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Research Article

Genetic variation in a closed line of the white shrimp *Litopenaeus vannamei* (Penaeidae)

Eloize Luvesuto, Patrícia Domingues de Freitas and Pedro Manoel Galetti Junior

Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP, Brazil.

Abstract

The culture of the marine shrimp *Litopenaeus vannamei* has recently boosted the Brazilian shrimp industry. However, it is well known that selection methods based solely on phenotypic characteristics, a reduced number of breeders and the practice of inbreeding may promote a significant raise in the genetic similarity of the captive populations, leading to greater disease susceptibility and impairing both the growth and final size of the shrimps. We used four microsatellite loci to investigate genetic variation in three generations (F_5, F_6 and F_7) of a closed and reared *L. vannamei* lineage. Although an accentuated heterozygosis deficit was detected, we also observed that the captive propagation of this lineage did not lead to a significant loss of genetic variability over the three generations studied. One possible reason for this is that the breeding conditions of this lineage were good enough to prevent any significant loss of genetic variability. However, three generations may have been insufficient to produce detectable changes in genetic frequencies in the loci studied. Alternatively, the microsatellite loci may have been non-neutral (biased) and related to the conditions in which the shrimps were kept, resulting in a similar allele pool in respect to these four microsatellites over the three generations studied. Any generalizations regarding microsatellite variation in closed shrimp lines may thus be incomplete and should be carefully analyzed.

Key words: broodstocks, inbreeding, penaeids, selection.

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Introduction

Culture of the Pacific white shrimp *Litopenaeus vannamei* has promoted the rapid growth and development of the Brazilian shrimp farm industry over the last few years, Brazil currently being the leading the shrimp aquaculture industry the Americas (ABCC, 2004). The shrimp *L. vannamei* is naturally distributed in the eastern Pacific from southern Mexico to northern Colombia and was introduced into Brazilian aquaculture during the early 1990's. However, in 1997 a Brazilian federal law restricted the importation of exotic aquatic species and all current *L. vannamei* broodstocks in Brazilian hatcheries have descended from the genetic material imported on several different occasions prior to 1997 (MAPA/SARC/DPA *et al.*, 2001). The renewal of the Brazilian *L. vannamei* broodstock gene pool is still hindered by importation restrictions, which may result in cultures of this exotic species suffering severe genetic damage unless an efficient genetic monitoring program is implemented (Freitas and Galetti Jr., 2002, 2005). The loss of genetic variability in Brazilian *L. vannamei* broodstocks over succeeding generations and inbreeding depression in small populations may contribute to the reduction of the survival, growth and reproduction rates (Gjedrem, 2005) and the capacity of the broodstocks to adapt to environmental changes (Sbordoni *et al.*, 1986).

A strict correlation between loss in genetic variation and shrimp production performance is well documented. In *Litopenaeus stylirostris*, low growth performance has been correlated with inbreeding in a closed line maintained in captivity for a long period of time (Bierne *et al.*, 2000). The high incidence of dwarfed shrimp observed during the harvest of several species is believed to be a consequence of the reduced genetic diversity in culture stocks (Benzie *et al.*, 1992, 1993) and, therefore, the efficiency of any selective breeding program is related to the level of genetic variability available in the population under selection (Goyard *et al.*, 2003; Gjedrem, 2005).

Microsatellite analyses have been used to assess genetic variation in several penaeid species, such as *L. vannamei* (Wolfus *et al.*, 1997), *Marsupenaeus japonicus* (Moore *et al.*, 1999), *L. stylirostris* (Bierne *et al.*, 2000), *Penaeus monodon* (Tassanakajon *et al.*, 1998; Brooker *et al.*, 2000; Pongsomboon *et al.*, 2000, Xu *et al.*, 2001), *Litopenaeus schmitti* (Maggioni *et al.*, 2003; Borrel *et al.*,

Send correspondence to Pedro Manoel Galetti Junior. Departamento de Genética e Evolução, Universidade Federal de São Carlos, Caixa Postal 676, 13565-905, São Carlos, SP, Brazil. E-mail: galettip@ufscar.br.

2004) and *Litopenaeus setiferus* (Ball and Chapman, 2003) but, however, little is known on the genetics of the Brazilian *L. vannamei* broodstocks (Freitas and Galetti Jr 2002, 2005; De Francisco and Galetti Jr, 2005; Gonçalves *et al.*, 2005). In the work described in the present paper we used microsatellite analysis to investigate the genetic variation in three consecutive generations (F_5, F_6 and F_7) of a Brazilian closed breeder *L. vannamei* lineage.

Material and Methods

Specimen selection

The *L. vannamei* specimens used in the analyses were obtained from a closed breeder lineage owned by the Valença da Bahia Maricultura shrimp hatchery and farm located in the town of Valença in the northeastern Brazilian state of Bahia. These broodstocks descend from a pool of native founder animals imported mainly from Panama (80%) and native and cultured *L. vannamei* from other countries such as Ecuador, Venezuela, Costa Rica and Mexico (20%). After adaptation to the conditions in captivity, males and females were selected and the F1 generation was obtained following the first spawning. Each year, a new generation was obtained after the selection and mating of individual *L. vannamei* from the previous generation. The choice of the breeders used to initiate the subsequent generations involved three individual body size selection phases, an initial selection of about 35 thousand post-larval shrimps, followed by a second selection round of 8 thousand to 12 thousand young shrimps (~80 days old) and a third selection of 4,000 adults (~240 days old) that were also free of necrosis, displayed perfect antenna and rostrum and adequate pigmentation of gills, uropods and spermatophores. After the third selection, 140 couples were transferred to maturation tanks, with only egged females being kept in collective spawning tanks. In 1999 we started sampling these shrimp for genetic analysis, and in the subsequent years we obtained pleopod samples from a total of 85 shrimps from the F_5, F_6 and F_7 generations, these samples being collected and stored in 1 mL of 95% ethanol at -20 °C. Genomic DNA extraction was performed according to Sambrook *et al.* (1989).

Amplification of microsatellite loci

Four primer sets, Pvan1758 and Pvan1815 (Cruz *et al.*, 2002) along with Lvan1 and Lvan7 (Freitas *et al.*, 2007), were used in a 25 µL reaction volume containing 50-150 ng of DNA, 0.2 mM of each dNTP, 7.5 pmoles μL^{-1} of each primer, 1.5-2.5 mM $MgCl_2$, 1 unit of *Taq* DNA Polymerase and 1 to 1.25x PCR buffer. The Master Mix (2.5X) kit (Eppendorf) was used for the Lvan1 and Lvan7 loci. The primers were 2'-chloro-5'-fluoro-7',8'-fused phenyl-1.4-dichloro-6-carboxyfluorescein (NED) fluorochrome-labeled for analysis in an automatic ABI Prism 377 DNA Sequencer (Applied Biosystems). Allele genotyping was performed using the GeneScan 2.1 and Genotyper 2.1 software (Applied Biosystems). The polymerase chain reaction (PCR) was performed in a PTC 100 thermocycler (MJ Research) for five minutes at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at the optimum annealing temperature of each primer and 1 min at 72 °C, with a final elongation for 20 min at 72 °C.

Statistical analyses

The expected heterozygosity (Levene, 1949) and F_{IS} (f) estimates (Weir and Cockerham, 1984) were calculated using the Genepop 3.4 software (Raymond and Rousset, 1995). The observed heterozygosis values in the analyzed generations were compared using the non-parametric Kruskal-Wallis test (Zar, 1999). The estimates of the exact *P*-values for the tests of conformity to the expectations of the Hardy-Weinberg equilibrium were calculated using the Markov-Monte Carlo chain randomization method (Guo and Thompson, 1992) and the values adjusted using the Bonferroni correction (Rice, 1989). The genotypic linkage disequilibrium was tested through the creation of contingency tables for all loci pairs in each population and posterior application of the Markov chain probability test. For all analyses, the chain parameters were as follows: dememorization number, 1000; number of batches 100; number of iterations per batch, 1000. The number of batches was sufficient for randomization, since it generated a low standard error, that is, less than 0.01. The pairwise comparisons for all populations with all loci were performed and combined throughout all loci using Fisher's method for the results of combined tests. The F_{ST} values (Weir and Cockerham, 1984) and significance of the F_{IS} (f) values were estimated using the FSTAT 2.9.3.2 program (Goudet, 2002). The null hypothesis (H_0) of the identical distribution of the allelic and genotypic frequencies throughout the populations was tested by calculating an unbiased significance estimate of the probability test (Raymond and Rousset 1995).

Results and Discussion

A total of 65 alleles were observed in the three generations studied, 13 for Lvan1, 17 for Lvan7, 21 for Pvan1758 and 14 for Pvan1815. No genotypic disequilibrium was found for any of the loci analyzed. The frequency of the most common alleles ranged from 0.139 for alleles 166, 174 and 184 in the Lvan7 locus to 0.35 for allele 135 in the Lvan1 locus. The Lvan1 135 allele was the only allele predominant in all three generations studied, but whether or not phenotype breeder selection is promoting its increased frequency is still an open question. In the three generations studied most loci showed a significant deviation from the Hardy-Weinberg equilibrium ($p < 0.012$) when the Bonferroni correction (Rice, 1989) was applied and the F_{IS} values obtained (Table 1) could be probably due to genetic drift, inbreeding and selection effects. Heterozygote deficit was

Table 1 - Number of *Litopenaeus vannamei* analyzed (n), observed heterozygosity (H_o), expected heterozygosity (H_e), number of observed alleles per locus per generation (NOA), frequency of the most common allele ($f_{(MC)}$), most common allele (MCA), inbreeding coefficient (F_{IS}) and its significance (p) for the three Brazilian *L. vannamei* broodstocks.

Broodstock and microsatellite loci	n	H_o	H_E	NOA	$f_{(MC)}$	MCA	F_{IS}	p
F5 broodstock								
Lvan1	20	0.35	0.80	7	0.350	135	0.569	0.0003
Lvan7	18	0.39	0.92	12	0.139	166/174/184	0.586	0.0001
Pvan1758	20	0.40	0.88	10	0.250	189	0.550	0.0001
Pvan1815	20	0.35	0.91	12	0.175	133	0.623	0.0001
Mean		0.37	0.88	10.3			0.583	0.0001
Deviation		0.03	0.056					
F6 broodstock								
Lvan1	19	0.68	0.77	7	0.342	135	0.112	0.2636
Lvan7	17	0.53	0.92	13	0.206	166	0.431	0.0001
Pvan1758	19	0.47	0.94	13	0.158	185	0.495	0.0001
Pvan1815	19	0.74	0.92	13	0.184	140	0.200	0.0128
Mean		0.60	0.88	11.5			0.319	0.0001
Deviation		0.12	0.08					
F7 broodstock								
Lvan1	20	0.45	0.84	9	0.300	135	0.470	0.0005
Lvan7	20	0.85	0.91	13	0.250	172	0.062	0.2833
Pvan1758	19	0.74	0.92	15	0.210	195	0.204	0.0172
Pvan1815	20	0.30	0.93	12	0.150	132	0.682	0.0001
Mean		0.58	0.90	12.3			0.355	0.0001
Deviation		0.25	0.04					

observed for all studied generations and could be related to null alleles, preferential mating and sampling errors (Cruz *et al.*, 2003).

The mean observed heterozygosity (H_o) values varied from 0.37 to 0.60 and were significantly different ($p = 0.018$) between the three generations but similar to the mean values reported for diverse cultured shrimp species (reviewed by Benzie, 2000). Although sampling effects and a large variance promoted by highly polymorphic markers should not be dismissed, these results could be explained by a raise in the frequency of rare alleles in older generations and the consequent contribution of these alleles to the formation of new genotypic classes. This is particularly significant for the locus Pvan1758, which showed 10 alleles in the F_5, 13 in the F_6 and 15 in the F_7 generations. The allele frequency distribution between the populations indicated a high similarity between populations in terms of the Lvan1, Lvan7 and Pvan1815 loci. However, the Pvan1758 locus showed a significant allele frequency difference when the F_5 x F_7 and F_6 x F_7 pairs were considered (Table 2). In addition, the diverse origin of the shrimps used during foundation of this closed lineage studied and the practices for the selection of breeders for the establishment of the subsequent generation could also favor an increase of heterozygotes if hybrid vigor is present.

The genotypic distribution analysis also detected no differentiation between the populations in relation to genotype frequency (Table 2), while a low F_{ST} value (F_{ST} = -0.004) indicated high homogeneity between the generations. However, it is well known that phenotypic selection, the employment of a reduced number of breeders for the establishment of subsequent generations and inbreeding all contribute to promoting a significant raise in the genetic similarity of captive populations (Freitas and Galetti Jr, 2005; Gjedrem, 2005). The small size of the broodstocks facilitates genetic drift and the consequent fixation of alleles, thus reducing diversity and allowing greater disease susceptibility, decreased growth and a smaller shrimp at harvest (Benzie, 2000; Bierne *et al.*, 2000). Despite contributing to the maintenance of phenotypic characteristics of commercial interest, increased inbreeding rates may result in genetic homogenization and a drastic reduction in heterozygosis (Goyard *et al.*, 2003; Freitas and Galetti Jr, 2005), although no such effects were detected in our study.

There are at least three possible explanations for our results. Firstly, it is possible that the captive breeding of this

Table 2 - Probability of the differentiation test in the distribution of the gene (*Ge*) and genotypic (*Go*) frequencies between *Litopenaeus vannamei* generations F_5, F_6 and F_7. Values of $p < 0.05$ reject the null hypothesis (H_0) that the allele distribution is identical throughout the generations.

Broodstocks	Microsatellite loci									
	Lvan1		Lvan7		Pvan1758		Pvan1815		All loci	
	Ge	*Go*	*Ge*	*Go*	*Ge*	*Go*	*Ge*	*Go*	*Ge*	*Go*
F5 x F6	0.227	0.467	0.173	0.598	0.221	0.253	0.263	0.626	0.020	0.620
F5 x F7	0.111	0.382	0.175	0.442	0.005	0.064	0.095	0.572	0.000	0.253
F6 x F7	0.196	0.334	0.636	0.858	0.003	0.044	0.592	0.868	0.014	0.339

closed *L. vannamei* lineage has indeed not yet resulted in a significant loss of genetic variability among the three generations studied. Secondly, three generations may not have been sufficient to detect genetic variation in the loci studied. Thirdly, the microsatellite loci studied were not neutral but all retained a certain degree of relationship with the conditions under which the broodstocks were reared, thus retaining a similar allele pool with respect to these alleles over the three generations studied. It thus seems that any generalization regarding microsatellite variation in a *L. vannamei* closed broodstock lines could be incomplete and should thus be carefully analyzed.

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