Assessment of genetic variation and productive markers through four progenies of the first introduced stock of cultured shrimp *Penaeus (Litopenaeus) vannamei* in Cuba

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Abstract The Pacific White shrimp, *Penaeus vannamei* is the only invertebrate cultured species in Cuba nowadays. Specific Pathogen Free (SPF) lines are imported from Shrimp Improvement System (SIS) in USA and genetic characterization is achieved once animals arrive. Monitoring crossings and progenies along the production process is also accomplished. The objective of this work was to seek both production and genetic tendencies in four progenies of the first introduced stock. Productive recorded data including yield, survival and final weight were computed. Four microsatellite regions were explored to characterize the four populations in culture. Both survival and yield of the first, second and tenth offspring generations were significantly different from the ninth one. This last stock offered the lowest values of the whole analyzed productive process. Genetic parameters remained similar for the second, ninth and tenth but significantly differed from the very first introduced stock. Relatedness coefficients suggest not related individuals and there is no evidence of a bottleneck effect for any of the progenies or the founder stock. In summary, it seems that inbreeding and genetic diversity is still not causing damages in animals that could influence the productive process.

Keywords *Penaeus vannamei*; culture; yield; relatedness; microsatellites; bottleneck

Background

The shrimp cultivation is the branch of the aquaculture that advances the shrimp’s ‘growth in captivity’. Nowadays this culture represents an opportunity for exportation, and an option of development for the revitalization of the fishing activity depressed by the over exploitation of the marine resources. According to FAO, 2010, the shrimps continue being in value terms, the main commercialized fishing product and this important industry had overcome an accelerated growth in the last decade of about 10 % annual in the last five years. Around 3.5 million of metric tons (MT) are currently harvest worldwide. Approximately 85 % of this total is produced in China and South East Asia, mainly in Thailand and Vietnam. Another 10% is produced in India and Bangladesh and the remaining 5 % in the Occidental Hemisphere (Newman, 2010).

For many years, the giant tiger shrimp, *Penaeus monodon*, was the main cultured species in the Oriental Hemisphere. At the beginning of the twentieth century, the industry reformed its production system to the Pacific White Shrimp, *Penaeus (Litopenaeus) vannamei* (Boone, 1931), and thus, this species was intentionally introduced in several Asiatic countries. Nowadays, almost the 65 % of the worldwide production belongs to this species and it is expected that this tendency would be maintained over years (Newman, 2010). A report from 2009 indicated that in Latin America and the Caribbean, the culture of the white shrimp represented the 24.3 % of the total cultivated aquatic species in the world (Mendoza-Ramírez, 2011). According to this same author, the main producer countries are Ecuador (194.628 MT), followed by Mexico (136.470 MT), Brazil (66.120 MT), Nicaragua (20.131 MT), Colombia (18.639 MT) and Venezuela (16.763 MT).

In Cuba this species was introduced for the first time at the end of the year 2003, as an alternative to make...
possible the stability of the shrimp industry (Tizol et al., 2004), replacing the main cultivated native species in the country *Litopenaeus schmitti*, that did not contribute to the projected productive yields. Since the very beginning of the *P. vannamei* culture, higher productions in the five shrimp culture Enterprise Base Units (EBUs) have been obtained. In that way, handling techniques, nutrition, health management and genetic breeding programs were altogether re-established for a suitable yield of this resource for the mentioned species, *P. vannamei*.

Many authors have reported a decline in genetic diversity when comparing natural populations with captive ones for different shrimp species such as in *Litopenaeus stylirostris* (Bierne et al., 2000a); *P. monodon* (Xu et al., 2001) and *P. vannamei* (Garcia et al., 1994; Wolfus et al., 1997). Nevertheless comparing several generations of cultured *P. vannamei*, non-significant fluctuations in genetic variability has been obtained (Cruz et al., 2004; Luvesuto et al., 2007; Perez-Enriquez et al., 2009; Souza De Lima et al., 2010; Vela-Avitúa et al., 2013). However, this last mentioned Mexican group (Vela-Avitúa et al., 2013) using more microsatellite loci as genetic markers and pedigree information found a non-significant decline in genetic diversity for two nonconsecutive generations of cultured *P. vannamei*. In neither of those cases productive markers have been shown neither so the decrease or maintenance of genetic parameters through different generations of the same line has not been proved. In order to use genetic management as a tool for farm producers the aim of this work is to seek both productive and genetic tendencies in four progeny stocks of the first introduction of *P. vannamei* used in Cuba for aquaculture.

1. Results

1.1 Productive marker of four descendant of the first founder stock of *P. vannamei* in Cuba for culture.

Two of the three productive indicators were variable among generations (Figure 1). Both survival (%) and yield (Kg/Hm²/cycle) of the first, second and tenth offspring generations (S1-1, S2-1 and S10-1), were significantly different (p ≤ 0.05) from the ninth one (S9-1). This last stock offered the lowest values of the whole analyzed productive process. On the other hand, the final weight (g) did not show significant differences (p ≥ 0.05) among the four studied progenies.

![Figure 1 Average productive markers: survival (%), yield (Kg/Hm²/cycle) and final weight (g) of four progenies of the first founder stock of *Penaeus vannamei* introduced and cultivated in Cuba. Dissimilar letters indicate significant differences in Kruskall-Wallis test (α=0.05)](image)

1.2 Genetic parameters of the first introduced stock of *P. vannamei* in Cuba for culture and three of its descendant

All averages of genetic parameters values: Allele number (Na), Allelic Richness (AR), Effective allele number (Ne), Private allele number (Np) as well as observed and expected heterozygosities (Ho and He respectively) are highest in the first introduced brood stock and remain similar from the second, ninth and tenth generations as it is shown in Figure 2.

This variation of the first introduced brood stock comparing to the rest of the descendant, is significant (p<0.05) for those parameters that involve quality of the alleles, but not for heterozygosities (Table 1).
expected heterozygosity, there is only significance between S0-1 and S9-1, but the difference (0.227) is very close to the upper confidence limit, CL (0.229).

A similar whole picture is found when a principal coordinates analyses (PCoA) is accomplished. Figure 3 shows a clear founder group (S0-1, in red) that shares information with the other three clusters, but it’s closer to the second generation (S2-1). The latest generations (S9-1, in blue; and S10-1 in yellow) are mixed together and slightly far from the other two stocks.

### 1.3 Inbreeding, Bottleneck effect and Relatedness coefficient of the first introduced stock of *P. vannamei* in Cuba for culture and three descendants.

The calculation by FSTAT program of the deviations from the equilibrium (Fis values) and its associate probability for the four mentioned stocks showed

| Allele Number Lower CL | S0-1 | S2-1 | S9-1 | S10-1 |
|------------------------|------|------|------|-------|
| Difference             | 4.000| 2.395| 2.651| 2.725 |
| Probability            | 0.012| 0.028| 0.018| 0.016 |

Table 1 Difference values and confidence limit (CL) in the null model of Monte Carlo analysis (10 000 permutations) of genetic parameters among the first stock (S0-1) of *P. vannamei* introduced in Cuba for the culture and its three descendant stocks: second (S2-1), ninth (S9-1) and tenth (S10-1) generations

| Expected Heterozygosity Lower CL | S0-1 | S2-1 | S9-1 | S10-1 |
|----------------------------------|------|------|------|-------|
| Difference                       | 2.300| 2.750| 3.000| <1.01 |
| Probability                      | 0.024| 0.013| 0.300| 0.278 |

Figure 2 Averages genetic diversity values: Allele number (Na), Effective allele number (Ne), Allelic richness (AR), Private allele number (Np), Observed heterozygosity (Ho) and Expected heterozygosity (He) for the first stock (S0-1) of *P. vannamei* introduced in Cuba for the culture and its three descendant stocks: second (S2-1), ninth (S9-1) and tenth (S10-1) generations.
positive values in all cases and either none of the populations is in Hardy-Weinberg equilibrium (Table 2).

The distributions of relatedness coefficients calculated by Queller and Goodnight, 1989 estimator between individual pairs of the four stocks showed highest frequencies in negative values. Only S9-1 showed a bimodal distribution with high frequency in negative values about -0.2 and other smaller peak in positive values around 0.5. (Figure 4)

The program COANCESTRY (Wang, 2011) lets to compare by a bootstrap method, the generated r coefficients distributions for pairs of individuals, resulting in statistical differences among all the analyzed stocks, except for S2-1 and S10-1, but the observed values are very near of the 95 percentile values of the confidence interval. Two examples of the output data after bootstrap comparisons are shown in Figure 5.

All Bottleneck program output tests (Sign test, Standardized difference test and Wilcoxon test) under the three models: Infinite Allele Model, IAM; Step Mutation Model, SMM and Two Phase Model, TPM had significance (p≥0.05) for the assumption of a heterozygosis excess. A typical L-shape graphic is obtained for every tested stock (Figure 6) but, however slight variations from the L-shape for S9-1 and S10-1 are observed.

2 Discussion

The sustainability of shrimp aquaculture industry is accomplished taking into a consideration of many facts, including handling, health and nutrition of captive species, and of course, the market price. Besides, aquaculture offers the opportunity to improve our basic knowledge about different species. In that way, genetics and molecular biology became in important tools not only to characterize natural and captive populations, but also to develop new treatments, improving diets and leading to basic and specific knowledge of molecular immunology, gene expression and genomics of different organisms with economic importance (Liu and Cordes, 2004). Several commercial domesticated strains of shrimps, particularly of penaeid have been developed in the world, for example in Hawaii, Venezuela, Colombia, Tahiti, México, Brazil and USA. These strains are currently available to producers who want to take advantage of selective breeding programs developed elsewhere in the world (Cuzon et al., 2004).

Although domestication and maintenance in captivity is an advantage for biomass production and market Table 2 Deviation from the equilibrium (Fis values) and its associate probability (pFis) for the first founder stock of P. vannamei (S0-1) and its descendants: the second (S2-1), nine (S9-1) and ten (S10-1) generation stocks calculated for the four loci: M1, Pvan 1758, Pvan 1815 and Pvan 0040. Calculations were made using the FSTAT program (version 2.9.3) software application. Bonferroni correction was used with α=0.00313 and 320 randomizations.

| Parameter | Stocks       |
|-----------|--------------|
|           | S0-1         | S2-1         | S9-1         | S10-1        |
| Fis       | 0.179        | 0.176        | 0.214        | 0.182        |
| pFis      | 0.0031       | 0.0063       | 0.0031       | 0.0031       |

Figure 3 Principal coordinates (PCoA) of genetic distances among the first founder stock (S0-1) of P. vannamei introduced in Cuba for the culture and three descendant stocks: second (S2-1), ninth (S9-1) and tenth (S10-1) generations.

Figure 4 Distributions of relatedness coefficients between individual pairs (calculated by Queller and Goodnight estimator) of the first stock of P. vannamei introduced in Cuba (S0-1) for the culture and its three descendant stocks: second (S2-1), ninth (S9-1) and tenth (S10-1) generations.

Table 2 Deviation from the equilibrium (Fis values) and its associate probability (pFis) for the first founder stock of P. vannamei (S0-1) and its descendants: the second (S2-1), nine (S9-1) and ten (S10-1) generation stocks calculated for the four loci: M1, Pvan 1758, Pvan 1815 and Pvan 0040. Calculations were made using the FSTAT program (version 2.9.3) software application. Bonferroni correction was used with α=0.00313 and 320 randomizations.
Figure 5 Two examples of 10000 bootstrap comparisons of Queller and Goodnigh relatedness coefficient distribution calculated using the Coancestry program. A: comparison between S0-1 and S10-1. B: comparison between S2-1 and S10-1. Note that in A the observed values (solid black line) are outside the distribution, so differences are significant. In B, the observed values are extremely closed to the 95% quartile.

Figure 6 Allele distribution by frequency ranges for the first introduced to Cuba (S0-1) and cultured stock of *P. vannamei* and its second (S2-1), ninth (S9-1) and tenth (S10-1) progenies.

In that way, genetic markers as allozymes (Rivera-García and Grijalva-Chon, 2006), RAPD (Freitas and Galetti, 2005; Rajakumaran et al., 2013), mitochondrial DNA (Robainas-Barcia and García-Machado, 2012) and microsatellites (Cruz et al., 2004; Luvesuto et al., 2007; Perez-Enriquez et al., 2009; Souza De Lima et al., 2010; Vela-Avitúa et al., 2013) have been used. Another molecular markers as AFLP has been used to map species genome (Wilson et al., 2002; Li et al., 2003)

In Cuba, both allozymes (García-Machado et al., 2001; Espinosa-López et al., 2003) and microsatellites (Espinosa-López et al., 2001; Borrell et al., 2007) have been used for characterization of natural populations (*L. schmitti* and *F. notialis*) and so, the creation of adequate brood stocks of the autochthonous species *P. schmitti*. Once the most sell in the world species, *Penaeus vannamei*, was introduced in the country for culture, it was characterized at each time (Borrell et al., 2006; Machado-Tamayo, 2006; Artiles et al., 2011a) as well as some crossings (Pérez-Belobarodova et al., 2012) using microsatellites by virtue of better properties such as codominance and high variability and also to compare among different stocks and recommend crossings to production. However, tendencies in yield and productive traits were not previously reported.
This is the first work that documents both tendencies for the first introduced stock in Cuba for culture of *P. vannamei* and some of its descendants.

### 2.1 Productive marker tendencies of four descendant of the first founder stock of *P. vannamei* in Cuba for culture.

The intentional introduction of *P. vannamei* in Cuba greatly increased the productive markers in the Enterprise Base Units (EBUs). Since 2006, these productive estimators have been showing irregularities as a consequence of culture system handling problems, mainly in food supply and nutritional content.

In fact, the fall in survival and yield for the ninth progeny of the founder stock, S9-1, could be due to a wrong handling process in grow out ponds for this culture cycle, specifically in food supply and quality of it. In a controlled experiment, Gaxiola et al., 2006 demonstrated that the change in diet composition have a long term effect in growth and survival of this species. In the present case, a change in the artificial diet (ECCAM, 2014, private communication) could lead to a decline in both productivity markers.

Even though significant differences were not detected for the final weight, a slight increase of the mean value (15 g) in the ninth offspring is observed at 120 culture days (Figure 1). In similar conditions in commercial culture ponds in Peru, using certified seeds from Ecuadorian laboratories, and good quality food supply, a final weight of 22 g was obtained (Mendoza-Ramirez, 2011).

The obtained values of survival for the first, second and tenth progenies of the first introduced stock (S1-1, S2-1 and S10-1) are higher than 50 %, comparable to two reports in USA (Sookying et al., 2011; Treece, 2015) and two studies in Costa Rica as well (Valverde-Moya and Alfaro-Montoya, 2013; 2014). Two main reasons could explain this. The first one is the origin of the lines. All stocks from SIS are Shrimp Pathogen Free (SPF) and besides, this imported line (The first introduction) was genetically manipulated for high growth (Tizol et al., 2004). The second one is the caring handling and the maintenance of a Sanitary Surveillance Program (Silveira, 2006) that avoids the viral diseases occurrence in Cuba since the introduction of *P. vannamei* (Artiles et al., 2011b).

The yield overcomes the 900 Kg/Hm$^2$/cycle, which is below the world standard average for the semi intensive culture. However, similar yields have been reported by some authors from Latin America region. That is the case of farms in Brazil (893.3 Kg/Hm$^2$/cycle) and a final weight of 11.3 g in 85 days in culture (Nunes et al., 2014) and Costa Rica (868 Kg/Hm$^2$/cycle) at 120 days in culture (Valverde-Moya and Alfaro-Montoya, 2014). Another report from Brazil is nearer of the average world standard, reaching 4.457 to 5,012 Kg/Hm$^2$/cycle, considered as a good profitable production level for this country (Alvino et al., 2014).

### 2.2 Genetic parameters tendencies of the first introduced stock of *P. vannamei* in Cuba for culture and three descendants.

There is an established consensus in shrimp genetic research that there is a decrease in genetic diversity parameters from wild to cultured shrimp stocks. For many penaeid species it has been very well documented with the use of different markers (Benzie, 2000; Xu et al., 2001; Meehan et al., 2003).

On the other hand, when comparing among generations in culture, although most of the authors do not find a significant variation (Cruz et al., 2004; Luvesuto et al., 2007; Perez-Enriquez et al., 2009), one researchers’ group does find a non-significant decrease although using different parameters, but employing microsatellites and pedigree information (Vela-Avítúa et al., 2013). In present work, a mix of those results has been obtained. In accordance with those authors, observed and expected heterozygosities (Ho and He respectively) are similar (Table I for values and significances and Figure 1 for tendency), with no drastic variations among generations. This is not surprising, since some authors have stayed that heterozygosity is an imperfect measure of genetic variability (Cruz et al., 2004; Souza De Lima et al., 2010) because high values of that parameter could be obtained with as few as two different alleles. Besides of that, Luikart et al., 2010 showed that He is less sensitive than allelic diversity when assessed soon after bottleneck. Neither surprising is the fact that as well as for the same cited authors, populations for most of the loci are in Hardy-Weinberg disequilibrium. This has been also widely documented for captive populations that do not strictly fit themselves to the statements of this law, *e.g.* Cruz et al., 2004; Luvesuto
A significant decrease in all parameters that involve quality of alleles has been proved when compared the first introduction (S0-1) with its tested descendants, but not among them (Table I for values and significances and Figure 1 for tendency). Cruz et al., 2004, assessed the genetic variability through consecutive generations (named G0, G1 and G2) in a two year Shrimp Breeding Program. They did not find differences in allele frequencies between G0 and G1, but they did between G1 and G2. Besides, a discrete decreasing tendency in effective allele number, but not in allele number, is observed. In general, as well as Luvesuto et al., 2007 in Brazil for the same species, P. vannamei, both groups of researchers did not detect genetic diversity reduction after three successive generations. Those authors characterized generations 5th, 6th and 7th of a closed and reared line of this species using four microsatellite regions. In a further work, Perez-Enriquez et al., 2009 did not find a decrease in the number of alleles between a 2000-2002 pool and the 2007 (a six to eight generation differences), but slight increase in this absolute number. Let’s note that although two of the loci, Pvan1758 and Pvan1815 (Cruz et al., 2002) were used in the previous work, in the further one; more new loci (TUMXLv8.256, TUMXLv10.312, TUMXLv9.103 and Lvan05) have been also used in this genetic status characterization of the cultured species in Mexico. Finally, Vela-Avitúa et al., 2013, using 26 loci and pedigree information, detected a decline in genetic parameters such as He, Na, Ne from a wild population to two captive generations (named GEN07 and GEN09), but those values however are similar between the one year different generations. When using effective population sizes based both in microsatellite and pedigree, a decline trend, even when was not significant between GEN07 and GEN09 was found.

Then, the question about the origin history of every stock that has entered into Cuba to be cultured is emerged. The answer will be inevitably uncertain since all the introductions have been bought knowing that here we have Specific Pathogen Free (SPF) shrimps and nothing else (except for the first introduced stock that it is known that has been genetically manipulated for high growth). This have been extremely helpful in preventing viral diseases occurrences in Cuba (Artilles et al., 2011b), but do not let to figure out about the previous history, as it would be the case of brood stock obtained from natural areas. So, the present S0-1, with the rest four introduced stocks from SIS, constitutes the founder stock of P. vannamei in Cuba. This fact is consistent to the panorama showed in PCoA analysis (Figure 3), and to the assignation exercise accomplished in Pérez-Beloborodova et al., 2012.

In addition to the absence of data about the previous history of the bought introductions (in that case before S0-1), there is more lack of information between this stock and its first descendant generation, due to no genetic analyzes were made at that time and neither samples were preserved. And so, taking into a consideration the processed data, there is an evident loss of alleles between the first introduced stock and its second generation pool. We cannot realize however if this loss was produced at that time or in a previous step, it is in S1-1. The non-analyzed data among the S2-1 and S9-1 seems not to have relevant meaning, because all the values remain similar for each subsequent descendants tested populations.

2.3 Inbreeding, Bottleneck effect and Relatedness coefficient of the first introduced stock of P. vannamei in Cuba for culture and three descendants.

In aquaculture, the mating among relatives is a common phenomenon that is not injurious itself, but its accumulation over generations could induce the expression of deleterious alleles once they are paired as recessives in homozygosis. This inbreeding depression could induce a decrease in productive performance traits, such as growth rate, survival, disease susceptibility as have been widely report in literature, e.g: (Sbordoni et al., 1986; Benzie, 2000; Bierne et al., 2000a; Bierne et al., 2000b; Hulata, 2001)

The evaluation of genetic diversity and inbreeding is so a potent tool for producers through all the process. The creation of genetic programs that help in taken choices for best crossings and breeders is a widely used strategy over the world. The inbreeding coefficient, Fis, is a measure of the intraspecific variation among populations, and so, an indirect extent of consanguinity. Positive values that are obtained in this work (Table 2) are indicative of heterozygous deficit as have been shown by most of the authors that have calculated it.
for captive population (Luvesuto et al., 2007; Perez-Enriquez et al., 2009; Souza De Lima et al., 2010; Vela-Avituá et al., 2013) and because of that, a Hardy-Weinberg disequilibrium is manifested for most of the loci in our work and in theirs. The Fst, a measure of interspecific variation among population is useful in our criterion, when those populations are in different geographical areas or when its origin in the time is different as is the case of comparison of descendant of different introduced stocks of P. vannamei for culture (Perez-Beloborodova et al., 2012). That is why this parameter was not used to estimate inbreeding among the first introduced stock and three of its descendants.

On the other hand, Queller and Goodnight estimator for relatedness was helpful to characterize those stocks in present work and in previous ones (Borrell et al., 2006; Artiles et al., 2011a; Perez-Beloborodova et al., 2012). Average media is not actually a good indicator because positive and negative values rates are obtained, but distributions for each stock offer a general picture about consanguinity. The mode of values is around zero or less for all the stocks, which indicates that most of the individuals are not related (Figure 4). Lower kurtosis is obtained in successive generations which indicate a logical increase in consanguinity over several generations. Besides, when distributions were compared, statistical differences were obtained among all stocks, except for one of the comparisons exemplified in Figure 5 together with another that showed significance.

The typical obtained L-shape when plotting the allele proportion versus the frequency range (Figure 6) means that not recent bottleneck effect has been produced (Allendorf and Luikart, 2007). More allele proportion at higher frequencies (mostly at frequency range one) would indicate the fixation of one or more allele at any or several loci, and thus, a recent bottleneck effect. The non-existence of this effect for none of the analyzed stocks in this work is consistent with the acceptable levels of genetic parameters, considering that more than ten progenies have been in culture because the previous history of this line is unknown. When using a similar procedure Nahavandi et al., 2011 detected a recent bottleneck effect in Wild tiger Shrimp Penaeus monodon in Malaysia, one of the major shrimp’s producer countries in the world maybe due to the over exploitation of this resource in natural areas. This fact highlights the importance of using Biotechnology in breeding programs as a tool to recommend and to guide crossings and matings.

2.4 General Remarks and perspectives.

The Pacific White shrimp, Penaeus (Litopenaeus vannamei) is the only shrimp cultured species nowadays in Cuba with a total catch around 3121.4 Tons in 2295 Hm². This estuarine resource constitutes an employment source and an important economic branch to the country. Due to the many advantages for domestication and the globalization of this species in the international market, all the shrimp aquaculture industry has been focus into its culture. Fortunately many genetic data about the Pacific white shrimp has been generated over the world and especially in shrimp producers’ countries.

In that way, basic and applied research could help to support shrimp productions and to maintain this economic and social object. This work offers a panoramic about the descendants of the first stock equally from the genetics and the productive point of view comparing trends of both performances. If we take into a consideration that no significant differences between the first and the tenth generations for final weight and yield were found while a significant increase in survival was established (Figure 1), and that genetic parameters remains more or less stable for the tested stocks (Figure 2 and table 1), it seems that there is not a negative influence of the inbreeding yet. This is substantially corroborated by the non-existence of a bottleneck effect (Figure 6) as well as by the fact that there is not a shift in the maxima peak of relatedness (Figure 4).

It is known that the first introduction of P. vannamei for culture is the most variable of the five that has been entered into the country (Artiles et al., 2011a). Thus, many questions still require answers and future work, such as: what would happen with the rest of descendants and crossings for genetic parameters and productive traits. For that reason a genetic characterization of the YAGUACAM’s brood stock it’s being currently done. Another important question is how many generations could lead to acceptable productions levels and the real correlation among phenotypic and genetic traits for Cuban stocks. Perez-Enriquez et al., 2009
have reported sufficient level of genetic variations after 10 years of management and about 20 generations. However those authors pointed out about the risk of possible inbreeding. In our opinion, more work on consanguinity levels should be done and the use of other estimators would be profitable. Besides, although a familiar selection program is perhaps not affordable in our economic conditions, it would be the best choice to combine classic and molecular genetics and in this sense, research could widely contribute to improve and increase production levels. A partial solution could be for the moment to improve molecular detection techniques, to expand the number of studied loci and the use of other indexes, such as the mentioned effective population size.

Of course, for suitable yields in productions a strict surveillance to nutrition, health and economic stimulation to shrimp farmers and directives are important factors always to take into a consideration. In that way, researchers from The French Institute Of Marine Science Research (IFREMER, from abbreviations in French) had report controlled experiments with different diets to raise offspring of the 25th generation of *P. vannamei* in clear water to 40 g (Cuzon et al., 2004). This is a punctual example on the importance of other factors in the growth and survival of animals, which definitely is the goal of the shrimp aquaculture industry.

### 3 Materials and Methods

#### 3.1 Productive markers record of several of the descendants of the first introduced founder stock in Cuba (S0-1).

The first founder stock (S0-1) of *P. vannamei* shrimps was introduced into Cuba in post-larvae stage from Shrimp Improvement System, in USA. After being held for a period in quarantine post larvae were planted in a 0.38 hm² pond at YAGUACAM Hatchery Centre, in Cienfuegos, Cuba in at a stock density of 0.5 individuals/m². A special diet was supplied to achieve the status of parents and so on, becoming the founder brood stock.

The semi intensive system is implemented in the four existing farms throughout the country. Post larvae are planted in commercial grow out ponds of around 8 – 10 hm² and at a stock density of 10 – 15 individuals/m². The pellet diet was supplied by Malta Cleytom from Microsoft Office Excel – tested in the fourers from The French Institute Of Marine Science Research (IFREMER, from abbreviations in French) had report controlled experiments with different diets to raise offspring of the 25th generation of *P. vannamei* in clear water to 40 g (Cuzon et al., 2004). This is a punctual example on the importance of other factors in the growth and survival of animals, which definitely is the goal of the shrimp aquaculture industry.

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#### 3.2 Stock nomenclature and sampling procedure for genetic analysis.

Nomenclature and number of individuals from each brood stock used for genetic analysis in this work is as follows:

- S0-1: 42 individuals of pure first introduction, previously genetically characterized by Borrell et al., 2006.
- S2-1: 21 individuals of the second generation of the so called first introduction, previously genetically characterized by Pérez-Beloborodova et al., 2012.
- S9-1: and S10-1: 30 individuals respectively of the ninth and tenth generations of the first introduction genetically characterized in this work.

Note that from the first generation of the first introduced stock (S1-1), genetic data was not obtained. Instead of the first descendants, samples of the very first stock (S0-1) were used to check genetic parameters tendencies. Pleopods and/or muscle tissue were randomly collected from adult shrimps at YAGUACAM Hatchery Centre, Cienfuegos, Cuba. The same quantity of males and females (1:1 proportion for sampling) was taken each time and tissue pieces were maintained in vials with absolute ethanol until used.

#### Table 3 Evaluated progenies of the Founder stock of white shrimp *P. vannamei* introduced into Cuba in 2003 and year of culture in commercial ponds of each one

| Founder stock | Descendants | Years of culture |
|---------------|-------------|-----------------|
| First introduction (S0-1) | S1-1 | 2004 |
| | S2-1 | 2005 |
| | S9-1 | 2012 |
| | S10-1 | 2013 |

The data was organized in Microsoft Office Excel 2007 according to: final weight (g); final survival (%) and yield (Kg/Hm²/cycle).

The analysis of the production performance of commercial ponds was made from the first offspring of these parental (S1-1). The Shrimp Culture Enterprise (ECCAM in Spanish) provided the historical production indicators records for the comparison of different descendants of the first founder stock. Descendants under study are show in Table 3. The two first and the two last progenies of the first founder stock until current moment of sampling were chosen for productive analyzes.
3.3 Microsatellite genotyping.
DNA was extracted with 5% or 10% Chelex – 100 resin (Walsh et al., 1991). Polymerase Chain Reaction (PCR) were carried out in Eppendorf thermo cycler for loci M1 (Wolfius et al., 1997) and Pvan 1758, Pvan 1815 and Pvan 0040 (Cruz et al., 2002) and were checked in 2 % agarose gel electrophoresis stained with ethidium bromide. The program was as follows: 94°C during 5 minutes, 35 cycles at 94°C for one minute, hybridization temperature variable depending of each loci during 30 seconds, 72 ºC 45 seconds (end of the cycle) and a final elongation at 72 ºC during 10 minutes. Hybridization temperatures by loci were: 50 ºC in Pvan 1815, 52-54 ºC in M1 and 45-48 ºC in Pvan 0040. A commercial mix of formamide/bromophenol blue was added to each vial with amplified products in 1:1 proportion. After that, denaturation of amplified products with colorant was carried out in the same Eppendorf thermo cycler and then was applied in 6% and urea 7 mol/L acrylamide – bis acrylamide vertical gels. The applied voltage was among 2500-3000 V and potency of about 80 W. After the electrophoretic run off, the gels were fixed with 10 % Acetic Acid solution, stained with 0.1 % silver nitrate- 0.05% formaldehyde and developed with 3 % sodium thiosulfate. Samples previously genotyped by Artiles et al., 2011a were used as controls for each microsatellite locus, with sizes ranging from 194 to 240 for M1, 140 to 146 for Pvan0040, 110 to 136 for Pvan 1815 and 174 to 188 for Pvan 1758. PGEM®, STR III, FFV and CTT were also included as conventional markers.

3.4 Statistics and calculations.
The analysis and interpretation of productive data was carried out using Statgraphics statistical package, version 3.5.2 (StatPoint Technologies, 2010) The normality was previously checked for a 0.05 significance level and when accomplished a simple ANOVA was used and the Multiple Range Test was applied if significant differences among means were found. Otherwise a non-parametric test (Kruskal-Wallis) was applied with the same significance level.

The number of alleles per locus, the frequency of each allele and the values for observed (Ho) and expected (He, according to Nei, 1978) heterozygosity for each locus, as well as whether the populations were in Hardy-Weinberg equilibrium, was calculated with the GeneAlEx (version 6.1) software application (Peakall and Smouse, 2006). Any locus with at least two alleles where the frequency of the most common allele did not exceed 95% was considered polymorphic (Graur, 2000).

Deviations from equilibrium were corroborated by calculating Fis (Wright, 1965), following the formula Fis = 1 - (Ho/He), with the FSTAT software application (version 2.9.3;Goudet, 2002). Although it depends on population size, it is unaffected by the presence of multiple alleles per locus, the number of individuals per population or the number of populations.

The genetic relatedness coefficient (r;Queller and Goodnight, 1989) for a single pair of individuals and the test of difference between groups was also calculated with COANCESTRY v1.0 (Wang, 2011). This coefficient is calculated for codominant markers, using the following formula:

\[r = \frac{\sum \sum (P_x - P_Y)}{\sum \sum (P_x - P_Y)}\]

where: x stands for the individuals; k for the loci; l for allelic positions (two for diploids and one for haploids), Px is the frequency of individual “x” for locus k and allelic position l, Py is the frequency of the allele “y” in the group or individual compared to x; and P* is the total frequency of the allele in the population. The genetic relatedness coefficient must be \(r \leq 0\) for non-related individuals; \(r = 0.25\) for half siblings and \(r \geq 0.5\) for full siblings.

The graphic of the distribution of relatedness coefficients was made in MatLab R2013a (8.1.0.604) program.

To check if there were variations in genetic diversity parameters, a 10000 iterations Monte Carlo analysis was performed using the Pop Tools v3.15 (add-insdel MS Excel) program.

To seek if a recent bottleneck effect would be produced, the Bottleneck program was run (Piry et al., 1998). All different test proposed by the authors were accomplished (Sign test, Standardized difference test and Wilcoxon test) under the three models: Infinite Allele Model, IAM (Maruyama and Fuerst, 1985); Step Mutation Model, SMM (Cornuet and Luikart, 1997) and Two Phase Model, TPM.
Author’s contribution
AA, LP and GE conceived of the study and designing of experiments and data records. AA drafted the manuscript and carried out PCR and electrophoresis with LB, who also computed relatedness results on COANCESTRY and Matlab. RC and LP carried out the sampling procedure and guided the productive process and recorded its data. All authors analyzed and interpreted the results, and read and approved the final manuscript.

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