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Research Article

Application of microsatellite DNA method in determining the genetic diversity of farmed broodstocks of *Penaeus vannamei* (Boone, 1931) in hatchery centers of Bushehr Province

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ABSTRACT. The aim of this study was a detection of different populations of Pacific white shrimp (*Penaeus vannamei* Boone, 1931) farmed broodstocks and to determine their genetic indexes in hatchery centers of Bushehr province. DNA was extracted from the muscle tissue of 30 broodstocks of 11 stocks using a commercial kit. The repeated sequences were amplified using ten specific primers by PCR in the Iranian Shrimp Center. The results showed that the number of alleles was 4.5-5.5 in the studied stocks. The most allele frequency was observed in the farmed broad stocks of the third stock in the H2 hatchery center, 5.2 ± 0/359. Also, the average of observed heterozygosity in the farmed broad stocks of the second stock in the H5 hatchery center obtained 0.669 ± 0.152, more than other stocks in all centers. Genetics indexes of the farmed broad stocks of the third stock in the H2 hatchery center were increased compared with other stocks in Bucher province. The inbreeding coefficients of the first stocks in the H1 and H3 hatchery centers were significantly more than others, respectively, 0.595 ± 0.105 and 0.547 ± 0.145. The increased inbreeding coefficients of the farmed broad stocks in these stocks can be referred to due to the small size of the founder population and biased selection programs. However, this amount is acceptable according to the information of the other hatchery centers in Bushehr province and other countries. Moreover, it was concluded that broodstock with higher diversity should be used to increase the genetic indicators in the hatchery centers.

Keywords: *Penaeus vannamei*; broad stock; genetic; diversity; inbreeding; microsatellite

INTRODUCTION

Due to the rapid growth of aquaculture in the last two decades, it was observed that the share of this industry in the production of seafood in the world increased from 3.9% of the total weight in 1970 to 27.1% in 2000 and 36% in 2006 (Pauly & Zeller 2017). Examining the data available in the world indicates that the level of global shrimp production in 2021 was at least 8.9% higher than in 2020, but the forecast for 2022 is a growth of more than 5% (Shen et al. 2021). Only 10% of the world's aquaculture production is estimated to be associated with genetic improvement programs (Gjedrem et al. 2012).

Penaeus vannamei is the most important worldwide in shrimp farming, contributing more than 71% of the total production (FAO 2014). *P. vannamei* is native to the eastern Pacific Ocean. It is commonly farmed in many countries.

Studying the genetic characteristics of shrimp samples and analyzing the genetic relationships between the breeders can be a suitable tool for managing gene-

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tic identification and genetic differences in breeding programs (Allegrucci et al. 1998, Sekino et al. 2002). Maintaining genetic diversity in shrimp populations requires the presence of genetic diversity in stocks in hatchery centers, similar biological patterns, and the use of wild and natural genetic resources (Williams & Johnson 2013). Controlling inbreeding and examining genetic diversity's increase or decrease in a selective breeding program is essential.

Microsatellites are simple repeating sequences of 1-6 nucleotides in different parts of the genetic material of DNA. Microsatellites are promising and sensitive in studying genetic variation, especially populations sampled over a reduced geographical scale or among closely related populations (Ding et al. 2009). The required information regarding genetic differences and different loci is obtained by examining them. According to the above information, it is possible to determine the level of genetic diversity and the relationships between the western white shrimp farmed brood stock in the hatchery centers of the country by using microsatellite molecular markers. The obtained data can be used regarding the best approach to produce the enclosed stem stocks with the highest genetic changes to control the inbreeding of the identified populations in hatchery centers. Therefore, this study aimed to identify different populations of western white shrimp farmed brood stock in hatchery centers of Bushehr Province and to determine their genetic indicators based on the historical information of foreign reserves.

MATERIALS AND METHODS

Sampling and history of different shrimp-farmed broodstock under study

In order to obtain information about the breeding history of western Pacific white shrimp (*P. vannamei*), the number of available stocks and the origin of breeding stock kept in Delaram (H1), Pars Abzistan (H2), Zadavuri Mand (H3), Hatham Shahrivar (H4) hatchery centers and Lian Gostar Talai (H5) located in Bushehr Province was obtained in 2022. The number of samples taken from each reserve was 30 pieces. After sampling, in order to increase the shelf life, the samples were stored in 70° alcohol at 4°C (Machado-Tamayo 2006).

Extracting and determining the quality of genetic material

DNA extraction of tissue samples from swimming leg muscle parts of western white shrimps was done. Then, the quality of the extracted genetic material was checked through electrophoresis on 1% agarose gel. In order to quantify, an A&E lab model spectrophotometer was used at wavelengths of 260 and 280 nm and the ratio 260:280 (García-Alegría et al. 2020).

Amplification of microsatellite sequences with specific primers

Amplification of the repetitive DNA sequences of the samples extracted through the technique polymerase chain reaction (PCR) using the thermocycler, Corrbet model was carried out by 10 pairs of specific primers for western white shrimp polymorphic made by Metabion, Germany, on the order of Pishgam Company (Cruz et al. 2002, Luvesuto et al. 2007) (Table 1).

The PCR product in a volume of 20 µL includes 2 µL of PCR Buffer, 1 µL of Mg Cl_2, 0.7 µL of NTP, 1 µL of forward primer, 1 µL of reverse primer, 0.3 µL Taq DNA, 13 µL double distilled water and 1 µL of template DNA was prepared (Freitas & Galetti 2002). The temperature program for the amplification of sites through PCR includes pre-denature at 94°C for 3 min, the second stage of denaturation at 94°C for 30 s, and annealing at a temperature of 60°C for 30 s, and extraction was set at a temperature of 72°C for 30 s for 30 cycles. In the end, the final extraction was done at 72°C for 5 min. Then, 5 µL of the PCR product was electrophoresed on an 8% polyacrylamide gel with a standard marker of 100-3000 base pairs for 2.5 h and finally, staining was done using 1% silver nitrate (Borrell et al. 2003).

Determining genetic indices for different shrimp stocks

After determining the locations on the gel, for each of them, the frequency values of true (Na) and effective (Ne) alleles, observed (HO) and expected (He) heterozygosity, similarity matrix, and genetic distance (Nei 1978, Nei & Kumar 2000), Hardy-Weinberg equilibrium, a genetic difference (Fst), inbreeding coefficient (Fis), gene flow (Nm) were determined by Gene Alex software (v.6). Also, an evolutionary topological tree between samples was calculated based on genetic distance using TFPGA software and differences between populations based on ANOVA test (Peakall & Smouse 2006). Finally, the data obtained from different reserves through SPSS statistical software using t-test and one-way analysis of variance (ANOVA) through Duncan's test, allelic frequency, genetic differentiation, inbreeding coefficient, and other indicators in foreign reserves were analyzed, and statistical analysis was done.

Table 1. The sequence of markers used to amplify repetitive sequences of microsatellites (microsatellites) of western white shrimp (*Penaeus vannamei*).

Prime	Sequence(5`→3`)	Open pair
Lvan01	F: GCCATAAACGCAAGACTGAG	146-136
	R: GCAGGTATACGGTCATGTGTA	
Lvan07	F: AAAGAGGAAGATGAGGAAG	223-189
	R: CCTCGGTTACGTATTTATTG	
Pvan0013	F: TGCTCTGGTAACGACAAACG	284-276
	R: AGACCTGTGGCGAAGTGC	
Pvan1758	F: TATGCTCGTTCCCTTTGCTT	189-163
	R: TTGAAGGAAAAGTGTTGGGG	
Pvan1815	F: GATCATTCGCCCCTCTTTTT	141-126
	R: ATCTACGGTTCGAGAGCAGA	
TUMXLv5.27	F: CAGACCCTAAATCTCCGTGC	182-166
	R: TGGAAAGGTCAGAGGTCACG	
TUMXLv5.38	F: CCTTTATGACTTCCCCCGAC	222-200
	R: CCGTACAGAAACGGAACGTC	
TUMXLv8.32	F: TTACCGCCTAAGAGCGAATG	228-216
	R: TGTCCTTTCGTACCAGTCAAG	
	R: CTAACCCAATATCGAATC	
TUDGLv5-7.33	F: TGCTAGAATGTCTTTCGAAG	126-115
	R: GTCTGGGGAAATCTTTAATG	
TUDGPv3-5.378	F: TCGGAAGGTGTCTTTCCAAAC	186-180

RESULTS

The results indicated that the number of alleles observed for the dominant data in the 11 farmed broodstocks was in the range of 4.5-5.5 alleles. The average number of true alleles observed in the farmed broodstock of the second stock of H1 and H5 was 5.500 ± 0.601 and 4.500 ± 0.453, the highest and the lowest number of true alleles observed compared to other stocks, respectively. No statistically significant difference was observed between the above-farmed broodstocks and others ($P > 0.05$) (Table 2).

Also, even though the average heterozygosity observed in the farmed broodstocks of the second stock of the H5 hatchery center is 0.669 ± 0.152 compared to others, there is no statistically significant difference between the heterozygosity observed in this stock and the farmed broodstocks of the first and second stock of H1, the second and third stock of H2 and the first stock of H3 were not observed ($P > 0.05$). However, the amount of this index of 0.438 ± 0.016 in the second stock of H3 was significantly lower than some stocks ($P < 0.05$) (Fig. 1).

Examining inbreeding showed that the level of this index has increased in the farmed broodstock of different stocks. However, the amount of this index in the first stocks of H3 and H1 was significantly higher than other stocks with the amount of 0.595 ± 0.105 and 0.547 ± 0.145, respectively ($P < 0.05$). However, the amount of this index in the first and second stocks of the H2 was significantly lower, with 0.359 ± 0.107 and 0.360 ± 0.085, respectively ($P < 0.05$) (Table 3).

Examining the Hardy-Weinberg equilibrium in order to show polymorphisms in different stocks indicated that all the examined locations of the second stock of H2 farmed broodstocks had significantly led to the stability and abundance of alleles ($P < 0.05$, $P < 0.01$, $P < 0.001$) but in other stocks kept in hatchery centers, they were observed significantly in only a few locations ($P < 0.05$, $P < 0.01$, $P < 0.001$)**.** It was also observed that there is the most significant genetic distance between the farmed broodstocks of the third stock of the H2 and other farmed broodstocks. Despite this, there was a small genetic distance between the first stock of H4 and H5 (Table 4, Fig. 2).

On the other hand, the highest level of genetic similarity was observed between the first and second stocks of H5 and between the first stock of H4 and the second stock of H2. However, a minor genetic similarity existed between the second stock of H2 and the first stock of the H1 (Table 5).

Nevertheless, it was observed that there is the highest level of genetic differentiation (Fst) between the second and third stock of H2, with the second stock

Table 2. Frequency of accurate and effective alleles in different western white shrimp stocks in hatchery centers in 2021. H1: Delaram, H2: Pars Abzistan, H3: Zadavuri Mand, H4: Hatham Shahrivar hatchery centers and H5: Lian Gostar Talai. S = number of stocks. Means having the same letter in the same row are not significantly different at $P < 0.05$.

Hatchery centers	Stock	Number of true alleles	Number of effective alleles
H1	1	4.8 ± 0.467^{a}	3.649 ± 0.423^{a}
	2	5.500 ± 0.601^{a}	3.989 ± 0.550^{a}
H2	1	5.400 ± 0.542^{a}	4.020 ± 0.528^{a}
	2	4.900 ± 0.674^{a}	3.591 ± 0.543^{a}
	3	5.200 ± 0.359^{a}	4.093 ± 0.297^{a}
H3	1	4.900 ± 0.504^{a}	3.793 ± 0.423^{a}
	2	5.200 ± 0.611^{a}	3.648 ± 0.466^{a}
H4	1	5.300 ± 0.539^{a}	3.488 ± 0.409^{a}
	2	4.800 ± 0.512^{a}	3.497 ± 0.421^{a}
H5	1	4.900 ± 0.458^{a}	3.321 ± 0.391^{a}
	2	4.500 ± 0.453^{a}	3.508 ± 0.422^{a}

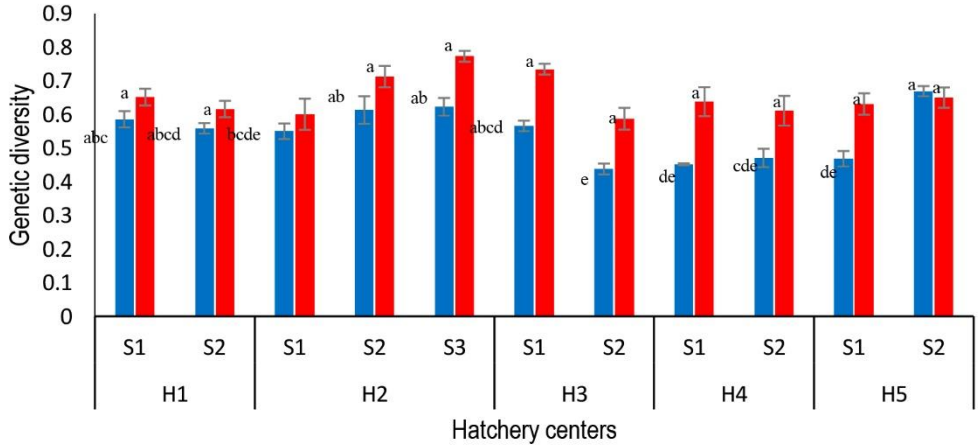

Figure 1. Values of genetic diversity of western white shrimp stocks in different hatchery centers in 2021. H1: Delaram, H2: Pars Abzistan, H3: Zadavuri Mand, H4: Hatham Shahrivar hatchery centers and H5: Lian Gostar Talai. S: number of stocks. Means having the same letter in the same row are not significantly different at $P < 0.05$.

Table 3. Inbreeding coefficient values in western white shrimp stocks in hatchery centers in 2021. H1: Delaram, H2: Pars Abzistan, H3: Zadavuri Mand, H4: Hatham Shahrivar hatchery centers and H5: Lian Gostar Talai. Means having the same letter in the same row are not significantly different at $P < 0.05$.

Hatchery centers	Stocks	Inbreeding coefficient
H1	1	0.547 ± 0.145^{a}
	2	0.426 ± 0.113^{b}
H2	1	0.360 ± 0.085^{c}
	2	0.359 ± 0.107^{c}
	3	0.424 ± 0.108^{bc}
H3	1	0.595 ± 0.105^{a}
	2	0.483 ± 0.117^{b}
H4	1	0.486 ± 0.094^{b}
	2	0.492 ± 0.112^{b}
H5	1	0.490 ± 0.094^{b}
	2	0.449 ± 0.101^{b}

of H3 and H1, and the lowest level of genetic differentiation between the first and second stock of the H5 and also between The progenitors of the first stock of the H4 were observed with the progenitors of the second stock of H1.

DISCUSSION

The results of the present study showed that the inbreeding coefficient was increased in the first stock of H3 and H1 compared to other hatchery centers. In a study conducted in Chinese hatchery centers, the inbreeding coefficient of the western white shrimp was calculated to be 0.07, which was different from this study (Ren et al. 2018). However, the number of genetic indicators of the third stock of H2 had increased compared to other stocks. Due to various reasons, including the lack of access to genetically improved

Table 4. The genetic distance of western white shrimp stocks in hatchery centers in Bushehr Province in 2021. H1: Delaram, H2: Pars Abzistan, H3: Zadavuri Mand, H4: Hatham Shahrivar hatchery centers and H5: Lian Gostar Talai. S: number of stocks.

H1S1	H1S2	H2S1	H2S2	H2S3	H3S1	H3S2	H4S1	H4S2	H5S1	H5S2	
0.000											H1S1
0.488	0.000										H1S2
0.406	0.257	0.000									H2S1
0.354	0.345	0.366	0.000								H2S2
0.788	0.751	0.638	0.760	0.000							H2S3
0.346	0.325	0.242	0.321	0.533	0.000						H3S1
0.382	0.439	0.298	0.348	0.742	0.302	0.000					H3S2
0.368	0.238	0.347	0.223	0.583	0.244	0.338	0.000				H4S1
0.379	0.398	0.395	0.424	0.573	0.483	0.467	0.381	0.000			H4S2
0.343	0.332	0.323	0.314	0.593	0.274	0.230	0.137	0.411	0.000		H5S1
0.495	0.421	0.405	0.421	0.493	0.420	0.368	0.285	0.229	0.217	0.000	H5S2

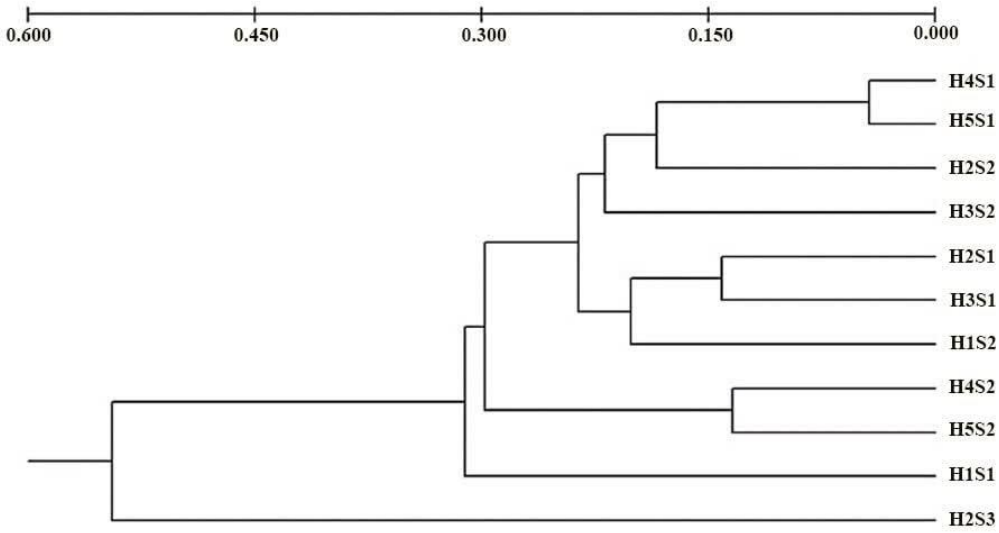

Figure 2. The phylogenetic relationship of different western white shrimp stocks in hatchery centers of Bushehr Province in 2021. H1: Delaram, H2: Pars Abzistan, H3: Zadavuri Mand, H4: Hatham Shahrivar hatchery centers and H5: Lian Gostar Talai. S: number of stocks.

specific disease-free brood stock, the rate of inbreeding in western white shrimp broodstock in Asian farms is relatively high. However, most importantly, the limited number of female shrimps with high fecundity that can spawn multiple times helps to increase the high inbreeding results (Ibarra et al. 2005, 2007). Together, these factors can significantly increase the risk of inbreeding in a captive population. The only real solution to this problem is for regional shrimp hatchery centers to initiate local breeding programs.

Similarly, one of the reasons for the increase in the inbreeding coefficient in the first stocks of H3 and H1 in Bushehr Province is the decrease in the number of participants and their incorrect selection. However, in the hatchery center of H2, due to keeping a large number of broodstocks and increasing the base population, the participation of individuals in cross-breeding had increased, so the number of genetic indicators in the broodstocks of this improved compared to other stocks. Also, the selection among the broodstocks made the genetic indicators stay the same in this hatchery center. The low distance and high genetic similarity of the first stocks of H4 and H5 were placed in a separate branch. However, there was the most significant genetic distance between the third stock of H2 and other stocks.

According to the results of this study, it was found that the number of observed alleles and the frequency

Table 5. Genetic similarity of western white shrimp stocks in hatchery centers in Bushehr province in 2021. H1: Delaram, H2: Pars Abzistan, H3: Zadavuri Mand, H4: Hatham Shahrivar hatchery centers and H5: Lian Gostar Talai. S: number of stocks.

H1S1	H1S2	H2S1	H2S2	H2S3	H3S1	H3S2	H4S1	H4S2	H5S1	H5S2	
1.000											H1S1
0.614	1.000										H1S2
0.666	0.773	1.000									H2S1
0.702	0.708	0.693	1.000								H2S2
0.455	0.472	0.528	0.468	1.000							H2S3
0.708	0.723	0.785	0.725	0.587	1.000						H3S1
0.683	0.645	0.742	0.706	0.476	0.739	1.000					H3S2
0.692	0.788	0.707	0.800	0.558	0.784	0.713	1.000				H4S1
0.685	0.671	0.674	0.655	0.564	0.617	0.627	0.683	1.000			H4S2
0.710	0.718	0.724	0.730	0.553	0.760	0.794	0.872	0.663	1.000		H5S1
0.609	0.656	0.667	0.657	0.611	0.657	0.692	0.752	0.795	0.805	1.000	H5S2

of specific alleles in different stocks of breeding progenitors in hatchery centers of Bushehr Province for the dominant data with the range of 4.5-5.5 alleles in different loci have significantly been reduced. Goyard et al. (2003) reported the number of alleles in wild populations in the range of 14-27 due to increased inbreeding and lack of proper selection of offspring in hatchery centers. It may occur after four to seven generations, and the frequency of alleles is significantly reduced. Therefore, the result of this study regarding the number of observed alleles is consistent with the previous study. Therefore, this problem can be improved to a certain extent by changing mating methods and implementing appropriate selection programs in the broodstocks in the hatchery centers of Bushehr province. Also, the tertiary stock of H2 was in the Hardy-Weinberg equilibrium. This issue can be due to the use of more broodstocks in reproduction as the base population and the increase in the participation of broodstocks in creating the next generations. However, in three to four sites investigated in other stocks, the balance was non-significant. Also, the results indicated that the stability and frequency of alleles in seven loci were non-significantly in the second stock of H5. It was considered that increasing null alleles could increase homozygosity and decrease heterozygosity in populations (Castric et al. 2002). This condition is observed more in populations that suffer from genetic bottlenecks, especially those kept in captivity or closed environments or centers where access to wild resources is impossible (Norris et al. 1999). This situation was present in the different stocks in the hatchery centers of Bushehr Province. Therefore the reason for the deviation from the Hardy-Weinberg equilibrium in some of the locations investigated in these breeders can be related to this issue.

Zhang et al. (2010) obtained no detectable change in gene frequencies or heterozygosity over the generations tested, implying that the loci have been unaffected by the selective pressures of culture or that these changes occurred early in culture and prior to the G05 generation in culture. Compared with results from the other populations, these data support the contention that proper breeding methods and genetic monitoring should be practiced in breeding programs.

CONCLUSIONS

According to the obtained results, the number of genetic indices of the third stock of H2 was increased compared to other stocks in the hatchery centers of Bushehr Province. Also, the selection among the farmed broodstock made the genetic indicators stay the same. Therefore, it is recommended that to improve the genetic indices and prevent regression caused by inbreeding, in addition to the selection of progeny based on genetic indices, outgroup matings between different reserves of progeny should be used in the hatchery center. This information can be applied to designing proper management guidelines for these genetic materials and future genetic improvement by selective breeding.

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