five microsatellite loci among 189 samples, derived from 35 families of 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.

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 [22]. 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. [22], 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 [23]. The allelic diversity (i.e., average number of alleles per locus) is a useful measure of genetic variability within a population, provided that comparisons are carried out between samples of similar size. In the 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. [24]. The TUMXLv9.178 locus had the most alleles (6 alleles), which is higher than the number reported by Meehan et al. [13]. Similarly, TUMXLv10.455 had 5 alleles, which is higher than 4 alleles reported by Meehan et al. [13]. In contrast, TUMXLv10.411 had 4 alleles, which is lower than 7 alleles reported by Meehan et al. [13]. CNM-MG 357 had 3 alleles, which is lower than 4 alleles reported by Pérez et al. [14]. Lvan 17 had 4 alleles, which is lower than 11 alleles reported by Marques et al. [15]. Based on the number of alleles as a key indicator of genetic diversity, our results were similar to those of Klongklaew and Songsangjinda [25], who studied the genetic diversity of *L. vannamei* broodstock from six populations: four from department of fisheries (DOF) hatcheries 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 an average number of alleles per locus (N_a) of 4.71±1.38, while the N_a value in this study was 4.400±1.140. These results indicated that the population possessed 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 at all loci (3-6 alleles). Similar results were found after seven generations of selection with 4 alleles and only 1.6 effectives [26]. 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. Since the ultimate goal of breeding programs is the selection of better performing individuals for a number of traits of interest, these results indicated a loss of genetic diversity. In order to broaden genetic diversity, it is advisable to introduce additional genetic variability to the breeding stock by crossing it with a different lineage obtained from different sources.

In all cases, H_o values were lower than the values for H_e. The average 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. [27] reported similar levels for these parameters for the F_1 generation of seven introduced populations in China. Zhang et al. [23] also reported comparable values. All loci are considered to suffer from a heterozygote deficit arising from inbreeding as a basis for the positive F_{is} values identified in the present study. This is commonly found in hatchery populations due to selection or inbreeding [28]. 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. This is similar to the findings by Lima et al. [29], 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 the state of 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.

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 did not indicate significant (p>0.05) differentiation among the 35 families (Table 3). Admixture model-based simulations to population structure suggested K = 2 as the most probable number of clusters, calculated following the Evanno criteria (Fig. 1). This suggests that our panel could be split into two clusters, cluster 1 (red bars) and cluster 2 (green bars) (Fig. 2). 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 indicated significant (p<0.05) differentiation among populations (Table 3). The PCA yielded comparable results to the STRUCTURE analysis (Fig. 3). Sub-population 1 (blue) comprised of 94 samples. Subpopulation 2 (orange) comprised of 85 samples. Ten samples (green), displayed a membership probability (Q value) ranging from 0.4 to 0.6, and were characterized as admixtures.

Population genetic structure describes how genetic variation is distributed within and among populations. This structure is shaped by factors such as different origins or different geographical sources, genetic drift, and selection. Understanding the population genetic structure aids in identifying distinct populations or germplasms within a species, which is crucial for developing effective management and conservation strategies. In this study, the F_{st} value, which was close to 0 and non-significant when considering 35 families, suggests that the population is actively exchanging genetic material through elevated levels of breeding. The Bayesian structural analysis identified two subpopulations, suggesting that the base population originated from two private hatcheries in Krabi province and Songkhla province. The results were comparable to the study of Huang et al. [27], which examined the genetic diversity of seedling samples gathered from seven culture populations across three primary shrimp production zones in Guangdong, China. They found that the seven populations were grouped into three clusters, revealing distinct genetic characteristics among the different cultured populations of *L. vannamei*.

Table 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 populations	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 populations	187	35.73	*F*st = 0.128	0.000*
Within individuals	189	51.44		

*p < 0.00000, after 1023 permutations.

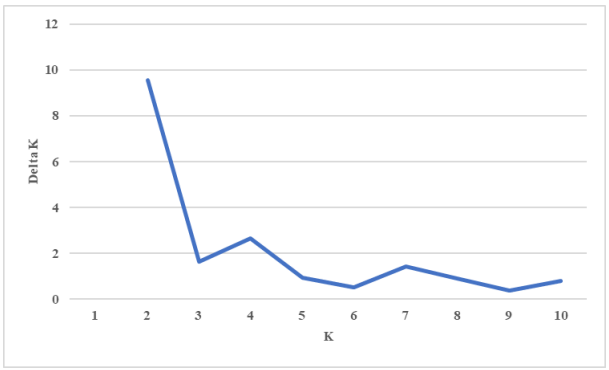

Fig. 1. Number of subpopulations calculated following the Evanno criteria.

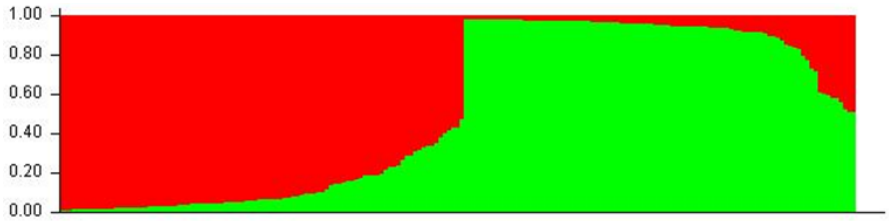

Fig. 2. Neighbor-joining tree based on Nei's genetic distances was colored according to membership probability (Q value) from STRUCTURE.

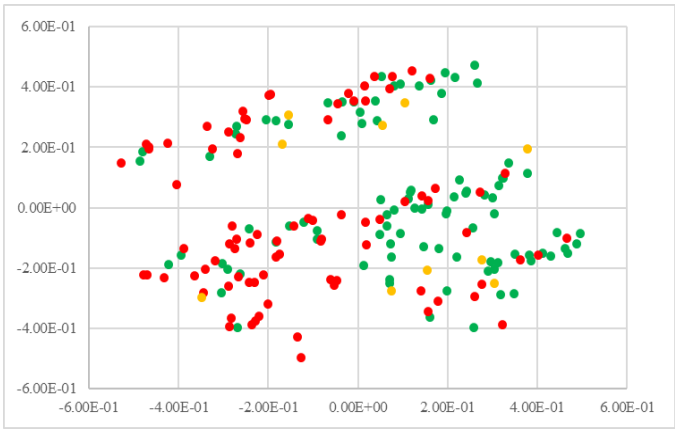

Fig. 3. Principal Component Analysis (PCA). Subpopulation 1 (red) comprised of 94 samples. Subpopulation 2 (green) comprised of 85 samples. Admixtures (orange) comprised of 10 samples.

4. Conclusion

The microsatellite 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 F_4 selective breeding lines. The heterozygosity observed indicates that the studied population possesses moderate genetic diversity. Signs of inbreeding were also observed, contributing to a reduction in genetic variation within the population and potentially slowing down the response to selection. These findings have significant implications for the management of *L. vannamei* in genetics improvement programs.

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