Low genetic variability in the fifth introduction of Litopenaeus vannamei in Cuba, as estimated with microsatellite markers

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The present work estimated the genetic variability and relatedness index of the fifth stock of Pacific white shrimp, Litopenaeus vannamei, imported into Cuba for farming purposes from the US Shrimp Improvement System (SIS). Genetic variability was estimated by genotyping 33 samples for four microsatellite loci: M1, Pvan 1815, Pvan 0040 and Pvan 1758. This stock had average expected and observed heterozygosities of 0.37 and 0.27 respectively; the lowest of all stocks previously introduced in Cuba. The above, together with the low amount of allelic variants detected for each microsatellite, was suggestive of low genetic variability. In addition, pairwise relatedness coefficients clustered around unity, indicating a high degree of consanguinity. Taken as a whole, the data suggests that this breeding stock should be crossed first with other individuals from a different source or with higher genetic variability.

Keywords: genetic variability, Litopenaeus vannamei, microsatellites, shrimp

Biotecnología Aplicada 2011;28:147-150

ABSTRACT

Limitada variabilidad genética de la quinta introducción en Cuba de Litopenaeus vannamei estimada con el uso de marcadores microsatélites. Se estimó la variabilidad genética y el índice de parentesco entre lotes de camarón blanco del Pacífico, Litopenaeus vannamei, introducidos por quinta ocasión en Cuba, procedentes del Centro de mejora del camarón, de Estados Unidos (Shrimp Improving System: SIS), para su cultivo. La variabilidad genética se estimó mediante el genotipo de 33 muestras con cuatro loci microsatélites: M1, Pvan 1815, Pvan 0040 y Pvan 1758. El quinto lote de Litopenaeus vannamei tuvo los valores promedios de heterocigosidad esperada y observada, más bajos de todos los introducidos en Cuba: 0.37 y 0.27, respectivamente. Esto, unido a la poca cantidad de variantes alelicas para cada región microsatélite, indica una escasa variabilidad genética. Los valores del coeficiente de parentesco alrededor de la unidad expresan una alta consanguinidad. Todo ello sugiere el cruce de los individuos de esta introducción con otros de diferente origen o más variables genéticamente.

Palabras clave: variabilidad genética, Litopenaeus vannamei, microsatélites, camarón

Introduction

Shrimp, as many other fishing resources, has received the impact of overfishing and environmental deterioration. Natural populations have shrunk, turning shrimp farming into a useful alternative for the obtaining of this important protein source.

During the year 2003, two stocks of Pacific white shrimp (Litopenaeus vannamei) from the US Shrimp Improvement System (SIS), denominated here as scotcks 1 and 2, were introduced for the first time in Cuba [1], followed by the gradual implementation of techniques for the handling, health, nutrition and assessment of the genetic variability of this species in hatcheries and farms previously involved with Litopenaeus schmitti, an autochthonous shrimp. Two additional stocks were introduced during successive years (denominated here as stocks 3 and 4), all characterized using microsatellite markers [2, 3].

A fifth stock of this species, obtained from the same source, was introduced in October 2008. Its genetic composition and allele polymorphism are yet to be examined, however. Although their consanguinity was low, the genetic variability of stocks 1 and 2 was already smaller than in natural populations [2]; stocks 3 and 4, likewise, exhibited a decreased genetic variability [3].

Using allobymes or microsatellites and a number of different shrimp species, it has been demonstrated that loss of genetic variability correlates with significant decreases in productivity (as for instance in Mar- supenaeus japonicus [4], Litopenaeus stylirostris [5], P. monodon [6] and L. vannamei [7, 8]). The use of microsatellite markers, however, is better suited for the follow-up and assessment of genetic variability in farmed populations, due to its higher resolution and sensitivity [2, 9].

The objectives of the present work, therefore, are to determine genetic variability and relatedness indexes in a sample from the fifth stock of L. vannamei introduced in Cuba, employing four microsatellite loci and comparing them with all of the previously introduced stocks.

Materials and methods

Sampling

Pleopod samples were taken from the fourth pair, between the exopodito and the endopodito, of 40 randomly chosen individuals evenly split between males and females. The sampling procedure was performed.

on previously acclimatized shrimp from the hatcheries of the Shrimp Genetic Center in Mariel, Cuba.

Genotyping of microsatellite loci
DNA extraction procedures, microsatellite loci selected for analysis (M1, obtained by Wolfus et al. [8], Pvan 1758, Pvan 1815 and Pvan 0040, isolated by Cruz et al.[10]), amplification programs and electrophoresis conditions for genotyping runs have all been described in Borrell et al. [2].

DNA was extracted with 5% Chelex resin, and the amplified fragments were separated under a running voltage of 2000 mV in 6% bis-acrylamide/acrylamide gels which were later stained with 0.1% silver nitrate-voltage of 2000 mV in 6% bis-acrylamide/acrylamide gels. DNA extraction procedures, microsatellite loci sequences (M1, obtained by Wolfus et al. [8], Pvan 1758, Pvan 1815 and Pvan 0040, isolated by Cruz et al.[10]), amplification programs and electrophoresis conditions for genotyping runs have all been described in Borrell et al. [2].

The present work analyzed the genetic variability of the fifth stock of L. vannamei introduced in Cuba for shrimp farming, using four microsatellite loci: M1, isolated from L. vannamei [8], and Pvan 0040, Pvan 1758 and Pvan 1815, isolated from the same species [10].

The table contains the main calculated parameters: number of alleles (Na), observed and expected heterozygosity (Ho and He) and deviations from Hardy-Weinberg equilibrium (Fst), together with the values estimated during previous studies. According to the results of the analysis of linkage disequilibrium with FSTAT (version 2.9.3), these loci are not genetically linked in L. vannamei [2, 9, 17].

Expected and observed heterozygosity
Average observed heterozygosity for the fifth stock of L. vannamei introduced in Cuba yielded a value of 0.271; the lowest figure compared to previous introduced stocks (Figure 1). In addition, this value is below all previous intervals reported by other researchers employing microsatellites to study peneaids shrimps.

A now classic review [9] on the use of microsatellite loci for natural populations from different species described observed heterozygosities that ranged from 0.425 to 0.964, yieldiing a mean of 0.666 which fell below the expected average (0.927). The same author observed heterozygosities ranging from 0.45 to 1.00 for three species of farmed peneaids, yielding a mean of 0.594, just below the mean for expected heterozygosity (0.674).

However, studies on P. stylirostris cultured for 22 and 24 generations [5] have produced much smaller values: Ho = 0.32 to 0.48; He = 0.46 to 0.61. Still, the values from the fifth stock introduced in Cuba (Ho = 0.271; He = 0.367) are below these figures (Figure 1), even though heterozygosity for all previous introductions of L. vannamei is within the above intervals. However, we agree that heterozygosity is an imperfect measure for variability, as it can yield high values with just two alleles, and, therefore, their quality also matters [17-19].

Results and discussion
Genetic variability of the fifth stock of L. vannamei compared to previous stocks


Table. Genetic variability parameters and deviation from Hardy-Weinberg equilibrium (FIS) for the four microsatellite loci isolated from Litopenaeus vannamei: M1 [8], Pvan 0040, Pvan 1758 and Pvan 1815 [10], in the five stocks introduced in Cuba for aquaculture *

* Data for the first and second stocks were taken from reference [2]; those for the third and fourth, from reference [3].

m: number of samples
Na: number of alleles
He: Expected heterozygosity according to Nei [11].
Ho: Observed heterozygosity

** p<0.01; *** p<0.001; ns: not significant.

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Allele frequencies and deviations from Hardy-Weinberg equilibrium

One single locus, *Pvan 0040*, is responsible to a large extent for the decrease in heterozygosity of the fifth stock, as it appears to be monomorphic in this case (as it did for the fourth *Litopenaeus vannamei* introduction). The other remaining three loci are still polymorphic, with five alleles each for *M1* and *Pvan 1758* and four alleles for *Pvan 1815*.

Allele frequencies for the loci of the fifth stock are shown in figure 2. Molecular weights for the observed alleles coincide within the intervals published by other authors, including those who first isolated them from a genomic library [10] and others who have later used them to characterize populations of this species [2, 3, 8, 17-19].

In the present work there were five *M1* alleles ranging from 206 to 240 bp; the most frequent were those of 240 bp (0.61) and 210 bp (0.28). This microsatellite has previously been used in isolation to estimate genetic variability for different populations [8], obtaining allele numbers ranging from 4 to 23. This underscores the usefulness of this locus for population genetics studies in this species. *M1* was also used for the genetic characterization of all previous introductions of *L. vannamei* into Cuba [2, 3].

The number of alleles per locus is also high for *Pvan 1815* and *Pvan 1758*, both in natural populations and in farming environments subject to genetic improvement programs [10, 17-19]. Still, allelic variants are often lost in the latter, due to factors such as the consanguinity that typically appears in farmed populations and, probably, experimental artifacts such as the appearance of null alleles due to misinterpretation of polymerase slippages or the Wahlund effect, caused by the mixing of populations.

When these microsatellites were first obtained in 2002 [10] from a small sample, the observed numbers of alleles per locus were 12 and 14 for *Pvan 1815* and *Pvan 1758* respectively, suggesting they represented excellent markers. Later studies employing these microsatellites for studies of other *L. vannamei* populations have found similar allele numbers [17-19]. Their allele number in the present study, however, is lower (4 for *Pvan 1815* and 5 for *Pvan 1758*) (Figure 2 and Table).

A retrospective look to previous stocks confirms that allele number has progressively decreased from earlier to later introductions for these and the other two loci. Finding out which specific alleles have been lost and determining the importance of these losses for future crossings or molecular marker-assisted selections would necessarily require, however, that all data be compared as a whole. As for locus *Pvan 0040*, which reappears here as a monomorphic allele and is

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one of the markers with the smallest number of allele variants. Although six alleles were found when it was first described [10], this study was only an initial report, based on a small sample. A later study from the same group where different populations were compared with the use of five microsatellite markers found 5 to 11 alleles for Pvan 0040 [17]. By the year 2009, however, this group had already stopped using this locus [19], employing Pvan 1758, Pvan 1815 and four new markers (Lvan 05 [20] and TUMXLv9.3, TUMXLv10.312 and TUMXLv8.256 [21]).

Using Pvan 0040 to characterize the two first stocks introduced in Cuba yielded 6 and 5 allele variants for stocks 1 and 2 [2], 5 alleles for the third, and, for the first time, only 1 allele for the fourth [3].

The results from the present work indicate that there is also a fixed Pvan 0040 allele for the fifth introduction of Litopenaeus vannamei in Cuba. Allele sizes in bp coincide between the fourth and the fifth introduction, despite the presence of disparities regarding sample sizes, methodologies for estimating genetic variability, reference molecular weight markers and positive controls. Future use of this marker for characterizing other stocks should, therefore, be reconsidered.

Although strict compliance with all conditions for reaching Hardy-Weinberg equilibrium is hard to come by in farmed populations, loci Pvan 1758 and Pvan 1815 do appear to be at equilibrium. M1 deviates from genetic equilibrium, however, mainly due to an excess of homozygotes as evidenced by the high value of FIS (Table). Previous studies [2, 3] of the introductions of L. vannamei in Cuba have evidenced significant deviations from genetic equilibrium, which in the present work take place only for M1. This phenomenon may be caused by a smaller sample size than that of other authors [2, 3], which would increase the probability of type I statistical errors; i.e. rejecting the null hypothesis when it is true.

Relatedness among the individuals of the fifth introduction of shrimps in Cuba
The analysis of the fifth introduction of L. vannamei shrimp in Cuba produced an anomalous curve with two maxima, corresponding to the region of highly related individuals (Figure 3). Note that the highest maximum corresponds to 1, that is, the highest possible degree of relatedness; the other maximum is at r = 0.15, close to that established by Queller and Goodnight [16] for half-brothers (r = 0.25). There is another peak to the left, although much smaller than the two others. Globally, average consanguinity yields a value of +0.037, implying that related individuals predominate in the sample.

The efficacy of the use of microsatellites and relatedness indexes to determine pedigrees was first shown while studying farmed turbot stocks with known consanguinities [22]. These authors obtained independent charts for related and non-related individuals, which implies that the frequency distribution of the relatedness indexes among individuals of the fifth introduction might actually correspond to the superimposition of these ideal curves. Most probably, the fifth introduced stock of L. vannamei actually constitutes a mixture.

Previous studies found genetic variability had decreased in stocks 3 and 4 of L. vannamei introduced in Cuba [3], but did not estimate genetic relatedness. However, since the first introductions, it became evident that the design of crossings using individuals from stock 2 (mean r = +0.1515) should not be undertaken without a methodology for following inter-individual genetic relatedness coefficients, which provide information on endogamy levels [2]. As mentioned above, average genetic relatedness in the present work is even higher, implying that these individuals should not be used as a breeding stock without an available technique for following up successive crossings.

Given the obstacles for the incorporation of individuals obtained from their natural environment into shrimp farming, the only available alternative for decreasing endogamy and raising genetic variability in L. vannamei stocks would be to measure these genetic parameters in the existing breeding stocks in Cuba, selecting the best according to all data.

Conclusions
Average expected and observed heterozygosity (0.37 and 0.27, respectively) of the fifth L. vannamei stock introduced in Cuba for shrimp farming were lower than those of previously imported stocks. This, together with the relatively small number of allele variants for each microsatellite region, indicates that its genetic variability is low. In addition, the fact that the genetic relatedness coefficient was close to unity indicates that its genetic consanguinity is high. According to the values obtained from the genetic parameters estimated in this work, it would therefore be necessary to cross this stock with others from the same source, in order to improve future production yields.

Figure 3. Distribution of relatedness coefficients (r) between pairs of individuals for the fifth batch of Litopenaeus vannamei imported into Cuba for shrimp farming, calculated following the procedure of Queller and Goodnight [16], using four microsatellite loci (M1 [8], Pvan 0040, Pvan 1758 and Pvan 1815 [10]).