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Genetic monitoring of broodstocks of the marine shrimp *Litopenaeus vannamei* in a closed rearing system in Pernambuco, Brazil

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Abstract

Loss of genetic variability can be detrimental to a population's survival traits and fitness. These effects are likely to be maximized in shrimp closed broodstock rearing systems where post-larvae are often produced from crosses of breeders collected from an associated grow-out farm after mass selection. Long-time broodstock management in closed systems is also expected to lead to reduction in or even complete eradication of genetic variability. The present work aimed at monitoring the genetic variability of a *Litopenaeus vannamei* hatchery in the state of Pernambuco (Brazil), during three successive replacements, using microsatellite markers. No significant genetic diversity losses have been observed through the values of mean heterozygosity ($H_o = 0.460$ and $H_e = 0.660$ in the first sample; $H_o = 0.420$ and $H_e = 0.620$ in the second sample; and $H_o = 0.600$ and $H_e = 0.660$ in the third sample). However, some alleles appear to have been lost after three replacements. The diversity level was considered to be high and is comparable to those reported for wild populations, suggesting that the original imported founder stock of Brazilian *L. vannamei* is likely to have had a high genetic diversity, possibly due to multiple origins.

Keywords: *L. vannamei*, broodstock, genetic monitoring, microsatellites

Introduction

Marine shrimp aquaculture is the most successful segment of world shrimp farming. In recent years, Brazil has become a world-level shrimp producer, mainly because of the introduction of the Pacific white shrimp, *Litopenaeus vannamei*, an exotic species in the Western Atlantic (FAO 2006). Little information is available about the genetic basis of past and present Brazilian commercial broodstocks. Limited historical records show that up to 1997, there were shrimp imports from Mexico, Guatemala, Panama, Colombia, Ecuador and Peru.

Mass selection is a common practice in shrimp aquaculture systems, and its use significantly improves production performance. However, the beneficial effects are quickly counterbalanced by a rapid growth in the inbreeding coefficient, which can affect fitness-related traits such as survival, reproduction and growth rate. The loss of genetic variability is often intensified by lacking or improper genetic management of the cultured populations, leading to inbreeding depression and a negative impact on production (De Donato, Manrique, Ramirez, Mayer & Howell 2005). Loss of genetic variability caused by inadequate breeding strategies has been described for *Penaeus stylirostris* (Bierne, Beuzart, Vonau, Bonhomme & Bedier 2000), *Penaeus monodon* (Xu, Primavera, De La Pena, Pettit, Belak & Alcivar-Warren 2001) and *L. vannamei* in Ecuador and

Mexico (Garcia, Faggart, Rhoades, Alcivar-Warren, Wyban, Carr & Sweeney 1994; Wolfus, Garcia & Alcivar-Warren 1997).

Significant loss of genetic diversity is detrimental to populations and affects their future sustainability (Dunham 2004). The effects are maximized in closed culture systems, where effective population size and the possibility of random mating are reduced. In addition to these usual concerns, for Brazilian shrimp aquaculture breeder imports have been banned since 1997 by the National Environmental Protection Agency (IBAMA), as a sanitary precaution to prevent the spread of exogenous pathogens, causing a break in the genetic input for the domestic broodstocks of the exotic *L. vannamei*.

Genetic variability monitoring programmes have been useful to follow changes caused by inbreeding, genetic drift or selection (Allendorf & Ryman 1987). To date, many types of molecular markers have been used to analyse the genetic parameters of natural and cultured populations; however, DNA microsatellites are quickly becoming a standard for their highly desirable characteristics of reproducibility, polymorphism and co-dominance (Schlötterer 2000). In addition to this, microsatellites are highly abundant and different loci can be alternatively selectively neutral or linked to traits of interest, making this class of marker suitable for a range of applications in aquaculture. The present study was aimed at using microsatellite loci to follow differences in genetic variability between lots of *L. vannamei*, along three successive broodstock replacements in a closed rearing system.

Materials and methods

Sample collection

Three sampling visits were successively conducted from February to October 2006, at a commercial *L. vannamei* hatchery, located in the state of Pernambuco, Brazil. At this facility, brodstocks are composed of 70 males and 70 females, with the same genetic origin. In each visit, 50 animals from the same broodstock were sampled in equal sexual proportions, comprising a total of 150 animals. The intervals between sampling visits were of approximately 4 months, according to broodstock replacement. For each animal, the fifth pair of pleopods was removed and immediately stored in 95% ethanol – a standard procedure to preserve DNA (Sambrook, Fritsch & Maniatis 1989).

DNA extraction

DNA was extracted following the standard PCI-proteinase K protocol of Sambrook *et al.* (1989) with a few modifications. Tissues were digested in Tris-HCl 100 mM, pH 7.5, 1% SDS with a final concentration of 428 $\mu g\,mL^{-1}$ of Proteinase K. The mixture was initially incubated at 50 °C for 2 h, then overnight, at 37 °C. DNA was then purified successively with phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). Each purification step has been followed by centrifugation at 10 000 *g* for 10 min. The supernatant was transferred to a 1.5 mL microtube and total DNA was precipitated using cold absolute ethanol; pellets were washed with 70% ethanol, air dried, resuspended in 50 μL TE (Tris-HCl 10 mM pH 8.0, EDTA 1 mM pH 8.0) and stored at − 20 °C.

Polymerase chain reaction

Three previously described microsatellite loci have been chosen, TUMXLv 5.38, TUMXLv 8.2 and Pvan 0013 (Cruz, Mejia-Ruiz, Perez-Enriquez & Ibarra 2002; Meehan, Xu, Zuniga & Alcivar-Warren 2003), based on the reproducibility of the amplicons under our laboratory conditions.

Polymerase chain reactions were conducted under the following conditions: 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. Each reaction included 1 U of *Taq* polymerase, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 μM of each primer and 100 ng of DNA in a final volume of 10 μL.

Polymerase chain reaction products were separated by electrophoresis in vertical 4% or 5% polyacrylamide gels, depending on the size of the fragments. Electrophoresis was conducted for 1 h and 30 min at 2000 V, 60 MA and 55 W. After electrophoresis, gels were fixed in acetic acid at 10%, dyed with silver nitrate at 0.1% and developed with sodium carbonate at 3%. Images were recorded using a scanner.

Statistical analysis

Statistical analysis were carried out mainly through GENEPOP software (Raymond & Rousset 1995). The number of alleles (A), observed (H_o), expected heterozygosities (H_e) and inbreeding coefficient (F_{IS}) have been calculated as estimators of genetic

diversity. Genetic differentiation among samples, linkage disequilibrium and departure from Hardy–Weinberg expected proportions have been tested through Fisher's exact tests based on Markov chains with 1000 dememorization steps, 100 batches and 1000 iterations per batch. Significance of multiple tests has been corrected by the Bonferroni method (Rice 1989). Effective number of alleles (a_e) was calculated according to Crow and Kimura (1970).

Results

Table 1 describes the genetic variability for the three microsatellite loci used in monitored *L. vannamei* broodstock populations. Average observed heterozygosity (H_o) varied from 0.420 to 0.600, while average expected heterozygosity (H_e) varied from 0.620 to 0.660. The average number of alleles (A) ranged from 4.33 to 5 in the three samples, while the mean effective number alleles (a_e), ranged from 3.29 to 3.79.

Table 1 Genetic variability of three microsatellite loci used in the monitoring of broodstock populations of *Litopenaeus vannamei*

	Population		
Locus	**Sample 1**	**Sample 2**	**Sample 3**
TUMXLv 5.38			
No of individuals	38	36	34
A*	8	8	6
a_e†	5.38	7.14	5
H_o‡	0.970	0.830	0.880
H_e§	0.840	0.870	0.810
TUMXLv 8.2			
No of individuals	38	40	41
A*	3	3	3
a_e†	2.33	2.63	2.70
H_o‡	0.180	0.280	0.610
H_e§	0.570	0.630	0.640
Pvan 0013			
No of individuals	38	43	39
A*	4	3	4
a_e†	2.27	1.59	2.17
H_o‡	0.240	0.160	0.310
H_e§	0.560	0.370	0.540
Average			
Individuals	38	39.67	38
A*	5	4.67	4.33
a_e†	3.33	3.79	3.29
H_o‡	0.460	0.420	0.600
H_e§	0.660	0.620	0.660

*Number of alleles.
†Effective number of alleles.
‡Observed heterozygosity.
§Expected heterozygosity.

All loci showed significant changes in allelic frequencies along the time. For locus TUMXLv 5.38, alleles 216 and 218 were not observed at the third sampling, despite being present at relatively high frequencies in the previous generations (Fig. 1a). For locus TUMXLv 8.2, there seems to be a trend involving the increasing presence of allele 230 with a corresponding decrease in allele 248 frequency along the time (Fig. 1b), while for loci TUMXLv 5.38 and TUMXLv 8.2, the frequencies for all alleles remained below 0.6 and different alleles are represented as most common in each sample. Locus Pvan 0013 showed allele 282 to always be present at a frequency higher than 0.6 (Fig. 1c). No significant linkage disequilibrium was observed, suggesting that the three loci were located in different chromosomes within the *L. vannamei* genome.

The average values of F_{IS} were 0.37 for sample 1, 0.39 for sample 2 and 0.13 for sample 3; there was little difference between the first and the second collection, but a marked decrease between the second and the third.

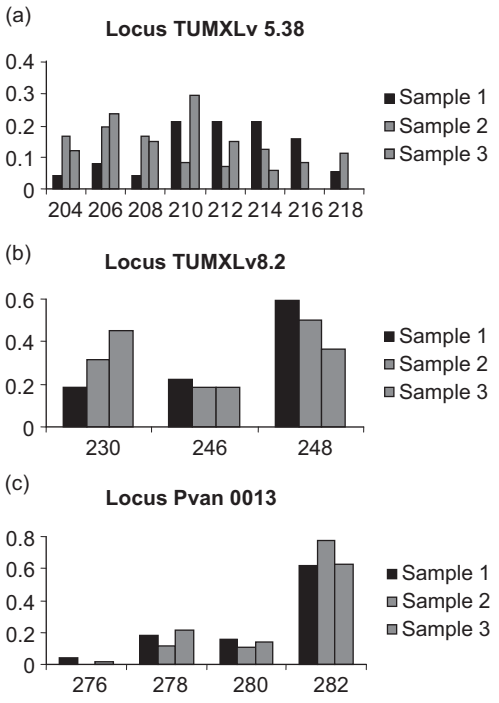

Figure 1 Histogram of allele frequency for the three microsatellite loci. For all loci, frequency differences among samples were highly significant ($P \ll 0.01$ through Fisher's exact test).

Discussion

Most *L. vannamei* hatcheries in Brazil have adopted a closed broodstock rearing system. This system consists in growing breeders in grow-out farms for 1 year, until they are mass selected to form a new broodstock. They are then taken to the associated hatchery for crossing and spawning and some of the progeny is transferred back to the grow-out farm. Broodstocks are replaced every 3–4 months and this cycle is usually repeated until a decline in reproduction performance is detected. At this stage, the genetic composition of the stock is in general considered to be one of the likely reasons for low performance; however, in most cases, not enough information about the history of the broodstocks is available to evaluate this hypothesis objectively. In addition to this, a generally accepted molecular marker standard is not yet available to allow absolute genetic variability estimations in shrimp culture. However, among the available markers, microsatellites have been used to detect variability changes in shrimp culture (e.g. Cruz, Ibarra, Mejia-Ruiz, Gaffney & Perez-Enriquez 2004; Tamayo 2006). Their versatility and reproducibility makes microsatellites the marker most likely to form a basis of a genetic monitoring system.

Heterozygosity is one of the most relevant parameters for captive populations because it determines the capability to deal with a wider biochemical environment both inside and outside the cell (Beaumont & Hoare 2002). A seminal study on six successive generations of *Penaeus japonicus* (F_1–F_6) showed a decrease in average observed heterozygosity (from 0.102 to 0.039) as estimated by allozymes (Sbordoni, De Metthaeis, Cobolli Sbordoni, La Rosa & Mattoccia 1986); however, most of the heterozygosity loss, in this case, was attributed to a bottleneck effect between F_1 and F_2, where only four F_1 breeders were used to produce F_2. More recently, Tamayo (2006) compared a parental stock (G_0) and its respective progeny (F_1) in *L. vannamei* through genotyping of four microsatellite loci and observed a significant decrease in heterozygosity from an observed heterozygosity (H_o) of 0.400 and an expected heterozygosity (H_e) of 0.600 in the G_0, to 0.290 and 0.440, respectively, in the F_1. In contrast, a study of *L. vannamei* reared during three consecutive generations (from G_0 through to G_2) found considerably less change in heterozygosity, with $H_o = 0.650$ and $H_e = 0.770$ for G_0, $H_o = 0.710$ and $H_e = 0.720$ for G_1 and $H_o = 0.720$ and $H_e = 0.710$ for G_2 (Cruz *et al.* 2004). In this case, the gradual increase in H_o values has been attributed to the introduction of new breeding males from the founding lineage into generation G_1.

In the present study, average values for H_o and H_e across three successive collections throughout one year did not show any significant reductions. Both H_o and H_e reached the lowest values in the second sampling and both reached higher values at the last sampling. The fluctuations observed for heterozygosity values are likely to reflect the short time taken between samples, the variability of the original founder stock, as well as the effective population size for establishing the broodstocks in the studied hatchery. The results observed were comparable to those of wild *L. vannamei* populations from Mexico to Panama, where average H_o and H_e were 0.320 and 0.675 respectively (Valles-Jimenez, Cruz & Perez-Enriquez 2005). No significant differences in heterozygosity values between hatchery and wild stocks have been described in several previous studies for fish species (O'Connell & Wright 1997; Coughlan, Imsland, Galvin, Fitzgerald, Naevdal & Cross 1998; Norris, Bradley & Cunningham 1999; Desvignes, Laroche, Durand & Bouvet 2001).

Heterozygosity has sometimes been regarded as a poor indicator of loss of genetic variability, when compared with the loss of rare alleles or with allele frequency fluctuations (Waples, Winans, Utter & Mahnken 1990; Beardmore, Mair & Lewis 1997). In the present study, the number of alleles per locus varied from three to eight, similar to Cruz *et al.* (2004), who found between one and nine alleles when analysing consecutive generations of *L. vannamei*. Meehan *et al.* (2003) characterized loci TUMXLv 5.38 and TUMXLv 8.2 in cultivated SPF *L. vannamei* populations and found eight and nine alleles respectively. In relation to our data, locus TUMXLv 8.2 seems to have lost six out of nine alleles, arguably a significant portion of its diversity. This is likely to be the result of a founder effect, in action when the species was imported to Brazil, to form the original broodstocks. A similar reduction in allele numbers has been recorded previously for locus Pvan0013. Only two alleles were present at this locus in cultured populations of *L. vannamei* (Cruz *et al.* 2002), while a maximum of six alleles have been typed in natural populations (Valles-Jimenez *et al.* 2005). In the present study, three out of the six alleles found by Valles-Jimenez *et al.* (2005) were present, from a total of four alleles. Allele 282 has been the most frequent in both studies (Fig. 1a). Finally, significant changes in allele frequencies were observed along the time. These seem especially important in locus TUMXLv

5.38, where two out of eight alleles may have been lost in a period of 12 months. Genetic drift could account for this kind of loss, because its effects tend to be stronger in populations with smaller effective sizes.

Inbreeding coefficient (F_{IS}) is a parameter that can range from 0 to 1, describing heterozygote deficit in a population (Hartl & Clark 1997). The average values found here for F_{IS} showed a decrease between the second and third samples – from 0.39 to 0.13, which reflects the effects of changes in heterozygosity. F_{IS} values that are not equal to 0 reveal inbreeding and, consequently, loss in genetic variability. Nevertheless, the value found for *L. vannamei* in natural populations of Central America described by Valles-Jimenez *et al.* (2005) was much higher ($F_{IS} = 0.53$) than that reported here. Tamayo (2006) found values of 0.3207 and 0.3580, for G_0 and F_1, respectively, which are closer to the present results (Table 1). De Donato *et al.* (2005) analysed 11 generations of mass selection in closed reproductive cycles in order to find out the effect of population size in the inbreeding coefficient (F^t) in a farm in Venezuela. These authors found an increase of 0.011–0.059 in this coefficient over a ten-years interval.

A loss of genetic variability was expected to occur in broodstocks of hatcheries that use a closed cycle system. However, our study found that in the short-term, no significant decrease was observed in heterozygosity and only slight effects on the number of alleles. Although the sampling strategy used here does not directly represent consecutive generations, the samples are consecutive in time and have the same genetic origin. The overall results suggest that large effective population sizes and high genetic diversity of the original founder stock may have played an important role in maintaining broodstock genetic variability in the hatchery studied.

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