Research Article

Genetic Characterization of Six Stocks of *Litopenaeus vannamei* Used in Cuba for Aquaculture by Means of Microsatellite Loci

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Four microsatellite loci were used to achieve genetic characterization of six stocks from *Litopenaeus vannamei* used for aquaculture in Cuba: second generation from first introduction (S2-1), first generation from the second one (S1-2), from the third one (S1-3), and the fourth one (S1-4) and the crossings from two parental population: first generation from the first with first generation from the third (S1-1 × S1-3) and first generation from the second with first generation from the third (S1-2 × S1-3). 66% (16/24) of genetic systems in total loci were in genetic disequilibrium. The four microsatellite loci were polymorphic for all six stocks. Major quantities of allelic variants correspond to locus Pvan 1758, which is at the same time that one where there are private alleles from first generation of the third. All F_{st} comparisons were significant. This indicates big differences between stocks. The highest values are those in which there is presence of the second introduction. This introduction and its descendants are also more consanguineous.

1. Introduction

In Latin America shrimp-producer countries, the Pacific white shrimp, *Litopenaeus vannamei*, is the most representative species, with about 90% of production. Native from East Pacific, and from the tropical American continent, this species has shown an excellent culture adaptation and has been more resistant to salinity, oxygen, and temperature fluctuations. That is why, in the last twenty years, *Litopenaeus vannamei*, has been introduced in many culture programs, and nowadays it is the second culture after *Penaeus monodon*.

In 2003 the first introduction of two stocks of White Pacific Shrimp, *Litopenaeus vannamei*, [1] was achieved in Cuba, imported from USA, Shrimp Improving System and so handling, nutrition, and health techniques are well established, as well as the assessment of genetic variation in farms that had before cultured the indigenous species *Litopenaeus*

schmitti. In total, five stocks have been introduced, and all of them have been characterized using microsatellite techniques [2–4].

Genetic studies have a capital importance in shrimp industry, in order to determine genetic variability level either in natural or cultured populations, but mainly to know when the latter could be enriched with new specimens [5]. It is moreover important to know the structure of natural population from which those specimens will be taken [6] and also to have good markers that allow population and family studies. It is clear that the priority should be given to the domestication and handling of broodstocks through the application of genetic techniques [7–9]. The aim of the present work is to characterize different cultured stocks of *Litopenaeus vannamei*, used in Cuba for aquaculture, by means of microsatellite markers.

2. Materials and Methods

2.1. Samples. Samples were taken from pleopods of 30 shrimps, specifically from the fourth pair, between exo-and endopodite. The same male and female quantity was taken randomly in shrimps from the Postlarvae Production Hatchery “YAGUACAM,” Cienfuegos, Cuba.

Individuals from first or second generation of introduced shrimps were taken for this genetic characterization as described below. Two first parental generations from first and second introduced stocks were randomly crossed with descendants of the first generation of the third introduced stock resulting in (S1-2 × S1-3) and (S1-1 × S1-3) as it is called in this work.

Thus, characterized lots were second generation from first introduced stock (S2-1), first generation from the second one (S1-2), from the third one (S1-3), and from the fourth one (S1-4) and the crossings: first generation from the first with first generation from the third (S1-1 × S1-3) and first generation from the second with first generation from the third (S1-2 × S1-3).

2.2. Microsatellite Genotyping. DNA isolation, amplification programs, and electrophoresis procedures were carried out as described in [2]. Used loci were M-1, isolated from *L. vannamei* by [10] and Pvan 0040, Pvan 1758, and Pvan 1815 obtained from the same species by [11]. As weight allelic controls, samples previously genotyped by [2], as well as PGEM, were used.

2.3. Statistics. Allele number by locus, allele frequency, and observed and expected heterozygosity for each locus, as well as, the Hardy-Weinberg equilibrium, were determined by GeneAEx 6.1 program [12]. A locus was considered as polymorphic if it presented at least two alleles and when the most common frequency of an allele did not exceed 95%.

The FSTAT statistical package version 2.93 [13] was used to calculate the linkage disequilibrium, and also the inbreeding coefficient within populations, F_{is} values [14], and pairwise F_{st} and P values between populations, after 1500 permutations. Significance levels were assessed through the Markov chains using 10000 Dememorisation, 100 batches, and 5000 iterations per batch using the Genepop Version 4.1.0 [15].

Assignment tests were used to clarify the belonging of individuals to the stocks. The Bayesian assignment test was performed [16] among shrimp stocks from Cuba using GeneClass 2 [17]. Simulations were run to determine the probability of assignment, using a probability of rejection set at $P < 0.05$.

Relatedness coefficient (r) according to [18] between individuals' pairs was calculated by GeneAEx 6.1 program [12]. The equation for this coefficient taking into consideration codominant marker is

$$r = \frac{\sum_x \sum_k \sum_l (p_{yl} - p^*)}{\sum_x \sum_k \sum_l (p_{xl} - p^*)}, \quad (1)$$

where x represents individuals, k all loci, l allelic positions (2 for diploids, 1 for haploids), P_x the frequency of the individual x for the locus k and allelic position l , P_y the frequency of the allele in the group or individual with it is compared x , and P^* the total frequency in the population.

Relatedness coefficient must be $r \leq 0$ to unrelated individuals, $r = 0.25$ to half sibs, and $r \geq 0.5$ to full sibs [18].

Relatedness means coefficients were compared with the Kruskal Wallis test (nonparametric ANOVA) by means of graphPad InStat version 3.00 [19]

3. Results

3.1. General Parameters of Genetic Variation of Six Stocks Descendants from Original Introductions: Hardy-Weinberg Deviation of Equilibrium. As it has been previously reported, *L. vannamei* shrimps introduced from SIS are characterized [2–4].

In the present work, the genetic variation analysis was performed to six relative stocks of those introductions by means of four microsatellite loci: M1, isolated from *L. vannamei*, and Pvan 0040, Pvan 1758, and Pvan 1815 also obtained from these species. Linkage disequilibrium was analyzed, and as it is known [2, 4, 11, 20], those loci for *L. vannamei* are not linked.

In Table 1 are shown the main genetic estimation parameters, such as number of alleles (N_a) and number of effective alleles (N_e), observed and expected heterozygosity (H_o and H_e), and so, the Hardy-Weinberg equilibrium deviations (F_{is} values). As several stocks are compared, private alleles are also provided (N_p). Note that first generation from second introduction (S1-2) is the one of less genetic variability, within first generation stocks, and even lesser than the only second generation stock analyzed. Highest variation stock belongs to crossing of first generation from the first with first generation from the third (S1-1 × S1-3). The lowest relative variation of any generations and stocks which included second introduction is remarkable and stocks which included second introduction.

Normally, the Hardy-Weinberg equilibrium conditions are not accomplished in cultures. In Table 1 it is observed that 66% (16/24) of genetic systems in total loci are significant in genetic disequilibrium by homozygotes excess.

3.2. Differentiation between Stocks. In Figure 1 frequencies of alleles of each microsatellite for all stocks are shown. Allele sizes are on range reported by other authors [2–4, 10, 20–22]. All loci were polymorphic and those with more allelic variants were Pvan 1758 (12 variants) and Pvan 1815 (9 variants), which is coincident with previous studies. Private alleles found in this paper corresponded to those loci. In the case of M1, distribution of frequencies is bimodal, with two predominant sizes, which includes the majority of stocks: 216, which contains all of them, and 202 in which it only lacks S1-4. In total there are eight allelic variants, and there are not private alleles for this microsatellite region.

TABLE 1: Genetic variability parameters and the Hardy-Weinberg deviation of equilibrium (F_{IS}) and its probability (Pfis) of four microsatellites loci isolated from *L. vannamei*, M-1 [10], Pvan 0040, Pvan 1758 and Pvan 1815 [11], in six different cultured stocks of *L. vannamei* used for aquaculture in Cuba. n : number of samples. Na: number of alleles, Ne: number of effective alleles, Np: number of private alleles, He: expected heterozygosity, Ho: observed heterozygosity. The associated probability was estimated using the Markov chains (10000 Dememorisation, 100 batches, and 5000 iterations per batch using Genepop program Version 4.1.0. Numbers in parenthesis are standard mean error.

Stock	N	Na	Ne	Np	Ho	He	Fis	Pfis
Locus M1								
S2-1	19	7	4.6	0	0.89	0.78	-0.115	0.001
S1-2	19	4	2.2	0	0.62	0.55	-2.212	0.660
S1-3	16	5	3.6	0	0.69	0.72	0.078	0.000
S1-4	22	3	2.0	0	0.59	0.50	-0.147	0.142
S1-1 $\times$ S1-3	23	5	3.0	0	0.91	0.67	-0.349	0.242
S1-2 $\times$ S1-3	25	4	3.1	0	0.36	0.68	0.485	0.000
Locus Pvan 0040								
S2-1	21	4	2.4	0	0.52	0.58	0.127	0.002
S1-2	21	2	1.3	0	0.24	0.21	-0.111	1.000
S1-3	19	4	2.5	0	0.16	0.59	0.746	0.000
S1-4	22	3	2.7	0	0.36	0.63	0.440	0.000
S1-1 $\times$ S1-3	23	4	3.9	0	0.56	0.74	0.261	0.000
S1-2 $\times$ S1-3	26	2	1.3	0	0.23	0.20	-0.111	1.000
Locus 1758								
S2-1	21	7	3.8	0	0.67	0.54	0.121	0.00
S1-2	21	4	3.5	0	0.67	0.71	0.088	0.000
S1-3	20	9	6.2	3	0.75	0.84	0.132	0.054
S1-4	22	7	3.9	1	0.68	0.74	0.108	0.009
S1-1 $\times$ S1-3	23	6	4.5	0	0.61	0.78	0.239	0.119
S1-2 $\times$ S1-3	26	6	1.8	0	0.73	0.64	-0.116	0.563
Locus 1815								
S2-1	21	3	2.1	0	0.14	0.52	0.737	0.00
S1-2	21	4	1.6	0	0.25	0.37	0.371	0.056
S1-3	20	6	3.8	0	0.65	0.74	0.147	0.006
S1-4	22	6	4.4	1	0.73	0.77	0.084	0.000
S1-1 $\times$ S1-3	23	8	3.8	0	0.52	0.74	0.315	0.000
S1-2 $\times$ S1-3	26	4	3.6	0	0.65	0.72	0.115	0.000
All loci								
S2-1	20.5(0.5)	5.2 (1.0)	3.2 (0.6)	0	0.56 (0.16)	0.66 (0.06)	0.176	0.000
S1-2	20.5 (0.5)	3.5 (0.5)	2.1 (0.5)	0	0.46 (0.13)	0.46 (0.11)	0.032	0.000
S1-3	18.7 (0.9)	6.0 (1.0)	4.0 (0.8)	3	0.56 (0.14)	0.72 (0.05)	0.250	0.000
S1-4	22.0 (0.0)	4.7 (1.0)	3.3 (0.5)	2	0.59 (0.08)	0.66 (0.06)	0.132	0.000
S1-1 $\times$ S1-3	23.0 (0.0)	5.0 (0.8)	3.8 (0.3)	0	0.65 (0.90)	0.73 (0.02)	0.131	0.000
S1-2 $\times$ S1-3	25.7 (0.2)	4.0 (0.8)	2.7 (0.5)	0	0.50 (0.12)	0.56 (0.12)	0.141	0.000

Pvan 0040 locus is that one which has less allelic variants, with only five. Its frequency distribution is unimodal and in 141 size all stocks are represented. Two other loci, Pvan 1758 and Pvan 1815, are unimodal and have private alleles that contribute to the highest quantity of allelic variants. Private alleles correspond to the first generations of the third and fourth introductions. Sizes and frequencies of appeared alleles (within parenthesis) are, for locus 1758: 182 (0.025), 184 (0.250), and 193 (0.025), which appeared in first generation of the third and 210 (0.023) from first generation

of the 4th. To 1815 there is only a private allele from first generation of the 4th: 111 (0.091).

The assignment test results are represented in Table 2. It revealed that 93.3% (125/134) from the shrimp were correctly assigned. S1-3 stock was the most failed assignment with only 80% of right assignment.

F_{st} calculations are shown on Table 3, comparing all stocks among them. It is observed that all comparisons are statistically significant. The biggest values are those that have the presence of the second introduction. On the other hand,

FIGURE 1: Allelic frequencies of loci M-1 [11], Pvan 0040, Pvan 1758 and, Pvan 1815 [12] for six *L. vannamei* stocks used for aquaculture in Cuba.

TABLE 2: Number of assigned individuals to the correspondent stock and/or to any other using the Bayesian assignment test [16] by GeneClass [17]. Simulations were run to determine the probability of assignment, using a probability of rejection set at $P < 0.05$.

Stock	Assigned to the same stock	Assigned to another stock	% of assignment to the same stock
S2-1	19	2	90,50
S1-2	20	1	95,5
S1-3	16	4	80
S1-4	21	1	95,4
S1-1 $\times$ S1-3	23	0	100
S1-2 $\times$ S1-3	26	1	96
Total	125	9	93.2

less F_{st} values are observed in comparisons between S1-3, S1-4, and S2-1.

3.3. Relatedness Values. Relatedness coefficients distributions between individual pairs of six stocks are shown in Figure 2, calculated according to [18]. All stocks, except S1-2 and crossing that contains it (S1-2 $\times$ S1-3), present unimodal distributions. Three of them present the mode in negative relatedness values (S1-3, S1-4, S1-1 $\times$ S1-3), and the only one from a second generation S2-1 presents the mode in zero. In the case of exceptions, for S1-2, one of the modes is near zero, while the second one is near positive values but

TABLE 3: F_{st} values estimated by FSTAT program [13] between *L. vannamei* shrimp stocks used for culture in Cuba. For all comparisons, P value = 0.00067; it was obtained after 1500 permutations also by the FSTAT program. Indicative adjusted nominal level (5%) for multiple comparisons is 0.003333.

	S2-1	S1-2	S1-3	S1-4	S1-1 $\times$ S1-3
S1-2	0.2163				
S1-3	0.1496	0.2387			
S1-4	0.1192	0.2120	0.1896		
S1-1 $\times$ S1-3	0.0667	0.1637	0.0962	0.0663	
S1-2 $\times$ S1-3	0.1639	0.1237	0.2030	0.1922	0.1282

lower than 0.5. For crossing (S1-2 $\times$ S1-3), the first mode is in negative values and the other one near zero. Mean relatedness coefficients values (Figure 2, right inside) have not significant differences and have standard deviation values. Those are for each stock, S2-1: -0.028 ; S1-2: 0.044 ; S1-3: -0.063 ; S1-4: -0.023 ; S1-2 $\times$ S1-3: -0.019 ; S1-1 $\times$ S1-3: -0.044 .

4. Discussion

4.1. Genetic Diversity within *L. vannamei* Stocks. The four microsatellite loci were polymorphic for all six stocks. The majority of expected and observed heterozygocities are on range according to many authors using microsatellites for penaeid shrimps [2–4, 20–26]. However, they are low and above range for one of the markers, Pvan 0040, which

FIGURE 2: Relatedness coefficient distributions between individual pairs from stocks of *L. vannamei* shrimps used for culture in Cuba, calculated according to [18], with 4 microsatellite loci: M-1 [10], Pvan 0040, Pvan 1758, and Pvan 1815 [11]. Right inside: mean values and standard deviation of relatedness coefficients for all stocks. *P* value for statistical comparison is 0.2426.

is the less variable loci of all stocks in which the second introduction is involved: in the first generation of the second introduction $H_o = 0.24$ and $H_e = 0.21$ and in crossing of first generation of second introduction with first generation of the first. Previously, [2–4] reported a great contribution of this locus to the decrease of total heterozygocities. Pvan 0040 was monomorphic for the original fourth and fifth introductions [3, 4], and so it could be considered that this locus is sufficiently sensitive to changes in different stocks.

Equilibrium deviations are significant in most loci by stocks, which in accord with other authors' results [3, 4, 20, 22].

Most of allelic frequency graphics are unimodals, with exception of M1 locus. The biggest quantities of allelic variants correspond to locus Pvan 1758, which is at the same time that one in which there are private alleles from first generation of the third. This is in agreement with genetic variability reported for that locus in previous works [20, 22, 25].

4.2. Differentiation between Stocks. Indeed all F_{st} comparisons were significant. They indicate big differences between stocks. The biggest values are those in which there is presence of the second introduction. In a similar way, other authors also obtained significant differences in natural populations as well as in cultured stocks [20, 22]. Using allozymes, the authors in [27, 28] also obtained a genetic differentiation in wild population of *L. shmitti* in Cuba and in wild and cultured *L. vannamei* stocks in Norwest Mexico, respectively. Those differences are in agreement with the assigning exercise, in which only first generation of the third stock had low percentages of assigning. It could be because there are

components of those stocks in others obtained by meanings of crossing with this third introduction.

Although studies with *L. vannamei* in Cuba indicate a decrease in genetic variability, existing stocks at the moment of this genetic characterization, even with little variations, seem, however, significantly different between them. The reason could be that in their origins they constitute genetic lines well diverse in their origins in respect to the main objective for production (e.g., illness resistance, increasing growth, etc.). However, once the selection pressure is increased, genetic variation is decreased as it could be seen in different genetic variability reports for several introductions [4, 29].

4.3. Relatedness Coefficients. Borrell et al. [2] pointed out the risk that could implicate using individuals from the second introduced stock for crossing with others without any methodology such as the following, of relatedness coefficient, that offers information about inbreeding levels. For the second stock, they obtained a mean relatedness coefficient indicative of more related individuals than those of the first one, similar to the results of this work, in which the most consanguineous stock is descendants of this second introduced stock. The highest value, indicative of a great relatedness, was obtained for the fifth introduced stock of *L. vannamei* in Cuba [4], and that is why the use of this molecular tool is of crucial importance for designing and following crossings. The rest of the stocks show means and distributions in agreement with individuals presumed not related, similar to that obtained by [30] for turbot, near zero. The second introduction and its descendants, as well as crossings that contain it, the same as the fifth [4], should be followed with this methodology, and also estimation of heritability for interested characters for culture would be profitable.

4.4. General Remarks. A progressive decrease in genetic variability is observed in successive introductions of *L. vannamei* into Cuba since 2003 and henceforth. It means that the first broodstock should be maintained and combined with others or new stock. Relatedness coefficients support the use of this stock and highlight the fact that the second stock should be carefully used in crossings because of their tendency to positive values.

However, the sustainability of the culture has been achieved, as well as maintenance of no viral diseases that have caused considerable losses in Latin America [31]. In that way, if surveillance program [32], starts with the premise of introducing SPF animals (Shrimp Pathogens Free) for health maintenance in cultures, a strategy of genetic management with microsatellite markers will warranty an adequate productive performance that does not drive to an irreversible endogamy. In agreement with [2], the maintenance of a common origin, besides of health warranty ever crossing stocks genetically different, avoids phenomena as outbreeding depression [33–35] that could damage productive yields in a short time.

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