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Full Length Research Paper

Assessment of the genetic diversity in five generations of a commercial broodstock line of *Litopenaeus vannamei* shrimp

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Genetic variation among and within five generations of an inbred commercial captive line of *Litopenaeus vannamei* and genetic distance among them were evaluated by random amplified polymorphic DNA (RAPD), using descriptive and genetic similarity analyses for dominant markers at single- and multi-populational level. One hundred individuals were analyzed and 56 polymorphic loci were identified with a set of six primers screened in a painel of 38 decamer primers tested. The values obtained for F_5, F_6, F_7, F_8 and F_9 generations revealed a progressive increasing of genetic similarity throughout the five stocks, confirmed by Nei genetic diversity analysis, which values decreased from 0.27 to 0.22 at F_5 and F_9, respectively. Significant allele frequency differences were observed at most analyzed loci. Strong correlation (R^2 = 0.9845) was observed between genetic similarity and generation time, and genetic similarity could reach closer to 100% at 18-19 generation of this line.

Key words: *Litopenaeus vannamei*, genetic diversity, genetic drift, inbreeding, RAPD.

INTRODUCTION

Loss of genetic variation in small populations can be a consequence of genetic drift and inbreeding, commonly observed within captive and/or threatened stocks (Sbordoni et al., 1986). The low levels of genetic variability may reduce the mean fitness of a population, affecting its viability, particularly, if parasites or competitors are present (Vrijenhoek, 1994). It is well known that the genetic variability declining in natural populations, associated to inbreeding and genetic drift, increases the probability of extinction of small populations, determining, in some cases, a process called extinction vortex in nature (Gilpin and Soulé, 1986).

Within captive populations, these effects is far well known (Bradford et al., 1958), including in aquaculture-based larger effective populations (Tave, 1993). It is important for a successful aquaculture to prevent such genetic variation losses.

Genetic variability reduction has previously been reported in reared shrimp. In *Marsupenaeus japonicus*, both inbreeding and genetic drift were implicated in a continuous reduction in the genetic diversity levels throughout five captive generations (Sbordoni et al., 1986).

It is already recognized that the current Brazilian shrimp farming activity involves an excellent management technology, with high productivity around 6.5 ton/ha/year (Rocha et al., 2004). Several advances were made after the introduction of the Pacific white shrimp, *Litopenaeus vannamei*, currently corresponding to 90% of all farmed shrimp in Brazil. The complete control of the reproductive cycle and post-larvae production have consolidated the Brazilian broodstocks, making it unnecessary for the importation of post-larvae and adult prawns, and avoiding the introduction of exotic pathogens (Batalha et al., 2002). Apart from the remarkable development of shrimp culture in

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Brazil, selective breeding programs have poorly been conduced (Galetti and Freitas, 1999). Most of hatchery laboratories develop management programs without any monitoring of the levels of genetic variability of their broodstocks.

In the present study, five generations of a closed line of the white-shrimp *L. vannamei* were analyzed by RAPD markers to assess the genetic variation within and between the cultured generations, as well as to infer the more likely causes leading to genetic variability reduction in these animals.

MATERIAL AND METHODS

Sample collection

A commercial line of *L. vannamei* owned by Valença da Bahia Maricultura shrimp hatchery and farm**,** located on the Northeastern region of Brazil in the municipality of Valença (Bahia state), was studied for five generations. The founder individuals of this line were obtained in 1995 by importation of native individuals mainly from Panama (80%), and native and farmed individuals from other countries including Ecuador, Venezuela, Costa Rica, and Mexico (20%). When adapted to captivity conditions, males and females were selected and, after the first spawning on captive conditions, the generation F_1 was obtained. At each year, a new generation was obtained after selection and mating of individuals belonging to the previous generation. The choice of breeders to originate the subsequent generations involved three phases: first, an individual body size selection of about 35 thousands of post-larvae (~PL21) followed by a second individual body size selection of 8-12 thousands young shrimps (~80 days old) also, free of necrosis and displaying perfect rostrum and antenna, and finally a third individual body size selection of 4000 adults (~240 days old) that are free of necrosis, displaying perfect antenna and rostrum and adequate pigmentation of gills, uropods and spermatophores. After this latter selection, 140 pairs, free of necrosis and other abnormalities, were transferred to maturation tanks and only egged females were kept at collective spawning tanks. The material sampling for genetic analysis was initiated at 1999. During the following years, samples of pleopods from shrimps belonging to generations F_5 (n=20), F_6 (n=20), F_7 (n=20), F_8 (n=20), and F_9 (n=20) were collected. The tissue samples were fixed in 1 ml of 95% ethanol and kept at -20°C.

Molecular analysis

Genomic DNA extraction followed phenol:clorophorm:isoamyl protocol (Sambrook et al. 1989). Ready-To-Go™ RAPD Analysis Beads Kit (Amersham Pharmacia Biotech) was used for amplification reactions in a PTC-100™ thermal cycler (MJ Research). To each bead containing thermostable polymerases (AmpliTaq™ DNA polymerase and Stoffel fragment), 0.4 mM of each dNTP, 2.5 µg BSA, 3 mM $MgCl_2$, 30 mM KCl, and 10 mM Tris (pH 8.3), 50 ng of template DNA, 25 pmol of a single RAPD primer and milliQ water to a 25 µl final reaction volume were added. PCR was programmed with a first cycle at 92°C for 4 min, followed by 35 cycles at 92°C for 2 min, 36°C for 1 min and 30 seconds and 72°C for 2 min with a final extension at 72°C for 3 min. The PCR products were run on agarose gel at 1.5% (for 3 h at 100 V) using 1X TBE buffer (Tris base, boric acid, EDTA) containing 0.5 µg/ml of ethidium bromide. The gels were visualized under UV light and a digital imaging system for electrophoresis gel documentation and analysis (Kodak Digital Science™ EDAS 290) was used. From the 38 arbitrary primers tested, only those with high reproducible amplification pattern were used for statistical analyses. After the selection of a set of six primers, the amplification profiles were established and new repeatability tests were carried out for each primer. The most representative loci were identified and used in statistical analyses.

Statistical analyses

In the RAPD analysis, dominance of alleles for the presumptive phenotypes, Mendelian segregation and populations under Hardy-Weinberg equilibrium, were assumed (D`Amato and Corach, 1996). A data matrix based on the presence (1) or absence (0) of a band for each selected locus was made involving all sampled individuals. A single- and multi-population descriptive statistics analysis for diploid data using dominant markers was performed by using the software Popgene version 1.31 (Yeh et al., 1999). Allele frequency difference among generations was calculated based on chi-square test was determined by studying multilocus genetic structure differences amongst strains. Percentage of polymorphic loci, gene diversity (Nei, 1973) and unbiased measure of genetic distance (Nei, 1972) were calculated. The Jaccard's similarity coefficient (Jaccard, 1901), which ignores the absence of bands as an indicator of similarity, was estimated by using the software NTSYS-pc version 1.8 (Rohlf, 1993). Jaccard´s coefficient divergences were tested by Mann Whitney and Kruskal Wallis test using BioEstat 2.0 software program (Ayres et al., 2000).

RESULTS

Sixty-four loci were selected for analysis and 87.5% were polymorphic. The F5 and F9 generations showed the largest and smallest number of polymorphic bands, respectively (Table 1). The Jaccard's similarity coefficients calculated for the sampled stocks revealed a remarkable increasing of genetic similarity levels throughout the five analyzed generations (P = 0.026) when values from F_5 and F_9 generations were compared. Analyses of genetic diversity have also suggested a higher genetic homogeneity amongst individuals from the latter generations. The mean heterozygosity declined from 0.267 at F_5 generation to 0.215 at F_9 generation (Table 2). Analyses of gene frequencies demonstrated significant differences (P<0.05) at most loci, as determined by homogeneity tests (Table 3). The fragments P2-a_1, P2-c_3, P3-j_{10}, P4-i_9, P5-e_5, P5-o_{15}, P6-i_9, and P6-a_1 demonstrated an increasing of their frequencies along the five generations, leading to a higher degree of homogeneity amongst individuals from latter generations. The P3-i_9 and P4-d_4 displayed progressive declined frequencies, while the fragment P4-b_2, present in F_5, F_6 and F_7, was absent in F_8 and F_9 generations. Other loci also underwent to a drastic frequency reduction at F_9 generation, becoming present in less than 8% of individuals, for instance P4-f_6 and P5-b_2. In contrast, two fragments, named P2-b_2 and P3-k_{11}, absent from F_7 generation, appeared again in the F_8 and

Table 1. Nucleotide composition, number of bands and polymorphic bands per generation of each primer used in the RAPD reactions.

Oligonucleotide	Nucleotide Sequence	Number of bands	Number of polymorphic bands per generation				
			F5	F6	F7	F8	F9
Primer 1	5' GGTGCGGGAA 3'	11	06	06	06	05	05
Primer 2	5' GTTTCGCTCC 3'	07	06	05	05	05	03
Primer 3	5' GTAGACCCGT 3'	12	10	11	09	11	11
Primer 4	5' AAGAGCCCGT 3'	10	05	05	06	06	04
Primer 5	5' AACGCGCAAC 3'	15	14	13	10	10	09
Primer 6	5' CCCGTCAGCA 3'	09	08	06	07	07	03
Total		**64**	**49**	**46**	**43**	**44**	**35**

Table 2. Jaccard's genetic similarity coefficient, sample size, polymorphic loci percentage, Nei's genetic diversity (h) and the standard deviation (sd) determined for each generation (F_5 to F_9), based on the results obtained with primers 1, 2, 3, 4, 5, and 6 in RAPD reactions.

Stock	Sample	Polymorphic loci	Jaccard's similarity	Nei genetic
F_5	20	76,56%	0.621	0.267±0.187
F_6	20	71.88%	0.662	0.259±0.188
F_7	20	67.19%	0.701	0.242±0.198
F_8	20	68.75%	0.749	0.253±0.196
F_9	20	54.69%	0.795	0.216±0.211

Table 3. Allele frequency and homogeneity test based on the determination of chi-square (X^2) and error probability (p), where $P < 0.05$ and df=4.

Fragments		Allele Frequency (f)					Homogeneity Test	
Locus	Size (pb)	F_5	F_6	F_7	F_8	F_9	X^2	p
P1-a_1	700	0.106	0.134	0.225	0.163	0.293	3.014	0.555
P1-b_2	670	0.329	0.194	0.225	0.408	0.553	7.517	0.111
P1-c_3	650	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P1-d_4	560	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P1-e_5	530	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P1-f_6	500	0.193	0.780	0.408	1.000	0.500	41.459	0.000^S
P1-g_7	430	0.258	0.194	0.513	0.613	0.553	20.761	0.000^S
P1-h_8	410	0.225	0.194	0.780	0.163	0.776	30.366	0.000^S
P1-i_9	400	0.553	0.776	0.684	0.776	1.000	11.647	0.200^S
P1-j_{10}	360	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P1-k_{11}	300	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P2-a_1	710	0.777	0.776	0.776	0.776	1.000	5.446	0.244
P2-b_2	700	0.780	0.258	0.000	0.025	0.000	14.101	0.007^S
P2-c_3	660	0.776	1.000	1.000	1.000	1.000	18.726	0.001^S
P2-d_4	640	0.258	0.106	0.329	0.134	0.163	4.368	0.358
P2-e_5	550	1.000	1.000	0.776	1.000	1.000	8.726	0.001^S

Table 3. contd.

P2-f_6	530	0.258	0.293	0.367	0.293	0.452	2.180	0.703
P2-g_7	500	0.452	0.408	0.408	0.776	0.225	12.937	0.012^S
P3-a_1	900	0.258	0.293	0.163	0.134	0.258	2.188	0.701
P3-b_2	850	0.780	0.163	0.613	0.051	0.293	22.984	0.000^S
P3-c_3	700	0.293	0.225	0.134	0.329	0.194	2.694	0.610
P3-d_4	660	0.134	0.163	0.134	0.025	0.329	7.260	0.123
P3-e_5	650	0.194	0.513	0.513	0.553	0.293	19.666	0.001^S
P3-f_6	640	0.258	0.106	0.163	0.293	0.553	11.942	0.018^S
P3-g_7	510	0.452	0.258	0.163	0.258	0.684	14.926	0.005^S
P3-h_8	500	0.258	0.500	0.329	1.000	0.553	26.998	0.000^S
P3-i_9	450	1.000	1.000	1.000	0.776	0.684	19.046	0.001^S
P3-j_{10}	410	0.225	0.408	0.452	0.684	0.776	15.670	0.003^S
P3-k_{11}	380	0.225	0.163	0.000	0.613	0.684	31.546	0.000^S
P3-l_{12}	340	1.000	0.776	1.000	0.776	1.000	14.734	0.005^S
P4-a_1	900	0.452	0.684	0.613	0.776	0.613	4.833	0.305
P4-b_2	840	0.780	0.500	0.500	0.000	0.000	32.362	0.000^S
P4-c_3	600	0.408	0.776	0.776	0.684	0.776	9.403	0.052^S
P4-d_4	520	1.000	1.000	0.776	0.776	0.776	10.330	0.035^S
P4-e_5	430	1.000	1.000	0.776	0.776	1.000	14.734	0.005^S
P4-f_6	400	0.253	0.134	0.106	0.134	0.078	1.922	0.750
P4-g_7	350	1.000	0.776	1.000	0.684	1.000	19.046	0.001^S
P4-h_8	290	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P4-i_9	270	0.776	1.000	1.000	1.000	1.000	18.726	0.000^S
P4-j_{10}	220	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P5-a_1	850	0.613	0.500	1.000	1.000	1.000	33.232	0.000^S
P5-b_2	800	0.258	0.293	0.258	0.329	0.078	4.084	0.395
P5-c_3	700	0.258	0.293	0.258	0.163	1.000	39.135	0.000^S
P5-d_4	660	0.367	0.553	1.000	0.500	0.452	20.032	0.000^S
P5-e_5	620	0.258	0.553	0.553	1.000	1.000	37.672	0.000^S
P5-f_6	540	0.553	0.780	0.134	0.194	0.134	17.146	0.002^S
P5-g_7	510	0.780	0.613	0.613	0.776	0.367	23.757	0.000^S
P5-h_8	500	0.253	0.131	0.513	0.367	0.452	18.153	0.000^S
P5-i_9	490	0.106	0.452	0.293	0.452	1.000	35.843	0.000^S
P5-j_{10}	470	0.293	0.408	0.323	0.452	0.106	6.651	0.155
P5-k_{11}	430	1.000	1.000	1.000	1.000	1.000	0.000	1.000
P5-l_{12}	390	0.613	0.776	0.684	0.408	0.684	6.593	0.159
P5-m_{13}	360	0.134	0.225	0.500	0.293	0.293	7.069	0.132
P5-n_{14}	310	0.500	0.408	1.000	1.000	1.000	31.368	0.000^S
P5-o_{15}	290	0.683	1.000	1.000	1.000	1.000	27.006	0.000^S
P6-a_1	800	0.612	1.000	1.000	1.000	1.000	33.585	0.000^S
P6-b_2	660	0.293	0.613	0.367	0.684	1.000	26.084	0.000^S
P6-c_3	630	0.163	0.225	0.134	0.408	1.000	10.044	0.040^S
P6-d_4	600	0.684	1.000	0.684	1.000	1.000	21.721	0.000^S
P6-e_5	530	0.452	0.134	0.613	0.776	1.000	35.7032	0.000^S
P6-f_6	500	0.613	0.367	0.452	0.684	1.000	20.471	0.000^S
P6-g_7	470	1.000	1.000	1.000	0.776	1.000	18.726	0.001^S
P6-h_8	460	0.513	0.293	0.106	0.258	0.258	5.931	0.204
P6-i_9	420	0.106	0.258	0.293	0.329	0.684	16.360	0.003^S

S: significantly difference, $P<0.05$.

Table 4. Genetic distance based on the Nei's unbiased measure.

Stock	Nei's Unbiased Measure of Genetic Distance				
	F_5	F_6	F_7	F_8	F_9
F_5					
F_6	0.044	*****			
F_7	0.060	0.038	*****		
F_8	0.112	0.084	0.081	*****	
F_9	0.139	0.116	0.122	0.077	*****

F_9 generations, what probably can reflect a sampling artifact. The results obtained from genetic diversity analysis demonstrated differentiated levels of genetic distance among the stocks (Table 4).

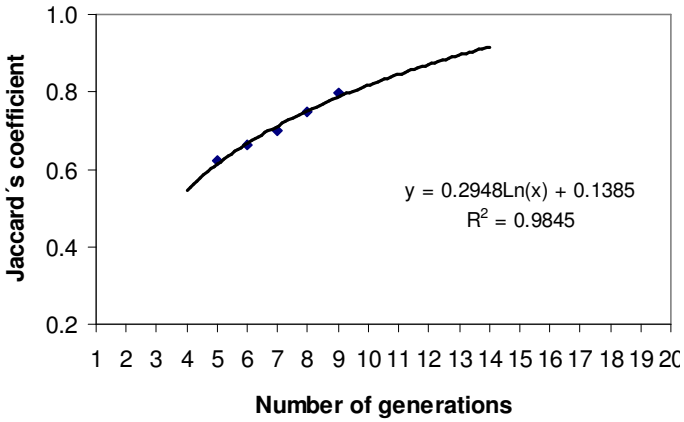

Figure 1. Jaccard´s similarity coefficient against number of generations in the broodstock line studied. Correlation (R^2) = 0.9845.

DISCUSSION

Allele frequency distribution along the five surveyed generations showed significant differences at most of the analyzed loci likely related to genetic drift and inbreeding. A putative random founder effect during the selection process of broodstock can lead to an effective loss of alleles in the following generations, just as verified for the fragment P4-b_2 absent in the generations F_8 and F_9. In contrast, several other alleles were only detected in the latter generations and could account for inbreeding effects, increasing their frequencies along the generations, although small sampling size effects could not be discarded.

Inbreeding effects should also lead to the observed decreased genetic diversity along the generations, verified by a strong positive correlation between genetic similarity and generation time (R^2 = 0.9845) and the genetic similarity could reach 100% near to 18-19 generation in this commercial line studied (Figure 1).

Similar findings have already been reported in other aquaculture organisms. Allozyme allele loss and heterozygosity reduction were reported when captive stocks of *L. vannamei* were compared with wild populations (Sunden and Davis, 1991). Allele losses were also revelead in reared lines of *Litopenaeus stylirostris*, and divergences observed between two studied lines indicated a probable founder effect since both stocks shared a common origin from a single wild population (Ramos-Paredes and Grijalva-Chon, 2003).

Founder effect and inbreeding have also been identified in *M. japonicus* populations, revealing a continuous reduction of allozyme polymorphism level throughout six captive generations, from 0.102 at F_1 to 0.039 at F_6 generation. An initial bottleneck which occurred on the first generation might have favored to a remarkable reduction of genetic variation levels in the following ones. Although the number of shrimp pairs used to produce the next generation ranged from 50 to 300, this study revealed that the effective number of parents contributing to each broodstock was as low as four (Sbordoni et al., 1986).

The commercial line here analyzed was formerly founded with animals originated from different countries (Panama, Ecuador, Venezuela, Costa Rica, and Mexico) and they have undergone a domestication process, currently reaching up to F_9 generation. Despite of this, diverse origin has contributed to a higher genetic diversification at the founder population since new gene pools were never introduced since then. The maintenance of this closed line throughout generations has favored the increasing of inbreeding and, conesquently, to a higher consanguinity level. Although, thus far, there were no evidence of performance losses regarding to fecundity and growth rates in the shrimp lineage studied here which could be accounted to inbreeding depression.

The increase of the effective number of mating pairs could be a useful strategy to diminishing the reduction rate of genetic diversity within this line, and cross-breeding using shrimp of different origins could recover

part of genetic variability lost in this commercial closed line.

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