Penaeus vannamei broodstock genetic diversity based on 16S rRNA gene mtDNA

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Abstract

Based on the 16S rRNA genome of mitochondrial DNA, this study examined the genetic diversity of *Penaeus vannamei* broodstocks to determine genetic indicators. This study included two populations, High Health (H) and Molokai (M), of the F3 and F5 generations, respectively. Extraction of DNA from samples was performed using a commercial kit. A pair of special primers amplified the 16S rRNA gene. Then, a 16S rRNA fragment of mitochondrial DNA was sequenced. The World Gene Bank has accession numbers LC468129.1 and LC468130.1 for these sequences. Based on the 486 loci identified, there are 484 conserved loci and two variable loci, each of which contains phylogenetic information under parsimony conditions, and 18 singletons. The similarity between LC468129.1 and LC468130.1 with LC121701.1 was 99.49 and 98.99%, respectively. In conclusion, Neighbor-joining algorithms identified each population with accession number LC468129.1 and LC468130.1 with 64% and 66% in separate branches, respectively.

Keyword: *Penaeus vannamei*, 16S rRNA, Genetic indicators, Phylogenetic

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Introduction

Penaeus vannamei belongs to the crustacean class Malacostraca, which is in the order Decapoda and family Penaeidae. This specie is spread all over the world because of having unique characteristics. Currently, it accounts for over 90% of the shrimp industry's production (Pazir et al. 2022; Ren et al. 2020). Panutrakul and Senanan (2021) state that the species' main habitat is along the coasts of the Pacific Ocean from southern Mexico to northern Colombia. The study of genetic characteristics of different populations of shrimp and an analysis of the genetic relationships between broodstocks can be useful tools in identifying genetic differences and genetic identification in breeding programs (Ren et al. 2020; Sun et al. 2022). Nowadays, molecular markers are used to determine the genetic indices of different shrimp populations, these markers can distinguish members or populations of the same species or genus. Markers can be divided into three categories: morphological, cytogenetic and molecular markers. The basis of morphological markers in genetic sequences. Morphological markers reflect genetic sequences and indirectly, these markers can also be considered genetic markers (Francisco and Galetti Junior 2005). A genetic marker is a segment of a gene or part of a DNA sequence that has a known location on a chromosome and can be used to identify individuals or species. Any trait that is different between people is due to the differences between their DNA sequences, and these differences can be used as genetic markers (Chawla 2011). Mitochondrial DNA has been widely used to determine the genetic structure of organisms, phylogenetic relationships, and gene flow patterns (Soares et al. 2021). In Peneus shrimps, mitochondrial DNA is a circular double-stranded molecule, as in other organisms. It contains two ribosomal RNA genes (12S rRNA & 16S rRNA), 22 tRNA genes, and 13 protein-producing genes. D-loops are replication control regions present in this genome (Peregrino-Uriarte et al. 2009). Most advanced and developed countries have reported genetic diversity studies using mitochondrial markers to study genealogies through mitochondrial genomes (Soares et al. 2021). There are several advantages of mitochondrial markers compared to other markers, including their ability to prevent recombination, maternal inheritance, haploid nature, and high mutation rate (Mendoza-Cano et al. 2013). Another advantage of the mitochondrial genome is its small size, which makes it easier to work with (Soares et al. 2021). This feature has introduced mitochondria as a useful tool to determine the phylogenetic relationships of organisms. This marker is a useful tool for determining the continuity of populations and the evolution of organisms and phylogenetic analysis (Mendoza-Cano et al. 2013). There are several mitochondrial markers, including 16SrRNA, 12SrRNA, COI, d-loop, and cytochrome B. Recently, the

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16SrRNA/tRNA region has been proposed as a mitochondrial marker (Soares et al. 2021). nowadays, to determine the phylogeny relationship between different species, including shrimps, COI and 16SrRNA mitochondrial markers were used. The 16S rRNA genome has a lower evolutionary rate compared to COI. This indicates that this marker is more conserved compared to COI (Thöny-Meyer et al. 1994). The use of 16S rRNA/tRNA region in determining phylogenetic relationships between Penaeidae species demonstrates a greater diversity level than that of COI and 16S rRNA (Calo-Mata et al. 2009). The mitochondrial genome can be used to determine genetic differences among shrimp populations. In addition to determining the level of molecular diversity and evolutionary relationships among organisms, phylogenetic analyses also determine the evolution rate of genes (Tamura et al. 2011). This study investigates the genetic diversity of the broodstocks of different generations of *P. vannamei* based on the 16S rRNA genome of mitochondrial DNA to determine genetic indicators including the amount of genetic distance, the amount of haplotype diversity, nucleotide diversity, Polymorphism and genetic differences was the of between them.

Material and method

Sample collection

This study was carried out on broodstocks of *P. vannamei* from two different populations at the Iranian shrimp research center (ISRC) in Bushehr Province. In this center, there are two populations: High Health (H) and Molokai (M). Based on previous data, these populations belong to the F3 and F5 generations, respectively. The private sector imported them previously from High Health Aquaculture Inc. and Molokai Broodstocks Company, respectively. A 16S rRNA gene analysis of two broodstock populations. Sampling was done from leg muscle tissue of broodstocks of different stocks.

DNA extraction

DNA extraction from the samples was performed with a commercial extraction kit. Briefly, to extract DAN from shrimp tissue, 170 µL of L buffer and 30 µL of proteinase K were combined with 30-35 mg tissue in 1.5 mL microtubes. The mixture was incubated at 56 C for 60 min to dissolve the tissue completely. This solution was centrifuged at 10000 rpm for 1 minute after adding 200 µL of buffer B to a filter micro tube. In the next step, the contents of the collection tube were removed from the tube and centrifuged at 14000 rpm for 2 minutes after adding 80 microliters of warm E buffer. Lastly, DNA was stored in a 1.5 microtube at -20 centigrade.

According to its instructions to determine the quality and quantity of DNA extraction, used agarose gel 1% and nanodrop A&B based on 260, 280 nm absorbance wavelengths and 260/280 and 260/230 Ratios, respectively.

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PCR and sequencing

A pair of special primers 16S rRNA -F (5'-GCCTGTTTAACAAAAACAT-3') and 16S rRNA -R (5'-CCGGTCTGAACTCAGATCATGT-3') (Francisco and Galetti Junior 2005; Simon et al. 1991) performed PCR amplification of the 16S rRNA gene with 529 bp. Amplification was carried out in 25 µL volumes by adding 12.5 µL of PCR master mix (Taq DNA Polymerase Master Mix - Ampliqon), 1 µL of each primer, 9.5 µL of sterile water and 1 µL DNA of each *P. vannamei* broodstock. Amplification of the 529 bp region was carried out using the following PCR conditions: initial denaturation of 94°C for 3 min followed by 35 cycles of denaturation, annealing, and extension at 94°C for 60 s, 51°C for 60 s, and 72°C for 60 s, respectively, and a final extension step was carried out at 72°C for 5 min (Francisco *et al*., 2005).PCR products were separated by 1% agarose gel so that bands around 529 bp length were excised (Francisco and Galetti Junior 2005; Simon et al. 1991).The sequencing of 16S rRNA fragment of mitochondrial DNA sequencing was performed according to the method of Sanger *et al*. (1977) by automatic sequencer ABI 377 (Applied Biosystems Inc.) by Seq/Teq/California/USA. the samples of mitochondrial DNA were sequenced based on 16S-Reverse primer. The accuracy of the obtained sequences was checked using Chromas Pro software based on the chromatogram of each sequence. Then the length of all sequencing was Unified by the BioEdit software.

Phylogenetic analysis and data analyses

In this study, ClustalW2 compared the sequences to each other and similar in NCBI online. ClustalW2 Multiple Alignment and estimating the transition/transversion rate by BioEdit and MEGA 7 software. Evaluation of genetic distance matrix conducted by Kimura-2-parameter based on the removal of gaps or basa Pairwise Considering the transition/transversion mutation, Uniform speed and homogenous pattern between individuals by MEGA7. The phylogenetic tree was drawn based on the Maximum Likelihood method based on 1000 Bootstrap by MEGA 7. We used BioEdite, MEGA 7.0 and DnaSP (Rozas et al. 2003) to analyze the distribution of data obtained from protected regions, molecular diversity indicators, such as the number of haplotypes, polymorphic sites, haplotype and nucleotide diversity, and variance based on Nei (Nei 1978).

Result

Results showed that nucleotide sequences of mitochondrial 16S rRNA regions of different samples had sequence lengths of 488-489 bases. Nucleotide BLAST software indicated 97-100% similarity with the World Gen bank registered sequences. These sequences were registered with accession numbers LC468129.1 and

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LC468130.1 in the World Gene Bank website.

Based on the alignment of the sequences of different populations, it can be concluded that they are all similar. As a result of the 486 loci identified, there are 484 conserved loci and two variable loci, which include phylogenetic information under parsimony conditions for both loci, and there are 18 singletons among these loci. Also, DnaSP analysis of 486 base pairs of mitochondrial 16S rRNA sequences of different generations of *P. vannamei* revealed that 3-6 haplotypes were identified among various generations. This was while polymorphism was in the range of 198-231. The amount of gene flow and gene differentiation between different generations of shrimp was -2.00 and -0.142, respectively (Table 1).

Table 1: Genetic indicators in two population of broodstocks of *P. vannamei*.

Population	Detected sites	Number of haplotypes	Number of polymorphs	The number of monomorphs	Singleton	Parsimonious	Haplotype diversity	Nucleotide diversity	homozygosity
High helath	486	1	0	486	18	3	0.0±0.00	0	1
Molokaei	486	1	0	486	18	3	0.0±0.00	0	1

According to the 16S rRNA sequence, the AT-rich region consists of 65-65.2% of the sequence, and its average content is 65.1%. This region also contained 32.7, 14.0, 32.3 and 21.0 % T, C, A, and G bases (Table 2).

Table 2: The percentage of base composition in the 16S rRNA region.

Population	T%	C %	A %	G %	AT %	Total
High Health (F0)	32.7	14.0	32.3	21.0	65.0	486.0
Molokaei (F0)	32.7	14.0	32.3	21.0	65.0	486.0
Avg.	32.7	14.0	32.3	21.0	65.1	485.6

Nucleotide abundance was calculated to be 35.20 % for A, 33.86 % for T, 11.51 % for C, and 19.44 % for G. The transition to transversion rate ratios for purines (K1) and pyrimidines (K2) are 5.48 and 18,896, respectively. Also, R and 4.483 were calculated as the tendency of transition to the overall transversion (Table 3).

Table 3: Maximum composite likelihood estimate of the pattern of nucleotide substitution.

	A	T	C	G
A	-	2.5	0.85	**7.85**
T	2.59	-	**16.03**	1.43
C	2.59	**47.16**	-	1.43
G	**14.22**	2.5	0.85	-

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Phylogenetic analysis of the genetic identity and distance matrix revealed a very significant similarity between different shrimp generations. There was a 99.49 and 98.99 % similarity between LC468129.1 and LC468130.1 with LC121701.1, respectively (Table 4). A comparison between the sequence of different generations of the study shrimp and the sequence recorded in NCBI indicated a genetic distance of 0.003 – 0.009 (Table 5).

Table 4: Genetic identity between different generations of *P. vannamei* with sequences registered in the World Gene Bank.

		1	2	3	4	5	6	7	8	9	10	11	12
1	LC121701.1		99.74	99.43	99.24	99.49	99.49	98.99	99.24	98.73	98.99	98.99	95.44
2	LC121704.1	99.74		99.71	99.49	99.74	99.74	99.23	99.49	98.97	99.23	99.23	95.63
3	LC121703.1	99.43	99.71		99.43	99.71	99.71	99.43	99.43	98.86	99.14	99.14	95.43
4	AY344192.1	99.24	99.49	99.43		99.78	99.78	99.31	99.54	99.08	99.35	99.35	96.09
5	**LC468129.1**	99.49	99.74	99.71	99.78		100	99.54	99.77	99.31	99.56	99.56	96.3
6	MK430849.1	99.49	99.74	99.71	99.78	100		99.54	99.77	99.31	99.56	99.56	96.3
7	HM014410.1	98.99	99.23	99.43	99.31	99.54	99.54		99.77	99.31	99.54	99.54	96.08
8	HM014411.1	99.24	99.49	99.43	99.54	99.77	99.77	99.77		99.54	99.77	99.77	96.31
9	HM014412.1	98.73	98.97	98.86	99.08	99.31	99.31	99.31	99.54		99.77	99.77	96.31
10	**LC468130.1**	98.99	99.23	99.14	99.35	99.56	99.56	99.54	99.77	99.77		100	96.73
11	MH300646.1	98.99	99.23	99.14	99.35	99.56	99.56	99.54	99.77	99.77	100		96.73
12	AF255054.1	95.44	95.63	95.43	96.09	96.3	96.3	96.08	96.31	96.31	96.73	96.73	

Table 5: Genetic distance between different generations of *P. vannamei* with sequences registered in the World Gene Bank.

		1	2	3	4	5	6	7	8	9	10	11
1	LC121701.1											
2	LC121704.1	0.003										
3	LC121703.1	0.003	0.000									
4	AY344192.1	0.003	0.000	0.000								
5	**LC468129.1**	0.003	0.000	0.000	0.000							
6	MK430849.1	0.003	0.000	0.000	0.000	0.000						
7	HM014410.1	0.006	0.003	0.003	0.003	0.003	0.003					
8	HM014411.1	0.006	0.003	0.003	0.003	0.003	0.003	0.000				
9	HM014412.1	0.012	0.009	0.009	0.009	0.009	0.009	0.006	0.006			
10	**LC468130.1**	0.009	0.006	0.006	0.006	0.006	0.006	0.003	0.003	0.003		
11	MH300646.1	0.009	0.006	0.006	0.006	0.006	0.006	0.003	0.003	0.003	0.000	
12	AF255054.1	0.049	0.046	0.046	0.046	0.046	0.046	0.043	0.043	0.043	0.039	0.039

According to Neighbor-Joining algorithms, there were each of population with accession number LC468129.1 and LC468130.1 with 64% and 66% in a separate branch, respectively (Figure 1).

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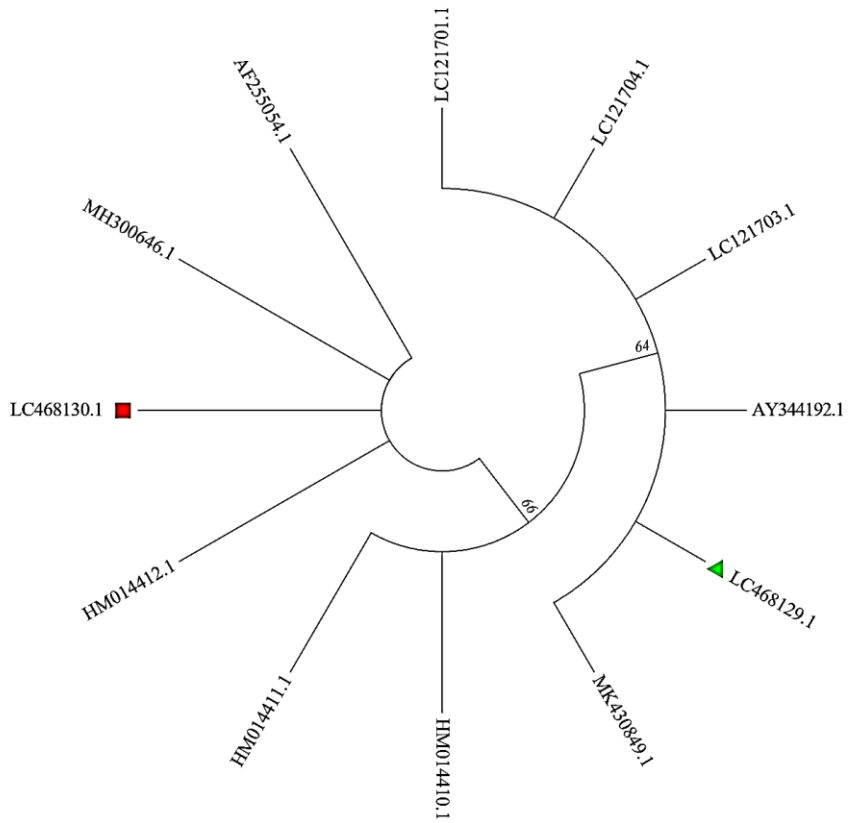

Figure 1: Neighbor-Joining phylogenetic analysis of mitochondrial 16S rRNA regions of different generations of *P. vannamei*

Discussion

There were 484 conserved positions out of the 486 aligned positions of the 16S rRNA gene sequence, of which two variable positions were found in both information positions out of the 486 aligned positions. Only two haplotypes and polymorphic loci were observed in all samples from different shrimp generations. In contrast, there was a small amount of nucleotide diversity among the generations of shrimp, indicating that nucleotide diversity is low in different generations of shrimp, hence the small value of this index. Genetic diversity values obtained from other research are consistent with these data. According to Shahrani et al. (2015), the 16S rRNA mitochondrial gene sequence was used in the study of *Metapenaeus affinis* in the Persian Gulf to calculate the average haplotype diversity among Bandar Abbas, Bushehr, and Abadan, and the average nucleotide diversity was calculated to be 0.002±0.003. Based on the results obtained from this study, it can be concluded that the amount of haplotype diversity in shrimps from the study areas was moderate. In contrast, the amount of nucleotide diversity was low. A comparison of the results of this study with those obtained by Sourinejad et al. (2014) on *Fenneropenaeus merguiensis* in Khorat Laft and Sirk in Hormozgan province indicates that genetic diversity in these shrimp is higher than in different generations in

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this study. There was also a significant reduction in genetic and nucleotide diversity. It can be stated that due to the keeping of *P. vannamei* in captivity and their non-native ness, as well as the lack of access to wild broodstocks due to the increase in gene flows between different generations, the amount of genetic diversity was extremely reduced. Due to the high conservation of the sites in the 16S rRNA region and its low evolution, the genetic distance between different shrimp generations was very low. According to Maggioni et al. (2001), the 16S rRNA region of the genome can show that the *F. subtilis* shrimp can have a different genetic pattern than other species. Unlike mitochondrial genomes located in the 16S rRNA region, the cytochrome oxidase I (COI) region can be used to differentiate shrimps genetically and determine the differences among shrimps. So that these sequences are able to show sufficient nucleotide diversity to determine the haplotype created in broodstocks. Mendoza-Cano et al. (2013) identified eight diverse loci for *F. subtilis* using the embryo cytochrome oxidase genome. However, in the comparison between this *F. subtilis* shrimp and *P. vannamei*, 70 different positions were identified. There is evidence that the COI gene provides an increased replacement rate, which can lead to the detection of genetic differences between populations of the same species (Gusmão et al. 2000).

According to the phylogenetic tree analysis based on Neighbor-Joining algorithms, despite the low genetic distance between different generations of shrimp, the shrimps resulting from the mating of High Health and Molokai shrimps are very similar to those of a zero generation of High Health shrimp. As a result, other shrimp generations were also placed on the same branch (Hosseini et al. 2004; Noroozi and Hosseini 2015). Gusmão et al. (2000) stated that due to the low genetic distance for five of the *P. vannamei* population (0.2-1%), they have a high similarity.

The genetic distance in the cytochrome oxidase region between *P. vannamei* and *Farfantepenaeus subtilis* is 10.94%, while it is 8.1% between *F. paulensis* and *F. subtilis* (Mendoza-Cano et al. 2013). The low genetic distance between generations may have been caused by mutation during their creation or by random genetic drift and or founder. Due to the lack of recombination in the maternal mitochondrial genome, it is expected to be highly susceptible to random events, such as genetic drift. In a population, haplotype diversity can range from zero (all individuals have the same haplotype) to one (all individuals have different haplotypes). Benzie et al. (2002) reported in a similar study conducted on *Penaeus monodon*, *P. vannamei* (Valles-Jimenez et al. 2004), *Farfantepenaeus duorarum* and *L. setiferus* (Bert and McMillen-Jackson 2004) that mitochondrial genome haplotype diversity was moderate to high. Although, the level of haplotype diversity was low (0.24) among

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Chinese shrimp (*Fenneropenaeus chinensis*). In general, for marine species, and particularly decapods, the high haplotypic diversity is attributed to their population size and dispersion, resulting in the maintenance of many unique haplotypes in the population (Zardoya et al. 2004). Additionally, effective population size and evolutionary dynamics (mutation, genetic drift, natural selection) may also affect genetic variation patterns at different levels (de Croos and Pálsson 2010). In this study, there were no differences in genetic diversity between generations of white-leg shrimp because there were no geographical separations and no spatial distances between reservoirs. Beacham et al. (2005) stated that due to the reduction of gene flows due to the presence of physical or natural barriers, with the increase of geographical distance, the genetic distance will also increase. The genetic differences between populations result from the integration of individuals in an area, while inbreeding leads to a gene pool unique to the same species (Piñera et al. 2007). Therefore, according to the results of the current research, it seems that there has been more transfer and exchange of gene flow between different generations of broodstock at this hatchery center.

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Conflict of Interest

To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

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