Assessment of genetic variability in two lots of white shrimp, *Litopenaeus vannamei* (Boone, 1931) introduced to Cuba

Master thesis

by

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Abstract

To monitor the genetic variability of *L.vannamei* shrimp-specie as founder stock and their correspondent first generation (F1), four microsatellite DNA loci species-specific for these animals were used. Data from allelic diversity (size, number and frequency of alleles) and levels of hetrozygozity were obtained as measures of genetic diversity.

The protocol used is belonging to the lab, from the Norwegian College of Fishery science (NFH) of the University of Tromsø (Uitø). The results indicated a decrease in some of measures for genetic diversity. The number of allele (Na) ranged from 1-9 and their effectives number of alleles (n_e) from 1.1 to 6.72. The mean value observed heterozygosity (Ho) by populations range from 0.29 to 0.63, which were lower than the expected one. Loss of genetic diversity may have originated from the selection of better performing individuals for a number of traits of interest. There is gap concerning to pedigree information from these animals, due to their origin place (USA).

Artificial breeding practices may result in a decrease of genetic variability in terms of allelic diversity but which is not necessarily detectable from the levels of heterozygosity. Thus, should be taking into account at moment to start a genetic breeding program with the animals tested.

This study highlights the importance of constantly assessing the genetic variability in the cultured shrimp populations in Cuba. Considering the low variability observed, it is suggested that the development of a sustainable selective breeding program in Cuba should be conducted only after application of measures aimed at enhancing the genetic variation (through exchange of broodstocks with others producers or sampling of wild specimens) and that the genetic information be constantly used to reduce the risks of further inbreeding.

1. Introduction

As fisheries approach their maximum sustainable yield worldwide, demand for aquaculture products is rapidly increasing. Within the Latin America and Caribbean area, six countries contributed with a 93% of the aquaculture production in 1997 – Cuba (6%) –, which it is different from the production in 1987 when Ecuador (48%), Cuba (12%), and Mexico (11%) were the dominant producers by countries (Hernández-Rodríguez *et al*. 2001). The same author estimated that Caribbean countries provided a 6% of the regional aquaculture volume produced, but only a 2% in terms of value, where Cuba among all countries in the area is responsible of 92% of such income.

In Cuba, aquaculture begins back in the 1920s, with the introduction of some foreign fish species like black-bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*) and common carp (*Cyprinus carpio*) for such activity. However, it is only during the 1960s when the aquaculture-activity retakes boom with the introduction of new foreign species like tilapia (*Oreochromis aureus*), catfish (*Clarias macrocephalus*) and tench (*Tinca tinca*). FAO (2006) reports values of aquaculture production until 2003s that display a steadily growth since the year 1967 until the 1999s, when dropped sharply.

The culture of shrimp in Cuba, as a part of this aquaculture industry started during the early 1980s, specifically in 1983 were this activity showed its profitability under scientific-technological base. Lately, in 1987 it becomes a commercial activity and it has been developed since with the indigenous shrimp species *Litopenaeus schmitti*.

So far, the potential to improve important quantitative traits including diseases resistance, growth, and food conversion efficiency by selective breeding strategies remains strong for aquaculture species. In order to avoid wild larvae dependency, improvements in husbandry, larval rearing, and larval nutrition, some genetic improvement of farmed shrimp have been done. The need for basic research has added emphasis to the development of genomic tools that can be applied generally to studies of shrimp health and disease. Although, there is still a knowledge gap in terms of genomic studies, it results in rapid increase in fundamental biology-knowledge of aquaculture species (Warr *et al*. 2004). Molecular techniques are important tools for the sustainable development of aquaculture activities, providing more accurate information on the genetic diversity of natural stocks and allowing genetic tagging of animals in breeding programmes.

The development of DNA-based genetic markers has had a revolutionary impact on animal genetics. With DNA markers, it is theoretically possible to observe and exploit genetic variation in the entire genome (Liu and Cordes, 2004). Ferguson (1994) considered that genetic variation is the basic resource of any successful commercial or rehabilitation culture program. The monitoring of genetic variation with molecular markers across generations is recommended to reveal changes in variability caused by genetic drift, bottleneck, inbreeding, or selection. Depression on genetic variability can provoke negative impacts on production traits of cultured shrimps. Unsuitable domestication (i.e., poor closed re-circulation system performance of broodstock with the resultant inadequate supply of quality post-larvae), limited choice of efficient and economical live feed, and inadequate water quality management have been identified as additional constrains to the development of shrimp culture.

Therefore, there is considerable scope for application of a wide range of the newly developing biotechnologies in an effort to overcome these problems. The use of microsatellites DNA loci as genetic markers constitutes one way to achieve these goals. They are currently used for many reasons that support their employment in different areas. The key factor leading to microsatellites their widespread adoption lies in the power that they provide to solve biological problems (Chambers and MacAvoy, 2000). Liu and Cordes (2004) made a study related to the use of genetic markers into aquaculture field and considered that microsatellites are still in their initial phase of growth.

1.1. Background of shrimp culture activity developed in Cuba

Through the years, *L. schmitti* was the species used by the shrimp sector in Cuba. The sector has been growing, increasing in numbers of farms and hectares destined for culture of shrimp with the improvement of the culture-technology. To date, there are 2 200 hectares of land devoted to such activity, shared by four shrimp-farms throughout the island that are mentioned below (Figure 1):

- SANROS
- CULTIZAZA
- CULTISUR
- CALISUR

The seed required for the culture is guaranteed mainly by YAGUACAM, a commercial shrimp's hatchery producing more than 90% of the post-larvae in Cuba. This production ensures sufficient amounts of post-larvae for each farm, and in combination with another hatchery (branch of CALISUR farm), constitutes keystones for the development of the shrimp culture-activity in Cuba. Together with these infrastructures, there is a feed factory – ALISUR- with the same goal of supply the amount of artificial feeds required in each shrimp farm. The system of culture employed by all shrimp farms has been the semi-intensive type. It is used for the cultivation of this species of shrimps ponds built in land, using pumps and planned pond layout for water exchange. So far, activities concerning shrimp culture in Cuba are managed by GEDECAM, a core enterprise situated at the Ministry of Fisheries (MIP), who's future projections on increase with the total of area for culture is until 6 000 hectares (Figure 2).

Researchers and producers from science institutions -mainly Fishery Research Center (CIP) and Marin Investigations Center (CIM) - and MIP had gotten some results from studies made in wild and domesticated populations alike. Most of these works were focused on improving performance, reducing cost-production or increasing quality of the product throughout all the culture system. Principal productivity indicators like post-larvae production and yields of shrimps showed a steadily growth since 1986 until 1991 (Alvarez *et al*. 1992). This growth is supported by an increase of 104% of production during the years 1988-1992, where among Caribbean countries Cuba was the biggest producer of shrimp (FAO, 1994).

However, data offered by CIP show that the main feature challenged, as result throughout all these years is the average-weigh of the cultured shrimp (figure 3). Here, there is a slope-down or declination in mean weigh of produced shrimp. Thus can be due to genetic and environment factors during cultivation-process of shrimps. The result was lower profitability for the shrimp-industry, because size (in reference to weigh) is a key feature on market area that together with lower prices could give lower income. EU represents the main market for these species produced in Cuba. Hence, the EU-market imports shrimp of a much lower unit value than the other two main markets (USA and Japan), probably because EU imports a lot of coldwater shrimp (from catches), generally smaller than the farmed warm water species and thus attains lower prices (Joseupeit, 2004). Economic improvement of the production was a common worry among the producers of the region, considering food and seed as most

expensive into cost production (FAO, 1994). The sustainability of *L. schmitti* became worst, when the shrimp prices dropped sharply in 2000 (Wikipedia, 2006).

In agreement with the present situation of the shrimp culture in Cuba, the introduction of the *Litopenaeus vannamei* constituted a necessary alternative that made possible the survival of shrimp-culture activity in the country. Productive and economic parameters were favourable indicators to the introduction of this new species. The *L. vannamei* is introduced for second time in Cuba, due to inexperience and favourable current prices for *L. schmitti* during the first time that was tried to introduce in. Shrimp culturists introduced this foreign species (*L. vannamei*) and replaced the culture of the native species (*L. schmitti*), with the objective to improve the production efficiency of the farms, because the new species presents a greater adaptation to local culture conditions (Tizol *et al.* 2004). Hence, it is easy to breed in captivity, but succumbs to the Taura disease. Therefore, the introduction for commercial purposes must take place under strict measures of sanitary and genetic conditions of exotic species, limiting the spreading of new pathogens and preserving the genetic variability in founder populations.

Figure 1. Infrastructure of shrimp culture in Cuba

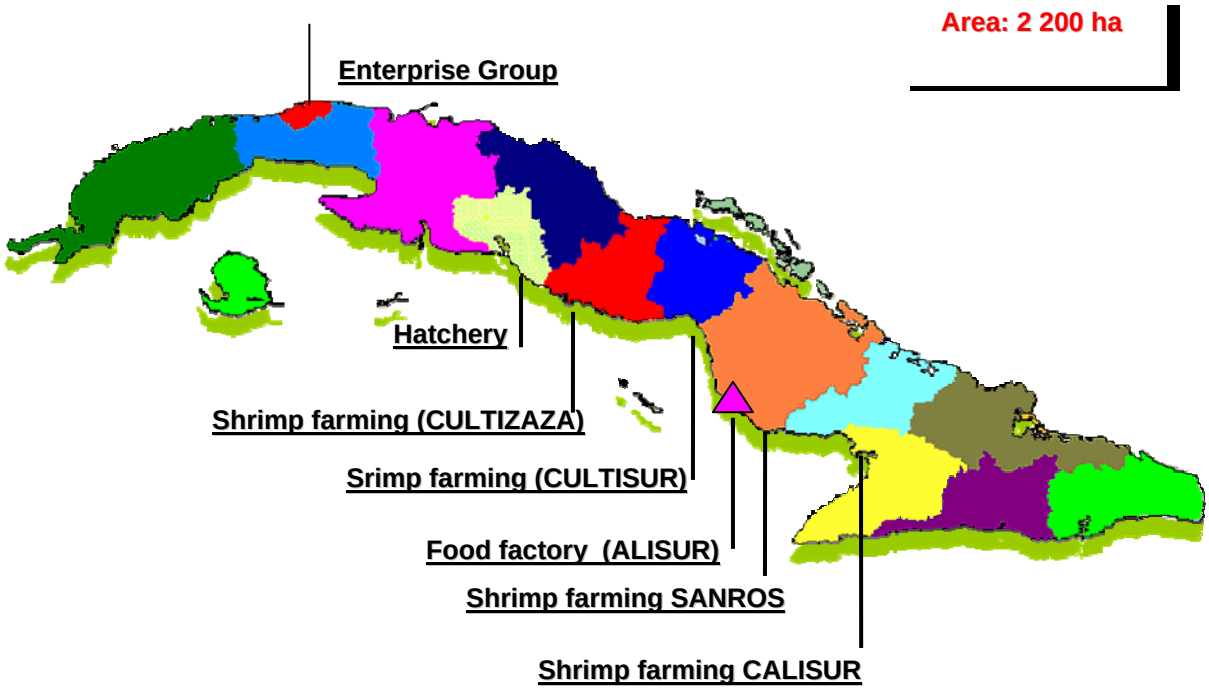


Figure 2. Further projections for the infrastructure of shrimp culture in Cuba

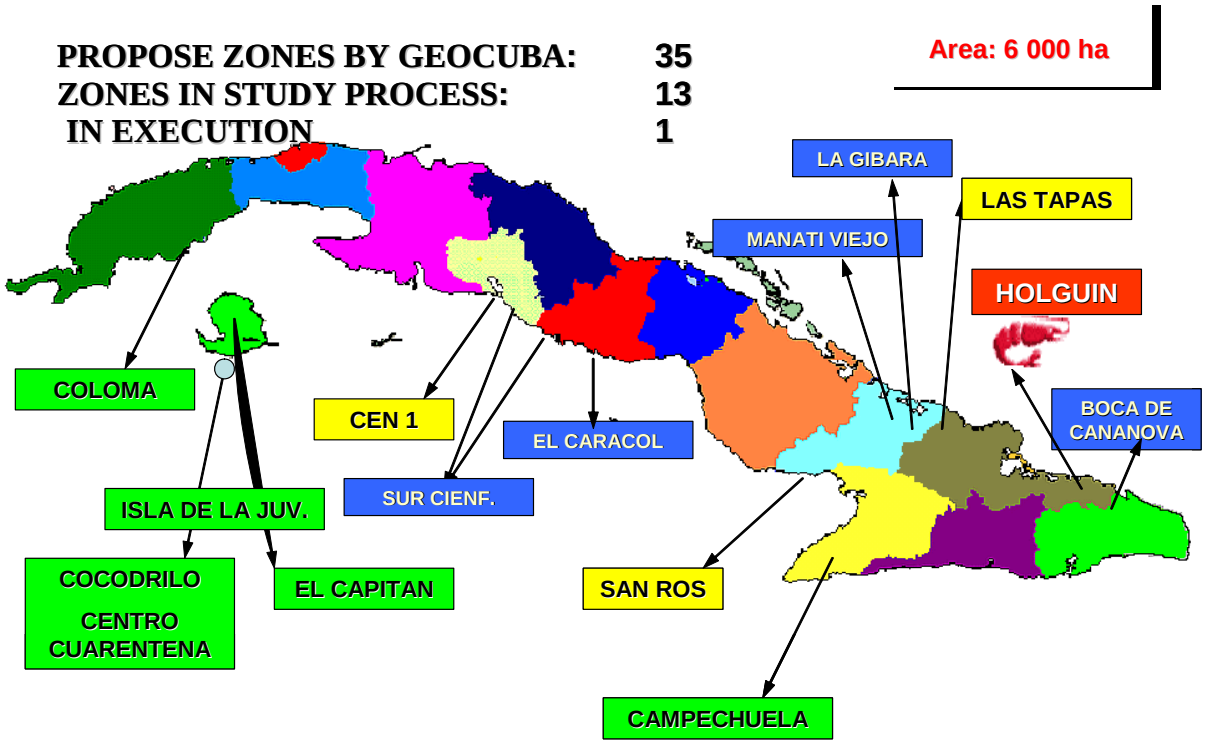
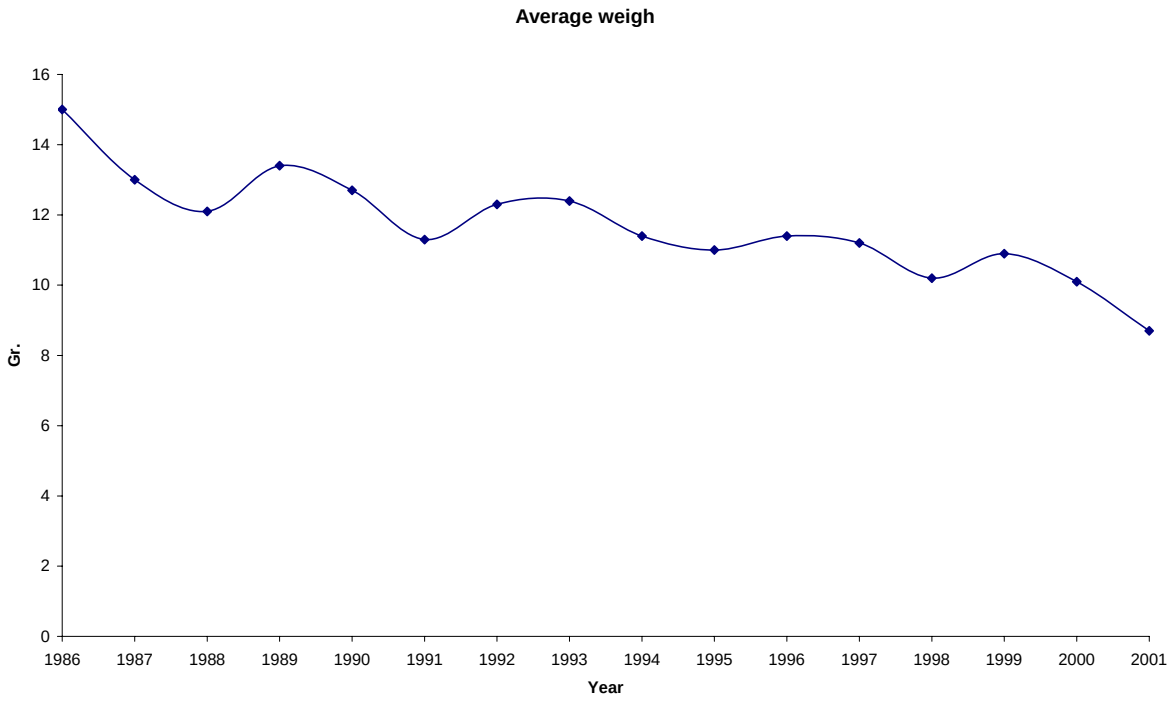


Figure 3. Average weight of *L. schmitti* performed through the years



1.2. *Litopenaeus vannamei*, the new species for cultivation

1.2.1. Characteristics of the species

Litopenaeus (Penaeus) *vannamei* (Figure 4) is native to the Eastern Pacific coast of Mexico, from Sonora and Central and South America as far south as to Tumbes in Northern Peru, in areas where water temperatures are normally over 20 ^{0}C throughout the year. The species grows to a size of 23 cm, and likes muddy bottoms at the depth of the shoreline down to about 72 meters (Holthuis, 1980). An assessment for populations-structure was made into wild populations, suggesting that populations of *L. vannamei* should be treated as genetically subdivided (Valles, 2005).

1.2.2. Taxonomical classification

Several common names have been attributed to this species, but its classification is known like it is describe by authors (Holthuis, 1980; Perez-Farfante and Kensley, 1997).

Kingdom Animalia -- animals
Phylum Arthropoda -- arthropods
Subphylum Crustacea -- crustaceans (Brünnich, 1772)
Class Malacostraca -- (Latreille, 1802)
Subclass Eumalacostraca -- (Grobben, 1892)
Superorder Eucarida -- (Calman, 1904)
Order Decapoda -- (Latrille, 1802)
Suborder Dendrobranchiata -- (Bate, 1888)
Superfamily Penaeoidea -- penaeoid shrimps (Rafinesque, 1815)
Family Penaeidae -- penaeid shrimps (Rafinesque, 1815)
Genus Litopenaeus -- (Perez-Farfante, 1969)
Species *Litopenaeus vannamei* -- (Boone, 1931)

Figure 4. *Litopenaeus* (Penaeus) *vannamei* (http://www.ars.usda.gov/is/graphics/photos)

1.2.3. Distinguishing features of *L. vannamei*

The rostrum is armed with dorsal and usually 2-4 (occasionally 5-8) ventral teeth, which are moderately long, and in the young distinctly surpassing antennular peduncle. Carapace has pronounced antennal and hepatic spines, and lacks orbital and pterygostomian spines. The postocular sulcus is absent. The telson is unarmed. Antennules lack a parapenaeid spine and the antennular flagellums are much shorter than the carapace. Male reproducers in mature stadium produce spermatophores extremely complex, consisting of a sperm mass encapsulated by a sheath, and bearing various attachment structures (anterior wing, lateral flap, caudal flange, dorsal plate), as well as adhesive and glutinous materials (Chow *et al*. 1991). The mature female has an open thelycum and sternite XIV bearing ridges, prominences, depressions, or grooves. Finally, this species has six nauplii stages, three protozeal stages, and three mysis stages in its life history (Kitani, 1986). The same author considered that the most distinguishable morphological character during larval stages is the development of supraorbital spines in the second and third protozoea stage.

1.2.4. Physiological aspects of *L. vannamei*

L. vannamei has the capacity to adapt to a wide range of salinities in the range of 0 to 50 ppt (Tizol *et al.*, 2004). Its life cycle consists of an oceanic planktonic larval stage, an estuarine potlarva-to-juvenile stage, and then returns to the marine environment as an adult to mature and spawn. The postlarvae migrate into estuaries, which is rich in nutrients and low in salinity. There, they grow up and juveniles eventually migrate back into open waters. Adults have

marine behaviour; being benthic animals living primarily on the sea bottom and where reach a mature stadium. Females have an egg production dependent on each individual size, found from 30 to 45 gr. of weight, producing between 100 and 250 thousand eggs by spawning, with a diameter of 0.22 mm approximately. The coloration is translucent white, thus it is most commonly known as the "white shrimp". The body of the species often has a bluish hue that is due to a predominance of blue chromatophores that are concentrated near the margins of the telson and uropods.

1.2.5. Close shrimp species

The *L. vannamei* are distinguished among other shrimp's peneidos by the presence of hepatica and antennae spine very pronounced in the cephalothorax, and by the presence of 8 or 9 teeth in the upper border of the rostrum. Also, *L. vannamei* can be distinguished from *L. schmitti, L. setiferus, L. occidentalis*, and *L. stylirostris* on the basis of the external genitalia. In these species, both the thelycum and the petasma, are more primitive that in species of other genera (Perez Farfante, 1969). Add that the five species of the genus *Litopenaeus* are restricted to American waters.

1.2.6. Aquaculture use of *L. vannamei*

The species -*L. vannamei*- was introduced to the Pacific Islands from the early 1970s, where research was conducted into breeding and their potential for aquaculture. During the late 1970s and early 1980s they were introduced to Hawaii and the Eastern Atlantic Coast of the Americas from South Carolina and Texas in the North to Central America and as far South as Brazil. Also, it was introduced experimentally to Asia from the years 1978-79, but commercially only since 1996s into Mainland China and the Taiwan Province of China, followed by most of the other coastal Asian countries in the years 2000-01 (Briggs, 2004). The dominant shrimp species in Latin America are the white shrimp (*L. vannamei*) and the blue shrimp (*L. stylirostris*). It constitutes a limiting factor for the future development of the shrimp-farming sector in Latin America and Caribbean, where predominance of *L. vannamei* culture are 90-95% (Hernández-Rodríguez, 2001; Wikipedia, 2006).

1.2.7. Breeding and selection

Production of specific pathogen free (SPF) animals and the development of specific pathogen resistant (SPR) strains are two complementary approaches that are currently possible using broodstock management programmes. SPF animals are produced by, selecting animals free of

known and detectable pathogens and raising them under controlled and strict sanitary conditions. The SPR animals are developed through selective breeding of animals known to be less susceptible to specific pathogens. The main benefit of this concept is the production of high health (HH) post-larvae, free of or resistant to known pathogens. If the immune or physiological traits are heritable, this translates into performance improvement at the farm level (Subasinghe *et al*. 1998).

However, many SPF stocks that have not been exposed to pathogens (specific or general) perform poorly when pathogens are present. As with most organisms, a lack of exposure to infectious organisms increases clinical susceptibility. Moreover, a potential harmful consequence of directional artificial selection is inbreeding (Ferguson, 1994).

1.2.8. Source of the studied animals

Shrimp Improvement System (SIS) is a commercial hatchery located in Miami, Florida, USA. The center has a developed program on genetic improvement of shrimp species. It based on family selection; they focus on selection for disease resistance and fast growth. The enterprise crossbreeds more than 50 families in each culture-section, resulting about 6 to 10 sections during the year. The families are tested facing familiar pathogens from the lab and commercial farms alike. When the results from the test are known, the best performances families are sent to bio-security installations were they are mixed into current populations on exploitation, integrating them to commercial production. Certificated institutions like Aquatic Animal Health Laboratory test all populations used in SIS from time to time, and up to date, their stocks have a historic background for more than 8 years without disease (disease-free). The working-program makes it possible to get lines resistant to new virus or mutation from them, in a period less than 12 month. These genetic programs get an advantage on inter-family variability for genetic gain from different lines.

So, with extensive information about this center (SIS), and with knowledge on what is going on regarding shrimp culture in the world; some specimens of *L. vannamei* were bought by Cuban shrimp culturists in SIS. General details about the introductions of this new species are shown in table 1 (information provided by the CIP). Researchers from the CIP headed each introduction, following international and national agreements concerning species introduction under FAO, OIE and Cuban regulations (FAO, 1996; Tizol *et al*. 2004).

Table 1. Introductions of *L. vannamei* by Cuban shrimp culturists

	First	Second	Third	Fourth
Date	10-2003	05-2004	12-2004	03-2005
Quantity	500 000	500 000	1 270	1 472
Age	Post-larvae (stage -10)	Post-larvae (stage -10)	Broodstock	Broodstock
Female	-	-	630	1008
Male	-	-	640	464

1.3. Microsatellite DNA marker loci

Microsatellite loci are sequences made up of a single sequence motif -comprised of very short (1-6 bp)-, repeated many times side-by-side in the nuclear genome of most taxa. They are found in all prokaryote and eukaryote genomes, present even in the smallest bacterial genomes (Tóth *et al*. 2000). They represent a unique type of tandem repetitive genomic sequences, which are abundantly distributed across genomes and demonstrate high levels of allele polymorphism. High polymorphism and the relative ease of scoring represent the two major features that make microsatellites of large interest for many genetic studies.

The analysis using microsatellite loci polymorphism is based in a PCR-based approach. The oligonucleotide primers are designed based on unique single-copy of a sequence flanking the microsatellite repeats. The PCR primer pairs are selected such that PCR products are of small molecular size (usually <300 bp). It provide relative ease in amplification from low-quality DNAs and also allowing for distinguishing small differences in molecular size of alleles among individuals by using polyacrylamide-gel electrophoresis or automated DNA sequencers. Ideally, each individual shows a single (homozygote) or two-band (heterozygote) DNA pattern, with one band inherited from each parent. PCR of microsatellites are usually multiplexed, such that two to five different microsatellites loci are amplified in a single PCR-reaction and analyzed simultaneously. One primer from each locus pair is fluorescently labeled, which result that each locus will have a different color, or alternatively the molecular size of the products of some loci in the multiplex differ, so that they do not overlap. The multilocus PCR-reaction products are then loaded together in the gel or capillaries of the sequencer and its detection system allows discrimination of the allelic products of each locus by color or molecular size.

1.3.1. Applications of microsatellite loci

In the field of aquaculture, microsatellites represent the markers of choice for genetic monitoring of farmed stocks in view of breeding programs. They allow the analysis of genetic variability and pedigree structure, to design beneficial crosses, select genetically improved stocks, minimize inbreeding and increase selection response (Chistiakov, 2005). Borrell *et al*. (2003) showed results obtained from the applications of microsatellites in the management of three cultured species: Atlantic salmon (*Salmo salar*), turbot (*Scophthalmus maximus*) and white shrimp (*Litopenaeus schmitti*).

Besides, mention that many finfish species have been tested also with the use of microsatellites loci markers, such as: turbot and rainbow trout (*Oncorhynchus mykiss*) (Estoup *et al*. 1998; Holm *et al*. 2001) Atlantic salmon (*Salmo salar*) (Norris *et al*. 1999), chinook salmon (*Oncorhynchus tshawytscha*) (Hedrick *et al*. 2000), Atlantic cod (*Gadus morhua*) (Delghandi, 2003), Atlantic halibut (*Hippoglossus hippoglossus*) (Jackson *et al*. 2003), common carp (*Cyprinus carpio*) (Vandeputte, 2003; Vandeputte *et al*. 2004), and artic charr (*Salvelinus alpinus*) (Ditlecadet *et al*. 2006).

1.3.2. Microsatellite marker loci in shrimp species

Vanavichit *et al*. (1998) using microsatellite as genetic markers in *Penaeus monodon*, considered that they are ideal for shrimp genome study and broodstock management. The primers used in this study resulted be polymorphic, and PCR products were generated ranging from 5-25 alleles per primer. Three out of ten *P.monodon* microsatellites isolated in this species also amplified alleles in *P.vannamei*, making them useful for genetic studies as potential for cross-species amplification (Xu *et al*. 1999). In the same way, Bierne *et al*. (2000) and Goyard *et al*. (2003) used heterologous loci from *P.vannamei* (Vanna01 and Vanna02) to assess the genetic bases of *Litopenaeus stylirostris*. In the same species (*P. stylirostris*), another study was run for variability test (Moullac *et al*. 2003).

Target species with these genetic markers has been the *L. schmitti* too. Bécquer *et al*. (2001) pointed out some issues on genetics studies done with the species, and compared the use of allozymes and microsatellite during the characterization of different populations (wild and domesticated), reaffirming that microsatellites are more genetic variable markers, resulting in a better characterization of shrimp populations. Meanwhile, Espinosa *et al*. (2001) determined the optimal conditions for the amplification of two loci (Lsch-1 and Lsch-2) into genetic-chromosome, using wild and domesticated species as well, getting a microsatllite library for *L.*

schmitti. Afterwards, they found that captivity affects the rostral teeth distribution. It is also showed using allozymes and microsatellite as molecular markers, a lesser on genetic variability for this species (Espinosa *et al*. 2002). Genetic distance and differentiation between populations exist in accordance with geographic location, marine currents, cycle life and ecological characteristic that have been demonstrated using microsatellites, which provided useful information on the good management of *L. schmitti* as conservation and breeding program alike (Borell *et al*. 2000; Maggioni *et al*. 2003).

1.3.3. Application of microsatellites in *L. vannamei*

Bagshaw and Buckholt (1997) discovered a novel combination of sequence elements associated to satellite/microsatellite into genome of marine shrimp *P. vannamei.* Using hybridization technique, sequence information of the 1259-bp B20 clone revealed two microsatelites and two candidate open reading frames (Garcia *et al*., 1996). In the same way, Meehan *et al*. (2003) presented results obtained by probe hybridization from isolate usable (polymorphic) microsatellite in this species. Besides, Cruz *et al*. (2002) made an isolation and characterization of five species-specific polymorphic microsatellites by screening procedure.

Microsatellites have been used for the assessment of the genetic variability of captive broodstock or wild population of *L. vannamei* as well (Wolfus *et al*. 1997; Cruz *et al*. 2004; Dueñas, 2005; Borrell *et al*. 2005; Valles *et al*. 2005 and Wang *et al*. 2006). Moreover, they were used in the examining genetic diversity of SPF *P. vannamei* within in selective breeding families (Garcia *et al*. 1994). On the other hand, they are being useful for optimization in the management of the species during massal selection process (Perez, 2003). Also, microsatellites are applicable in study of inheritance for allelic variation in this species (Zhang and Xiang, 2005).

1.4. Aim of the study

The ability to close the life cycle of *L. vannamei* and produce broodstocks within the culture ponds, permit work on domestication and genetic selection looking for favourable traits of aquaculture importance. Between such traits are growth rate, disease resistance and rapid maturation of the species. Through these means, domesticated stocks of SPF and SPR shrimps have been developed, and are currently commercially available from USA (Subasinghe *et al*. 2003; Briggs, 2004). Although it is know that to obtain such level of SPF or SPR certification

in shrimp, a hard genetic works is required; still unclear knowledge of genetic quality from that broodstocks exists. Genetic diversity analysis using microsatellites is done in the first and second group of *L. vannamei* (Table 1) introduced in Cuba (Dueñas, 2005; Borrell *et al*. 2005). However, as consequence of that gap it is necessary to find out which is the genetic value that such animals bought in USA have. Pedigree information from generation to generation no exists, resulting in a general lack of information need to establish a viable selective breeding program.

Therefore, this study tends to monitor genetic variability in the founder stock and up to a first generation (F1) using four species-specific microsatellite DNA loci of *L. vannamei* as genetic makers, determining the potential of these animals for the initiation of a breeding program for long term.

2. Materials and Methods

2.1. Sample collection

Samples from farmed white shrimp *L. vannamei* were collected from YAGUACAM, a commercial hatchery located in Yaguanabo, Cienfuegos, Cuba. (Figure 1). A total of 310 tissues-samples of these specimens were collected randomly from four different populations in July 2005 (Table 2). These were two parental stocks (3P and 4P), corresponding to the third and fourth introduction of *L. vannamei* to Cuba from USA (Table 1), and their respective F1 offspring (3F and 4F). In this study, F1 generations are denoted like 3F and 4F in correspondent to parental stock.

Broodstocks were first place into maturation tanks (10 m^3), using 85 females and 120 males by tank, separated by sex and with a photoperiod of 14:10 hours (light/dark). Mature females were then subjected to unilateral eyestalk ablation and stocked with males in a proportion of 10 males for 1 female (10:1). The F1-generation (3F and 4F) had produced by individuals spawning of already copulated female by males. Black cylinder-conic tanks of 200 L for spawn and 100 L for egg incubation were used.

Samples collected from parental groups were individually weighted. Besides, all samples were collected from different age classes and body tissues as described in table 2. All collected tissues were stored in 95% ethanol and shipped to Norway for genetic analysis at the Norwegian College of Fishery science (NFH) of the University of Tromsø (Uitø).

Table 2. Data from samples collected of *L. vannamei* in Cuban hatchery

Population	Stadiums	Mean Weight (gr.)	No. Specimens	Tissue	Introduced
3P	Adult	Female 42 Male: 32	75	Pleopods	Yes
3F	Juvenile	-	80	Tail	No
4P	Adult	Female 42 Male: 32	75	Pleopods	Yes
4F	Post-Larvae (PL)	-	80	Whole PL	No

2.2. DNA extraction

Total genomic DNA was extracted from each sample using an in-house extraction protocol of extraction (see Appendix 1). First, tissues were washed with 300 µl High TE buffer (0,1M Tris, 0,04M EDTA* pH 8,0) followed by 650 µl of salt lysis buffer proteinase K SDS (0,01M Tris, 0,4M NaCl, 0,002M Na_2EDTApH 8,3). The mixture was then incubated overnight at 50 ^{0}C in a Termaks oven (16 hours). DNA was extracted in two successive steps: first, the samples were centrifuged to remove all debris (step 3 – 4, Appendix 1), and then they were centrifuged to precipitate the DNA pellet (step 5 – 7, Appendix 1). The latter was then washed in 250 µl of 70% ethanol, resuspended in TE buffer (0,01M Tris, 0,001M EDTA* pH 8,0), and preserved at -18 ^{0}C.

2.3. Microsatellite loci amplification

The targets DNA were amplified by polymerase chain reaction (PCR), using four species-specific microsatellite loci (primers) known to be polymorphic in *L. vannamei*: *Pvan1815*, *Pvan0040*, *Pvan1758* (Cruz *et al*. 2002) and *M1* (Wolfus *et al*. 1997). Primers were obtained from Applied Biosystem UK (see Appendix 2). The PCR-process was performed in 10 µl volumes, containing the above primers, *Taq*-polymerase and target-DNA sample using the procedure given in Appendix 3. PCR amplifications were run in a Gene Amp® PCR System 2700 (Applied Biosystems), using the following temperature cycling conditions: start with 1 min at 95 ^{0}C; 32 cycles of 20 sec at 95 ^{0}C, 30 sec at 50 ^{0}C, and 1 min at 68 ^{0}C, final extension 10 min at 68 ^{0}C. 2 µl of PCR product was mix with 8 µl of deionised formamide and size standard. In order to obtain single stranded DNA fragments, this mix was heated for 5 min at 95 ^{0}C and then rapidly cooled to 4 ^{0}C. Separation of the PCR products was performed using an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.4. Genetic diversity and departure from Hardy-Weinberg equilibrium

Samples size (N) used by each primer and the mean number of the alleles (Na) was calculated using Fstat program (v.2.9.3). The effective number of alleles (n_e) was estimated as the reciprocal of the expected homozygosity (Hartl and Clark, 1997).

$$n_e = 1 \Big/ \sum p_i^2 \text{ or } 1 \Big/ \hat{F}$$

Where:

$$\sum p_i^2 = \hat{F} = 1 - H_o$$

It can be interpreted as the equilibrium value of the proportion of homozygous genotypes. Hence, homozygosity and heterozygosity are opposite sides at the same coin.

The observed heterozygosity (H_o) values were calculated directly from the observed genotypes and the expected heterozygosity (H_e) values were determined for each locus using GENEPOP program (version 3.4) (Raymond and Rousset, 1995).

F_{IS}-inbreeding coefficient values that reveal the reduction in the average proportion of heterozygous genotypes within populations were estimated. Departures from Hardy-Weinberg equilibrium (HWE) were tested using "Hardy-Weinberg test". The association between alleles were evaluated by 2-locus linkage disequilibrium analysis. In all these cases, the tests were evaluated using GENEPOP program (Raymond and Rousset, 1995). The Markov chain method was employed to estimate the probability of significant deviation (*P*-values). Pairwise F_{ST} values between populations were calculated in Fstat program (Goudet, 2001) to determine population genetic differentiation using the Wright's F-statistics. The levels of significance for the tests, including all pair-wise comparisons were adjusted using a sequential Bonferroni correction (Rice, 1989). Differences among populations regarding mean number of alleles (Na), effective number of alleles (n_e), and observed heterozygosity (H_o) were analysed using paired t-test (T-student test) using Excel software. Differences were accepted as significant when the probability value < 0.05.

3. Results

3.1. Genetic diversity and departure from Hardy-Weinberg equilibrium

The genetic diversity of *L. vannamei* from four populations using four species-specific microsatellite loci is shown in table 3. Two microsatellite loci, *Pvan0040* and *Pvan1758* were difficult to amplify in the multiplex system, whereas the remaining two loci (*Pvan1815* and *M1*) allowed correct genotyping of all or nearly all samples (Table 2).

The mean number of alleles (Na) and mean effective number of alleles (n_e) per locus across populations ranged from 4.5 to 6.75 and 1.48 to 3.81 respectively. The number of alleles per locus was higher in *Pvan1758* with 5-9 alleles, followed by *M1* (5-7 alleles) and *Pvan1815* (4-8 alleles). One locus (*Pvan0040*) resulted monomorphic in the population 4P whereas an extra allele was found in its respective F1-progeny (4F). The presence of two extra alleles was also found in the 3F progeny group compared to their parents (3P) for the *M1* locus. It could be associated to the difficult to amplify all the samples by each primer. The mean observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.29 to 0.63 and from 0.45 to 0.66 respectively (Table 3). Samples from the third introduction (3P and 3F) showed higher mean Na than those from the fourth introduction (4P and 4F). The population 4P and 4F showed the highest mean n_e, H_o, and H_e.

Among populations, Na values ranged from 1 to 9, n_e values from 1.1 to 6.72, and H_o values from 0.06 to 0.85. In all populations at all loci, n_e was lower than observed Na with exception of the monomorphic locus (*Pvan0040*) in 4P population. In nearly all cases, the H_o-values were lower than the H_e-values, indicating a general deficit of heterozygous types for the loci under study. Only in locus *Pvan1758* tested in 4P population the H_o-value resulted higher than the H_e-value (Table 3), indicative of an excess of heterozygous types. There were not significant differences ($P>0.05$) in H_o values compared to H_e values by populations (Table 4).

All analysed populations departed from Hardy-Weinberg equilibrium (HWE) across loci ($P<0.05$). In 14 out of 16 possible tests, were observed significant departures from HWE ($P<0.05$) after the sequential Bonferroni procedure due to deficit of heterozygous. Only with exception the 3F population at *Pvan1758* locus that was in HWE equilibrium ($P=0.16$) (Table 3).

Table 3. Genetic diversity found in four *L. vannamei* populations at four loci

Populations		Locus				Mean
		Pvan1815	*Pvan0040*	*M1*	*Pvan1758*	
3P	Sample size (N)	71	48	67	46	58
	No. of alleles (Na)	8	5	5	9	6.75
	Effective Na. (n_e)	2.29	1.1	1.72	2.3	1.85
	Observed heter. (H_o)	0.5638	0.0625	0.4179	0.5652	0.4023
	Expected heter. (H_e)	0.5683	0.3502	0.7652	0.7471	0.6077
	P	0.0408	<0.0001	<0.0001	<0.0001	0.0102
3F	Sample size (N)	80	59	80	66	71.25
	No. of alleles (Na)	6	4	7	5	5.5
	Effective Na. (n_e)	1.54	1.13	1.25	2	1.48
	Observed heter. (H_o)	0.3500	0.1186	0.2000	0.5000	0.2922
	Expected heter. (H_e)	0.4205	0.2057	0.6173	0.5407	0.4461
	P	0.0001	<0.0001	<0.0001	0.1609	0.0403
4P	Sample size (N)	72	58	67	47	61
	No. of alleles (Na)	4	1	6	7	4.5
	Effective Na. (n_e)	1.53	1	3.19	6.72	3.81
	Observed heter. (H_o)	0.3472	n.a.	0.6866	0.8511	0.6283
	Expected heter. (H_e)	0.5092	n.a.	0.7313	0.7493	0.6633
	P	0.0043	n.a.	<0.0001	0.0014	0.0019
4F	Sample size (N)	80	55	80	62	69.25
	No. of alleles (Na)	4	2	6	6	4.5
	Effective Na. (n_e)	1.43	1	2.5	3.87	2.6
	Observed heter. (H_o)	0.3000	n.a.	0.6000	0.7419	0.5473
	Expected heter. (H_e)	0.4222	0.0364	0.6899	0.7544	0.6222
	P	0.0007	0.0092	0.0054	0.0085	0.0060

P = probability of significant deviation from Hardy-Weinberg equilibrium (following Markov chain procedure, *P*=0.05).
n.a. = not available.

Table 4.Comparative test between mean observed heterozygosity and expected heterozygosity across all loci by population

(H_o - H_e).	Population	*P*
Observed heterozygosity H_o	3P	0.072
	3F	0.174
versus	4P	0.693
Expected heterozygosity H_e	4F	0.148

All analysed populations showed a total of positive values during the estimation of F_{IS} test (Table 5). It means that there were complete reductions of heterozygous in all populations under study. Likewise, in nearly all loci the F_{IS} values estimated were positive with the exception in *Pvan1758* locus at 4P population (F_{IS} = -0.136). Coincidently to results in table 3, where it is such position (*Pvan1758* locus at 4P population) the H_o-value is higher than H_e-value due to excess of heterozygous. All F_{IS} values were different from zero (P<0.05), being only at locus *Pvan1758* in 3F population no different from zero (P>0.05).

Table 5. F_{IS} value estimated for the four populations of *L. vannamei*

Population	Locus				Total
	Pvan1815	*Pvan0040*	*M1*	*Pvan1758*	
3P	+0.009	+0.822	+0.454	+0.243	+0.3207
3F	+0.168	+0.423	+0.676	+0.075*	+0.3580
4P	+0.318	n.a.	+0.061	-0.136	+0.0817
4F	+0.289	+1	+0.130	+0.016	+0.1433

n.a. = not available.

Pairwise F_{ST} values estimated, indicated little and moderate genetic differentiation among the populations studied (Table 6). Significant levels (P<0.05) for F_{ST} parameter is present among almost all populations. All results different from zero except paired between 4P and 4F populations. It means that there is practically no genetic differentiation between these two populations. Population 3F resulted be the most different genetically among all populations.

Table 6. Pairwise F_{ST} estimates among four populations of *L. vannamei*

Population	3F	4P	4F
3P	0.0842*	0.0378*	0.0277*
3F		0.1145*	0.1125*
4P			0.0065 (N.S.)

N.S. = not significant from zero.

The linkage disequilibrium analyses for alleles were highly significant (P<0.0001) for almost all pair of loci (Table 7). No significant 2-locus linkage disequilibrium was detected (P>0.0001) at *M1* and *Pvan0040* pair, which indicate no association between these two genotypes. In general can be considered as linked the genes in this study with high significant value (P<0.0001).

Table 7. Results from the comparison test of linkage disequilibrium by pair locus, with four species-specific primers of *L. vannamei*

Pair locus		*P*
M1	*Pvan1758*	<0.001
M1	*Pvan0040*	0.05
M1	*Pvan1815*	<0.001
Pvan1758	*Pvan0040*	<0.001
Pvan1758	*Pvan1815*	<0.001
Pvan0040	*Pvan1815*	<0.001

Between successive generations, there were no significant differences (P>0.05) related to the Na-value (Table 8). Besides, a comparative test between parental groups (3P and 4P) is added shown no significant values (P>0.05) for Na-values as well. In the same way, no significant differences (P>0.05) were found respect to n_e-values for any case alike. However, the H_o in 4P group was significant higher (P=0.046) than in 4F group. It means a reduction of heterozygous in their correspondent F1. In case of the relation 3P-3F analyses, the H_o was not significant (P=0.178). Significant lower (P<0.001) resulted the H_o in 3P compared to 4P group, which showed significant differences between the parental broodstocks (Table 8).

Table 8. Pairwise comparisons test between the two successive introductions (3P and 4P) of *L. vannamei* shrimp-species and their consecutives generations (3P and 3F; 4P and 4F)

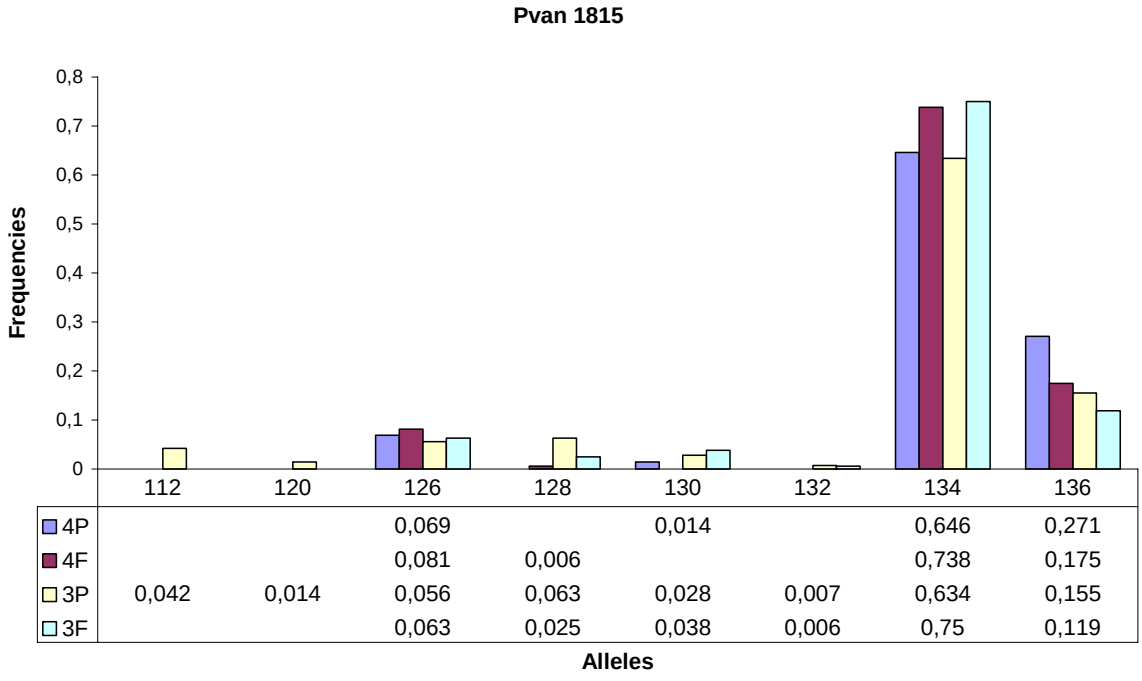
Indicator	Populations	*P*
Number of alleles (Na)	3P-4P	0.153
	3P-3F	0.391
	4P-4F	1
Effective number of alleles (Ne)	3P-4P	0.220
	3P-3F	0.107
	4P-4F	0.284
Observed heterozygosity (H_o)	3P-4P	<0.001
	3P-3F	0.178
	4P-4F	0.046*

3.2. Allelic frequencies differences

The frequency distribution of microsatellite alleles varied among the four populations. The more common alleles were 134 bp, 144 bp, 187 bp and 213 bp across populations and loci. Likewise, among all these common alleles 144 resulted be, the one showing the highest frequency with 1.000 (100%). Exclusive alleles (refer to those which are no present in other groups) were more representatives in samples analysed corresponding to the third introduction, characterized by a lost of these alleles into their correspondent progeny (F1).

3.2.1. Locus *Pvan1815*

It showed 8 alleles that are represented in the follow figure 5 with their respective frequencies. Most common allele resulted be 134 bp for all populations, which together with the 136 bp resulted be the more common allele (see below). Two exclusive alleles were found in the 3P group -112 bp and 120 bp-, which did not show in the corresponding descendants group (3F).



	112	120	126	128	130	132	134	136
4P			0,069		0,014		0,646	0,271
4F			0,081	0,006			0,738	0,175
3P	0,042	0,014	0,056	0,063	0,028	0,007	0,634	0,155
3F			0,063	0,025	0,038	0,006	0,75	0,119

Figure 5. Allelic frequencies for *Pvan1815* locus

3.2.2. Locus *Pvan0040*

This locus showed the lowest number of alleles (n = 6), which are given together with their frequencies in the follow figure 6. Most common allele and alleles through all groups resulted be the same at 144 bp in all cases (see below). It is showed two exclusive alleles, 134 bp and 148 bp for 3F and 3P populations respectively.

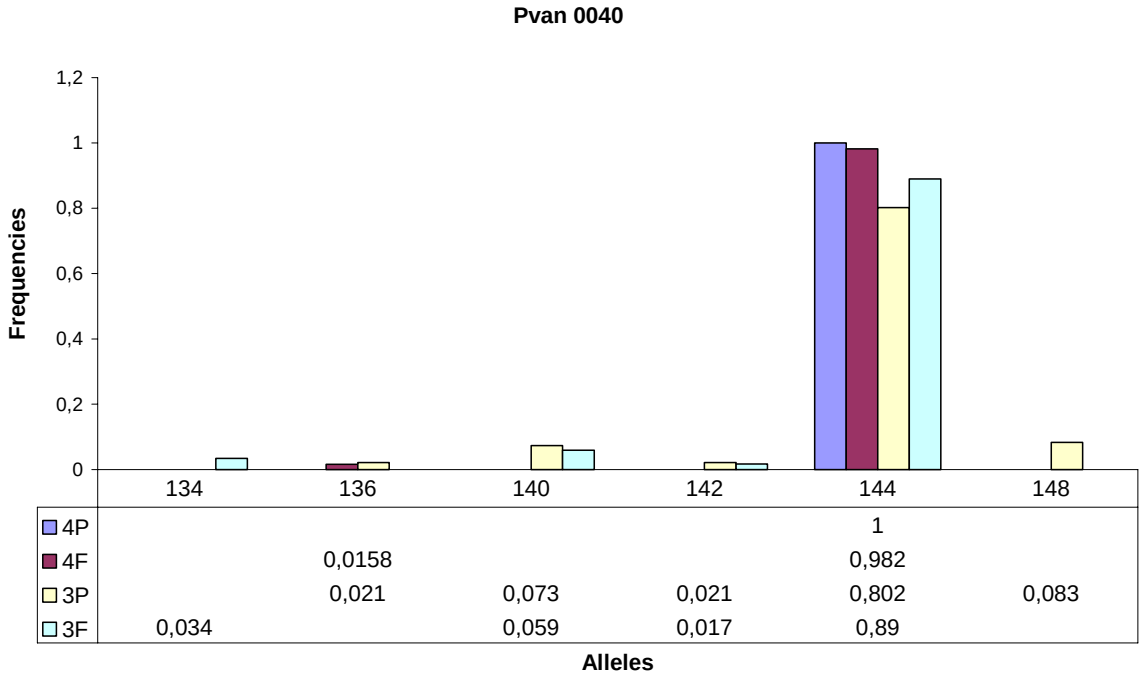
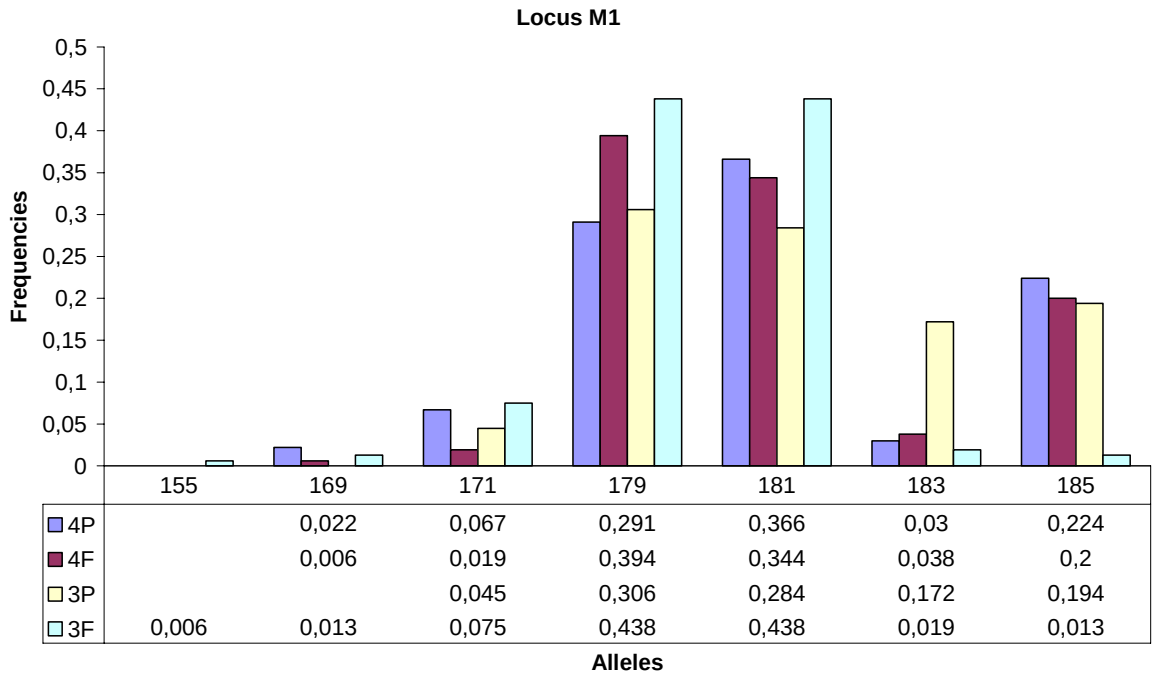


Figure 6. Allelic frequencies for *Pvan0040* locus

3.2.3. Locus *M1*

It showed 7 alleles whit their respective frequencies that are shown in the follow figure 7. The most common alleles were 179 bp for 4F, 3P and 3F populations and 181 bp for 4P and 3F populations (see below). In case of samples from the 3F group, the highest frequency is shared between two alleles (179 bp and 181 bp). The more common alleles were 179 bp, 181 bp and 185 bp in this locus. An exclusive allele was gained in descendents (3F group) from reproducer of third introduction broodstock at 155 bp but with a low frequency (0.006).

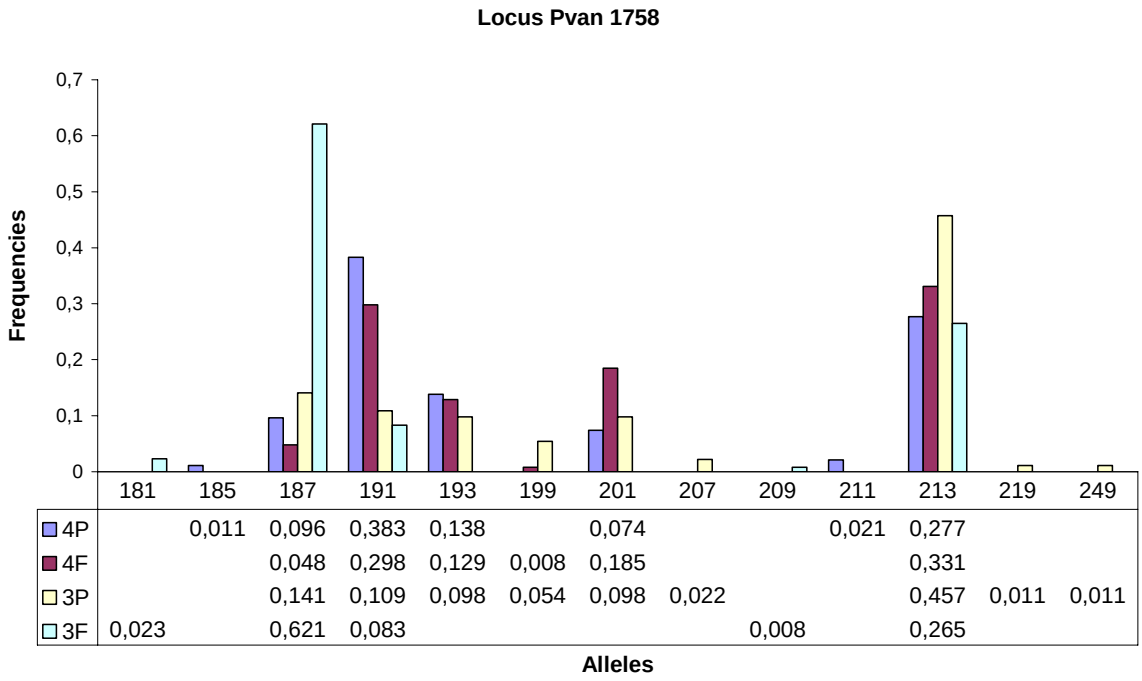


	155	169	171	179	181	183	185
4P		0,022	0,067	0,291	0,366	0,03	0,224
4F		0,006	0,019	0,394	0,344	0,038	0,2
3P			0,045	0,306	0,284	0,172	0,194
3F	0,006	0,013	0,075	0,438	0,438	0,019	0,013

Figure 7. Allelic frequencies for *M1* locus

3.2.4. Locus *Pvan1758*

It was observed 13 alleles, which are shown in the follow figure 8 with their respective frequencies. The most common allele resulted be 187 bp and 191 bp for 3F and 4P populations respectively, and 213 bp for 4F and 3P populations alike (see below). The more common alleles were 187 bp, 191 bp and 213 bp. There were several exclusive alleles (7) showed by all populations and distributed among all of them with exception of 4F group. For each population it was represented as: 4P (185 bp and 211 bp), 3P (207 bp, 219 bp and 249 bp), and 3F (181 bp and 209 bp); all of them with low frequency that ranged between 0.008 and 0.023 frequencies.



	181	185	187	191	193	199	201	207	209	211	213	219	249
4P		0,011	0,096	0,383	0,138		0,074			0,021	0,277		
4F			0,048	0,298	0,129	0,008	0,185				0,331		
3P			0,141	0,109	0,098	0,054	0,098	0,022			0,457	0,011	0,011
3F	0,023		0,621	0,083					0,008		0,265		

Figure 8. Allelic frequencies for *Pvan1758* locus

4. Discussion

In this work, I attempted to investigate the genetic variability in two broodstocks of shrimp (*L. vannamei*) introduced to a Cuban hatchery from USA and their respective F1 progenies by use of microsatellite markers. The specific loci were selected from the literature as the most highly variable for these species (Wolfus *et al*. 1997; Cruz *et al*. 2002; Borrell *et al*. 2005; Dueñas *et al*. 2005).

4.1. Genetic diversity: number of alleles and heterozygosity

The *Pvan0040* locus in one of the parental groups (4P) resulted to be monomorphic whereas the number of alleles reported in the literature at this locus is Na=7 (Valles, 2005). This can be explained by the particular inbred nature of the stocks introduced to Cuba which has lead to fixation through several generations of selection. The presence of a monomorphic locus in the analysis of farmed populations have been reported for shrimp *L. stylirostris* (Bierne *et al*. 2000), two commercial traits of Artic charr (*Salvelinus alpinus*) (Ditlecadet *et al*. 2006), and wild populations of Atlantic salmon, *Salmo salar* (Norris *et al*. 1999).

The allelic diversity (i.e., mean number of alleles per locus) is a useful measure of genetic variability within a population, provided that comparisons are carried out between samples of similar size. The mean number of alleles per locus in this study (1-9) was slightly lower than reported in other studies of farmed *L. vannamei*. Using similar primers, Borrell *et al*. (2005) and Dueñas (2005) observed a number of alleles between 3 and 12 in specimens belonging to the first and second introductions of *L. vannamei* to Cuba. Elsewhere, Cruz *et al.* (2004) using only two primers (*Pvan1758* and *Pvan1815*) obtained values from 7 to 10 alleles when analyzing two consecutive generations (G_o until G_2) within a shrimp-breeding program. Moreover, a range of 4-23 alleles was reported at *M1* locus in analysing the genetic diversity of a further shrimp breeding project (Wolfus *et al*. 1997). In specimens from wild populations of *L. vannamei* it has been reported a range from 2 to 16 numbers of alleles per locus using five species-specific primers (Valles *et al*. 2005).

In this study a problem arose with the presence of extra alleles observed in the F1 progenies (3F and 4F) belonging to the introductions for *Pvan0040* and *M1* loci. This could be related to the possibility that individuals carrying these alleles in the parent populations (3P and 4P) were not sampled or, did not produce usable results after amplification due to sample degradation or specific PCR inability (Suden and Scott, 1991; Wolfus *et al*. 1997).

In other shrimp species, Goyard *et al*. (2003) using two microsatellite loci from *L. vannamei* (Vanna01 and Vanna02) found a range of 1 to 9 alleles per locus in farmed *L. stylirostris*. The same authors compared these results to those of wild populations (14-27) showing a significant loss after 4 and 7 generations of selection. Bierne *et al.* (2000) found in the same species (*L. stylirostris*) a range of 2-4 alleles per locus in hatcheries after 22 and 24 generations with microsatellites. Similarly, the loss in average number of alleles per locus in cultured populations of *P. monodon* (12.8) compared to wild populations (23.9) (Xu *et al*. 2001).

The effective number of alleles per locus found in this work varied from 1 to 6.72 and was lower than the observed number of alleles. Similar results were found after seven generations of selection with 4 alleles and only 1.6 effectives (Wolfus *et al*. 1997). But they are lower than in other results, where they reach values until 10.5 (Valles *et al*. 2005) and 15.7 in wild populations (Wolfus *et al*. 1997). Taking into account that the effective number of alleles decrease into the next generation, is necessary to consider that many alleles have showed low frequencies (value <0.01). Since background on the origin, number of generations in captivity, and mating management of the individuals we used as founder stock are uncertain, is leading us to suspect that in previous management of these individuals was a loss of genetic diversity. Some alleles are represented in very small frequencies and the risks of increased homozygosity are therefore high.

There were no significant differences in successive generations (3P-3F and 4P-4F) but still the pedigree of the specimens is unknown and, assuming that they are already selected for specific traits –increased growth and disease-resistance- is evident to assume an artificial selection addressing to small population size (bottleneck). One way to improve performance in a trait is cull out individuals with poor characteristics. The consequence of this approach is the potential loss in genetic variability arising from restricting the number of breeders. At this level, an effective population size gives us the degree to which genetic drift can change allele frequencies from one generation to the next, due only to the random effects of broodstock's sampling. Suden and Scott (1991) pointed out that genetic variation could be lost through genetic drift, but there is an inverse relationship between genetic drift and effective size of the breeding population. Small effective population sizes and poor management breeding programs are generally blamed for loss of genetic diversity in farmed stocks (Norris *et al*. 1999).

Nevertheless, maintaining pedigree information on all individuals in the breeding program and using this information to schedule mating designs, it will ensure only a minimum increase in variability every generation. Bernhard and Olesen (2002) estimated during a stochastic simulation that reducing the number of broodstock pairs might increase the rate of inbreeding to as much as 6-8% per generation.

Although the genetic variability of various wild populations of *L. vannamei* has been demonstrated (Valles, 2005), the allelic diversity available in founder populations is likely equivalent to that of domesticated populations in this work. The loss of genetic diversity in domesticated stocks previously observed in *L. vannamei* (Suden and Scott, 1991; Donato *et al*., 2005); *L. schmitti* (Espinosa *et al*., 2001) *L. stylirostris* (Goyard *et al*., 2003) common carp (*Cyprinus carpio*) (see Vandeputte, 2003) Atlantic halibut (*Hippoglossus hippoglossus*) (Jackson *et al*., 2003) and resumed for different populations of penaeid prawns (see Benzie, 2000) is confirmed by the present study.

Because of the closed production cycle used in hatcheries, a decrease of genetic variability was expected for *L. vannamei* strains. Similarly to other studies, the mean observed heterozygosity value obtained by population was smaller than the expected one (Benzie, 2000; Goyard *et al*. 2003; Soto-Hernández and Grijalva-Chon, 2004; Dueñas, 2005). The mean values (0.29 to 0.63) obtained in this study resulted lower compared to results revealed in the same species (*L. vannamei*) with mean values from 0.45 to 0.67 (Borrell *et al*., 2005) and 0.49 to 0.72 (Dueñas, 2005). Such results were lower than similar studies in farmed *L. vannamei*, where Cruz *et al*. (2004) and Wolfus *et al.* (1997) estimated for all their populations giving 70% and 98.1% of observed heterozygosity respectively. Also, Benzie (2000) shown in three different culture shrimp species that observed heterozygosity over all loci could range from 0.45 to 1.

The present heterozygosity values revealed in this study are in the range found in wild populations using microsatellites for *L. vannamei*, which ranged from 0.045 to 0.614 (Valles *et al*. 2005). Studies using allozymes have reported lower values of heterozygosity than those using microsatellites (Suden and Scott, 1991; Soto-Hernández and Grijalva-Chon, 2004). Despite, the values reported for wild populations of *L. vannamei* are lower than those reported for other wild populations where ranged from 0.425 to 0.964 (see Benzie, 2000).

Considered as amount of genetic variation through generations is perceived that individuals from the fourth introduction and their progeny (4P and 4F) showed H_o = 0.63 to 0.55 and H_e = 0.62 to 0.66. These values are in the same range found in a study realized to individuals from the first introduction of *L. vannamei* realized in Cuba with values of H_o = 0.72 to 0.49 and H_e = 0.6 to 0.83 (Dueñas 2005). However, results from the third (3P and 3F) introduction resulted lower than both results (H_o = 0.40 to 0.29; H_e = 0.45 to 0.61). In spite of the loss of heterozygosity observed in both groups studied through successive generations, the first group mentioned above (4P and 4F) apparently showed higher average level of heterozygosity than individuals from the third introduction (3P and 3F) concerning to heterozygosity values.

The loss of heterozygosity through generations has been observed in other shrimp studies. Over a period of 11 generations on a cultivated strain of *L. vannamei*, the coefficient of inbreeding per generation grew steadily (Donato *et al*. 2005). In the same species (*L. vannamei*), but after seven generations of captive breeding, a lack of genetic diversity was revealed (Wolfus *et al*. 1997). These researchers suggested that further depletion of genetic diversity could occur if appropriate management of this population was not performed. Bierne *et al.* (2000) showed in *L. stylirostris* a significant heterozygosity deficiency after 17 generations of selection. A consistent and progressive reduction of heterozygosity levels was also observed from the F1 through the F6 generations using allozymes in hatchery stocks of *Penaeus japonicus* (Sbordoni *et al*. 1986). The authors attributed these results to a severe bottleneck effect in the founder population. Benzie (2000) taking this example occurred in *P. japonicus*, highlighted the potentially damaging effects of uncontrolled inbreeding and the need to maintain reasonably high levels of effective population sizes.

One might expect a reduction in the amount of genetic variation in cultured stocks, simply because of founder or bottleneck effects in the establishment of broodstoock populations, and possibly as a result of domestication. Population bottleneck and founder effects may cause a reduction in the number of alleles, a distorted distribution in numbers of molecular differences among alleles, and an increased linkage disequilibrium, resulting in a serious loss of heterozygosity (Hart and Clark, 1997).

Cruz *et al.* (2004) found different results during a 2-generation breeding program (H_o = 0.65 to 0.72; H_e = 0.71 to 0.77), where introduced new variability from the same original founder stock into the broodstock population during the second generation. The same authors

considered that heterozygosity is an imperfect measure of variability, since it is insensitive to the number of alleles. Allelic diversity appears to be a more sensitive measure of genetic variability than observed heterozygosity (Norris *et al*. 1999; Ditlecadet *et al*. 2006). Xu *et al*. (2001) explained in their paper the importance of heterozygosity, but underline the fact that it is not a perfect index. The changes in heterozygosity are less evident because the number of alleles or allelic frequencies can affect them, and a heterozygosity reduction due to allele loss can be compensated by an increase in the frequency on the remaining alleles.

The probability of loosing an allele is related to its frequency. When a population goes through a bottleneck, many low frequency alleles may be eliminated, or contribute little to heterozygosity. In a bottleneck of short duration could severely reduce the number of alleles (by eliminating rare alleles) without having much effect on heterozygosity (Norris *et al*. 1999). This is explained by Goyard *et al*. (2003) in *L. stylirostris*, whom considered that the number of spawners participating in the maintenance of the populations is very small and is likely to increase inbreeding. Besides, Wolfus *et al.* (1997) considered in their study that an inbred population could have been descended from the genetic contribution of only two alleles.

4.2. Deviation from Hardy-Weinberg equilibrium and inbreeding coefficient

The analysis of genotypes frequencies revealed deviations from Hardy-Weinberg equilibrium in this study, due to deficits of heterozygotes as it is showed by results of the estimations for the inbreeding coefficient (F_{IS}). Goyard *et al*. (2003) and Valles *et al*. (2005) mentioned four potential causes that may induce to these deficits of heterozygotes. The first is a technical artifact linked to the occurrence of null alleles (i.e., the failure to amplify one of the alleles in an individual or miscoring due to stutter bands). This phenomenon has been reported by Pongsomboon *et al*. (2000) in *L. monodon* species, which also took into consideration small sample size. They used 50 samples that normally adequate for allozyme studies, but may not cover all possible genotypes generated by microsatellites. In our case due to the amount of samples used here and that we did not realized “null allele test”, we can not rule out the possibility of null alleles or sampling errors as source of deficit of heterozygous. Although, mentioned that not all samples were amplified correctly by the primers used.

Other potential causes suggested in agreement to Castric *et al*. (2002) are based on the biology of the studied population: (i) “Whalund effect” (ii) inbreeding and (iii) selection of specific alleles. Heterozygosity deficiency could result from a subdivision of the local

population into isolated and differtiated reproductive units (Whalund effect). This effect could overestimate the expect rate of heterozygosity due to the fact that our samples are already issued for several genetically distinct populations, which probably leads us to the false conclusion that they have less heterozygote than it should be at Hardy-Weinberg equilibrium. Most individuals collected for this assessment could be derived from a small number of progenitors, since are selected for specific traits, mating could then be biased toward relatives (inbreeding). Vandeputte, (2003) pointed out that the lower variability indicated in many cases of domesticated strains, is due to small effective numbers of breeders result in some level of inbreeding. The selection of specific alleles is not possible to exclude it and could have happened during the reading-process of such alleles after the separation of the PCR products.

4.3. Linkage disequilibrium and pair wise comparisons

Changes in the distribution of genotypes (almost all genes linked), shown to be totally different from wild populations. Further show that these candidate broodstock are becoming genetically distinct from their population of origin. In regions of tight linkage could be explained by either "selective sweep" characterized by the overrepresentation of the favoured allele or, "background selection" where reduce the number of chromosomes that can contribute to the ancestry of remote generations (see Hartl and Clark, 1997). The tighter the linkage, the greater the reduction in polymorphism due to background selection. In spite of results found in this work, we can not reject the usefulness of the markers used in this study, since they have been worked successfully in others studies. We can assume that the results obtained here can be related to previous management, which still are unknown for us. This assumption is supported by Castric *et al*. (2002) whose pointed out that when inbreeding is present in a population, we would expect that all loci were correlated (linkage disequilibrium).

Paired comparisons showed significant genetic differences between all populations, with the exception of 4P and 4F pair populations. This may have resulted from different founder populations and different selection procedures. As some specimens tested in this work are already selected for increased growth and disease resistant strains mixed in the same pool, probably they have nearly fixed some alleles through successive generations. In this regard, values reported of paired comparisons by Benzie (2000) from five main shrimp aquaculture species showed ranges from 0.007 to 0.031. The same author considered that these species have been found to be strongly structured, not only with respect to identity of genotypes, but

also with respect to levels of genetic variability. Values found for 4P and 4F pair (0.0065) resulted lower than the lowest value showed in other species (Benzie, 2000).

5. Conclusions

Multiple measures decreased (Na, n_e, Ho) indicating that this genetic distinction may be the result of a loss diversity. Since the ultimate goal of breeding programs is the selection of better performing individuals for a number of traits of interest, these results indicated a loss of genetic diversity.

A possible small number of breeders may endanger the cultivated strains from these populations. It is necessary to have well control over possible matings in a future breeding program involving these specimens.

Artificial breeding practices may result in a decrease of genetic variability in terms of allelic diversity but which is not necessarily detectable from the levels of heterozygosity. It is necessary to distinguish between quantitative and qualitative differences in diversity, when genes and alleles at low frequencies are being considered. This may explain why the ranges of heterozygosity values between cultured and wild populations overlapped each other, although cultured populations were less diverse than wild stocks.

This study highlights the importance of constantly assessing the genetic variability in the cultured shrimp populations in Cuba. Considering the low variability observed, it is suggested that the development of a sustainable selective breeding program in Cuba be conducted only after application of measures aimed at enhancing the genetic variation (through exchange of broodstocks with others producers or sampling of wild specimens) and that the genetic information be constantly used to reduce the risks of further inbreeding.

References

Alvarez, J.S., Perez, M. and Toledo, J. (1992). The state of aquaculture in Cuba and profiles of nutrition and alimentation. In: The nutrition and alimentation in the aquaculture of Latin America and Carribean. FAO–Italia gcp/rla/102/ita. Available at: http://www.fao.org/docrep/field/003/AB487S/AB487S00.htm#TOC

Bagshaw, J.C. and Buckholt, M.A. (1997). A novel satellite/microsatellite combination in the genome of the marine shrimp, *Penaeus vannamei*. Gene, 184, 211-214.

Becquer, U., Diaz, R., Hernandez, N., Romo, J., Rodríguez, T., Azanza, J., Guerrero, C. and Espinosa, G. (2001). Genetic in shrimp culture *Litopenaeus schmitti* in Cuba: Results and Perspective. VI Ecuadorian Congress of Aquaculture and V Ecuadorian Congress of Aquaculture & Exposition. Hilton Colon, Guayaquil, Ecuador, October 24-27.

Bernhard, B. and Olesen, I. (2002). Designing aquaculture mass selection programs to avoid high inbreeding rates. Aquaculture, 2004, 349-359.

Benzie, J.A. (2000). Population genetic structure in penaeid praws. Aquaculture Research, 31, 95-119.

Bierne, N., Beuzart, I., Vonau, V., Bonhomme, F. And Bédier, E. (2000). Microsatellite-associated heterosis in hatchery-propagated stocks of the shrimp *Penaeus stylirostris*. Aquaculture, 184, 203-219.

Borrell, Y.J., Espinosa, G., Vázquez, E., Sánchez, J.A. and Blanco, G. (2005). Genetic characterization of the first stocks of *Litopenaeus vannamei* introduced in Cuba for aquaculture. Paper developed by Biochemistry department from University of Havana and Department of Functional Biology in Aquatic Genetic Lab from University of Ovide, Spain.

Borrell, Y., Blanco, G., Vásquez, E., Alvarez, J. Pineda, H., Espinosa, G. Fernandez-Pato, C., Martinez, C. and Sánchez, J.A. (2003). The variability of microsatellite and their application into aquaculture. CIVA 2003, 104-1056.

Borrell, Y.J., Espinosa, G., Vázquez, E., Sánchez, J.A. and Blanco, G. (2000). DNA microsatellites variability and genetic differentiation among natural populations of the Cuban white shrimp Litopenaeus schmitti. Paper developed by Biochemistry department from University of Havana and Department of Functional Biology in Aquatic Genetic Lab from University of Ovide, Spain.

Briggs, M., Funge-Smith, S., Subasinghe, R. and Philips, M. (2004). Introductions and movement of Penaeus vannamei and Penaeus stylirostris in Asia and the Pacific. FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS REGIONAL OFFICE FOR ASIA AND THE PACIFIC. R. P. 2004/10. Bangkok, FAO: 99.

Castric, V., Bernatchez, L., Belkir, K. and Bonhomme, F. (2002). Heterozygote deficiencies in small lacustrine populations of brook charr *Salvelinus Fontinalis* Mitchill (Pisces, Salmonidae): a test of alternative hypotheses. Heredity, 89, 27-35.

Chistiakov, D., Hellemans, B. and Volckaert, F. (2005). Microsatellites and their genomic distribution, evolution, function and applications: A review with special reference to fish genetics. Review, Aquaculture, article in press, doi: 10.1016/j.aquaculture.2005.11.031.

Chow, S., M. M. Dougherty, W. J. Dougherty and P. A. Sandifer. (1991). Spermatophore Formation in the White Shrimps Penaeus Setiferus and P. Vannamei. Journal of Crustacean Biology 11(2): 201-216.

Cruz, P., Ibarra, A.M., Mejia-Ruiz, H., Gaffney, P.M. and Pérez-Enríquez, R. (2004). Genetic variability assessed by microsatellites in a breeding program of pacific white shrimp (*Litopenaeus vannamei*). Marine Biotechnology, 6, 157-164.

Cruz, P., Mejia-Ruiz, H., Perez-Enriquez, R. and Ibarra, M. (2002). Isolation and characterization of microsatellites in Pacific white shrimp Penaeus (*Litopenaeus*) vanamei. Molecular Ecology Notes, 2, 239-241.

Delghandi, M., Mortensen, A. and Westgaard, J. (2003). Simultaneous analysis of six microsatellites markers in Atlantic cod (*Gadus morhua*): A novel multiplex assay system for use in selective breeding studies. Marine Biotechnology, 5, 141-148.

Ditlecadet, D., Dufresne, F., Rose, N. and Ulrich, P. (2006). Applying microsatellites in two commercial strains of Artic charr (Salvelinus alpinus): Potential for a selective breeding program. Aquaculture, doi: 10.1016/j.aquaculture.2006.03.016.

Donato, M., Manrique, R., Ramirez, R., Mayer, L. and Howell, C. (2005). Mass selection and inbreeding effects on a cultivated strain of Penaeus (*Litopenaeus*) in Venezuela. Aquaculture, 247, 159-167.

Dueñas, S. (2005). Thesis of grade: Genetic variability of microsatellite in the first stoks of *L. vanamei* introduced in Cuba for culture. Department of biology from University of Havana.

Espinosa, G., Becquer, U., Borrell, Y., Romo, J., Diaz, R., Azanza, J. and Rosa de Dios (2002). Morphological and genetic variability of natural and cultured population of the Cuban White Shrimp Litopenaeus schmitti. CIVA 2002, 778-784.

Espinosa, G., Pager, M., García, E., Borrell, Y., Corona, N., Robainas, A. and Deutsch, J. (2001). Microsatellites from the White Shrimp *Litopenaeus schmitti* (Crustacea, Decapoda). Biotecnología Aplicada, 18 (4)232-234.

Estoup, A., Gharbi, K. SanCristobal, M., Chevalet, C. Haffray, P. and Guyomard, R. (1997). Parentage assignment using microsatellites in turbot (*Scophthalmus maximus*) and rainbow trout (*Oncorhynchus mykiss*) hatchery populations. Can. J. Fish. Aquat. Sci., 55, 715-725.

FAO (2006). FIGIS (Fisheries global information system). http://www.fao.org/

FAO (1994). Diagnosis about the state of aquaculture in Latin America and Caribbean. Diagnostico sobre el estado de la acuicultura en America latina y el Caribe. FAO-ITALIA, GCP/RLA/102/ITA. Available at|:
http://www.fao.org/docrep/field/003/AB484S/AB484S00.htm#TOC

FAO. (1996). Precautionary approach to capture fisheries and species introductions. Elaborated by the technical Consultation on the precautionary Approach to Capture Fisheries (Including Species Introductions). Lysekil, Sweden, 6-13 June 1995. FAO Technical Guidelines for Responsible Fisheries. No. 2, 54.

Ferguson, M. (1994). The role of molecular genetic markers in the management of cultured fishes. Review in Fish Biology and Fisheries, 4, 351-373.

Garcia, D.K., Faggart, M.A, Rhoades, L., Alcivar-Waren, A., Wyban, J.A., Carr, W.H., Sweeney, J.N. and Ebert, K.M. Genetic diversity of cultured Penaeus vannamei shrimp using three molecular genetic techniques. Mol. Biol. Biotechnol., 3(5):270-280.

Garcia, D.K., Dhar, A.K., Alcívar-Warren, A. (1996). Molecular analysis of a RAPD marker (B20) reveals two microsatelites and differential mRNA expression in Penaeus vannamei. Mol. Mar. Biol. Biotechnol, 5(1): 71-83.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3).

Goyard, E. Arnaud, S., Vonau, V., Bishoff, V., Mouchel, O., Pham, D., Wyban, J. and Boudry, P. (2003). Residual genetic variability in domesticated populations of the Pacific blue shrimp (*Litopenaeus stylirostris*) of New Caledonia, French Polynesia and Hawaii and some management recommendations. Aquatic Living Resources, 16, 501-508.

Hartl, D. and Clark., A. (1997). Principles of population genetics, third edition.

Hedrick, P. Rashbrook, V.K. and Hedgecock, D. Effective size of winter-run Chinook salmon based on microsatellite analysis of returning spawners. Can. J. Fish. Aquat. Sci. 57, 2368-2373.

Hernández-Rodríguez, A., Alceste-Oliviero, C., Sanchez, R., Jory, D., Vidal, L. & Constain-Franco, L.-F. (2001). Aquaculture development trends in Latin America and the Caribbean. In R.P. Subasinghe, P. Bueno, M.J. Phillips, C. Hough, S.E. McGladdery & J.R. Arthur, eds. Aquaculture in the Third Millennium. Technical Proceedings of the

Conference on Aquaculture in the Third Millennium, Bangkok, Thailand, 20-25 February 2000. 317-340. NACA, Bangkok and FAO, Rome

Holm, L. Loeschcke, V. and Bendixen, C. (2001). Elucidation of the molecular basis of a null allele in a Rainbow trout microsatellite. Marine Biotechnology, 3, 555-560.

Holthuis, L.B. (1980). FAO species catalogue. Shrimps and prawns of the world. An annotated catalogue of species of interest to fisheries. FAO Fish. Synop., 125 (l): 271.

Jackson, T.R., Martin-Robichaud, D.J and Reith, M. (2003). Application of DNA markers to the management of Atlantic halibut (*Hipoglossus hippoglossus*) broodstock. Aquaculture, 220, 245-259.

Josupeit, H. (2004). An Overview on the World Shrimp Market. GLOBEFISH. World Shrimp Markets, Madrid, Spain, October 26-27.

Kitani, H. (1986). Larval Development Of The White Shrimp Penaeus Vannamei Boone Reared In The Laboratory And The Statistical Observation Of Its Naupliar Stages. Bulletin of the Japanese Society of Scientific Fisheries 52(7): 1131-1139.

Liu, Z.J. and Cordes, J.F. (2004). DNA marker technologies and their applications in aquaculture genetics. Review, Aquaculture, 238, 1-37.

Maggioni, R., Rogers, A.D. and Maclean, N. (2003) Population structure of *Litopenaeus schmitti* (Decapoda: Penaeidae) from Brazilian coast identified using six polymorphic microsatellite loci. Molecular Ecology, 12, 3213-3217.

Meehan, D., Xu, Z., Zuniga, G. and Alcívar-warren, A. (2003). High frequency and large number of polymorphic microsatelites in cultured shrimp, Penaeus (*Litopenaeus*) vannamei [Crustacea: decapoda]. Short communications, Marine Biotechnology, 5, 311-330.

Moullac, G., Goyard, E., Saulnier, D., Haffner, P., Thouard, E., Nedelec, G., Goguenheim, J., Rouxel, C. and Cuzon, G. (2003). Recent improvements in broodstock management and larviculture in marine species in Polynesia and New Caledonia: genetic and health approaches. Aquaculture, 227, 89-106.

Norris, A.T., Bradley, D.G. and Cunningham, E.P. (1999). Microsatellite genetic variation between and within farmed and wild Atlantic salmon (*Salmo salar*) populations. Aquaculture, 180, 247-264.

Perez Farfante, I., and Kensley, B. (1997). Penaeoid and sergestoid shrimps and prawns of the world: keys and diagnoses for the families and genera. Mémoirus du Muséum nationale d'Histoire naturalle. Paris, France.
http://www.itis.usda.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=551682

Perez Farfante, I. (1969). Western Atlantic Shrimps of the Genus Penaeus. Fishery Bulletin 67(3): 461-591

Pongsomboon, S., Whan, V. Moore, S.S. and Tassanakajon, J. (2000). Characterzation of tri- and tetranucleotide microsatellites in the black tiger prawn, *Penaeus monodon*. ScienceAsia, 26, 1-8.

Raymond, M and Rousset, F. (1995). Genepop (version 1.2) – Population-genetics software for exact test and Ecumenicism. Journal of heredity, 86, 248-249.

Rice, W.R. (1989). Analyzing tables of statistical test. Evolution 43 (1): 223-225.

Sbordoni, V.E, Matthaeis, E. Sbordoni, C.M., Rosa, G. and Mattoccia, M. (1986). Bottleneck effects and depression of genetic variability in hatchery stocks of *Penaeus japonicus* (Crustacea, Decapoda). Aquaculture, 57, 239-251.

Soto-Hernández and Grijalva-Chon, J.M. (2004) Genetic differentiation in hatchery strains and wild white shrimp *Penaeus* (*Litopenaeus*) *vannamei* (Boone, 1931) from northwest Mexico. Aquaculture International, 12, 593-601.

Subasinhe, R.P., Curry, D., McGladdery, S.E. and Bartley, D. (2003) Recent Technological Innovations in Aquaculture. In, Review of the state of world aquaculture. FAO Fisheries Circular No. 886, Rev. 2, 59-74.

Subasinhe, R.P., Bartley, D.M., McGladdery, S. and Barg, U. (1998). Sustainable shrimp culture development: biotechnological issues and challenges. In: Flegel, T.W. (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Suden, S.L. and Scott, K.D. (1991). Evaluation of genetic variation in a domestic population of *Penaeus vannamei* (Boone): a comparison with three natural populations. Aquaculture, 97, 131-142.

Tizol, R., Jaime, B., Laira, R., Pérez, L., Machado, R. and Silveira, R. (2004). Introduction in Cuba of *L. vannamei*. Quarantine I. Paper below Fishery Research Center (CIP).

Tóth, G., Gáspári, Z. and Jurka, J. (2000). Microsatellites in Different Eukaryotic Genomes: Survey and Analysis. Genome Research, 10, 967-981.

Valles-Jimenez, R., Cruz, P. and Perez-Enrique, R. (2005). Population genetic structure of pacific white shrimp (*Litopenaeus vannamei*) from Mexico to Panama: Microsatellite DNA variation. Marine Biotechnology, 6, 475-484.

Vallez, R. (2005). Thesis: Studies on genetic structure of white shrimp, (*Litopenaeus vannamei*), from Oriental Pacific inferred by microsatellite analysis and DNA mitocondrial. Post-grade studies. La Paz, Mexico, Biology Investigations Center from Northeast. 74.

Vanavichit, A., Wuthimaethavee, S., Taechayinkphibool, D., Kruthoo, P.S., Lumubol, P. and Tragoonrung, S. (1998). Development of simple sequence length polymorphisms in the black tiger shrimp (*Penaeus monodon*). In Flegel, T.W. (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Vandeputte, M. (2003). Selective breeding of quantitative traits in the common carp (*Cyprinus carpio*): a review. Review, Aquatic living Resources, 16, 399-407.

Vandeputte, M., Kocour, M., Mauger, S., Dupont-Nivet, M., Guerry, D., Rodina, M., Gela, D., Vallod, D., Chevassus, B. and Linhart, O. (2004). Heritability estimates for growth-related traits using microsatellite parentage assignment in juvenile common carp (*Cyprinus carpio* L.). Aquaculture, 235, 223-236.

Wang, H.X, Wu, C.G., Zhang, L.S. and Xiang, J.H. (2006). The application of microsatellite markers for parentage determination in selective breeding of pacific white shrimp (*Litopenaeus vannamei*). Yi Chuan, 28(2): 179-83.

Warr, G., Liu, J., Rexroad, C., Trumble, B., Gross, P., Benzie, J., Wilson, M., Gaffeney, P., Yant, R., Kocker, T., Mileham, A., Waldbieser, G., Hedgecock, D. and Westerman, M. (2004). Genomic enablement of aquaculture species. A White Paper Submitted by the Aquaculture Genome Coordinating Committee on behalf of the 300 authors.

Wikipedia (2006). Shrimp farm. Available at: http://www.wikipedia.org/

Wolfus, G.M., Garcia, D.K., Alcívar-Warren, A. (1997). Application of the microsatellite technique for analyzing genetic diversity in shrimp breeding programs. Aquaculture, 152, 35-47.

Xu, Z. Primavera, J.H., Pena, L. Pettit, P., Belack, J. and Aicivar-Warren, A. (2001). Genetic diversity of wild and cultured black tiger shrimp (*Penaeus monodon*) in the Philippines using microsatellites. Aquaculture, 199, 13-40.

Xu, Z., Dhar, A.K., Wyrzykowski, J. and Alcivar-Warren, A. (1999). Identification of abundant and informative microsatellites from shrimp (*Penaeus monodon*) genome. Short communication, Animal Genetics, 30, 150-156.

Zhang, L.S. and Xiang, J.H. (2005). Apreliminary study on the inheritance of microsatellite in two selective breeding families of shrimp (*Litopenaeus vannamei*). Yi Chuan, 27(6): 919-24.

Appendixes

Appendix 1.Extraction of DNA from tissues

1. Dissect out the tissue of interest and wash with 300 µl High TE buffer to get rid of the remaining ethanol.
2. Pipette off the High TE buffer and add 650 µl of Salt lysis buffer.
   For extracting 72 samples you need 46,5 ml Salt lysis buffer
   548 µl Proteinase K
   1,95 ml 20% SDS sollution
   Incubate the samples at 50º Celsius over night and mix from time to time.
3. Add 350 µl of the saturated NaCl solution to each sample and mix gently. Mix them on the thermo mixer for 10 minutes.
4. Centrifuge the samples at 12000 rpm for 10 minutes.
5. Carefully pipet off the supernatant into a clean 1.9 ml eppendorf tube. Take a new pasteurpipet for every sample to prevent cross contamination.
6. Add 1 ml of Isopropanol to each tube and mix gently. The DNA will precipitate and you can see it as a white fluffy material.
7. Centrifuge the samples at 12000 rpm for 10 minutes.
8. Discard the supernatant (keep the pellet).
9. Wash the pellet in 250 µl of 70% ethanol.
10. Pipet off the ethanol.
11. Resuspend the DNA pellet in TE buffer. Adjust the amount of TE buffer you add according to the pellet size. On average add 500-600 µl to each pellet.

The samples are now ready for PCR or storage at -20º Celsius.

Appendix 2. Characteristics of primers used in the study

Primer	Sequence	Molecular Weight	Length (№ base)	nmol
M1	F: GTG TGT TGC GGA ATC GAA R: CTA ACC CAA TAT CGA ATC	5412	18	10
Pvan1758	F: TAT GCT CGT TCC CTT TGC TT R: TTG AAG GAA AAG TGT TGG GG	6301	20	10
Pvan0040	F: TTT ACG ATC AGA TTG TTC R: GAA ATA GAA AAT AAA GAA C	6191	18	10
Pvan1815	F: GAT CAT TCG CCC CTC TTT TT R: ATC TAC GGT TCG AGA GCA GA	6166	20	10

Appendix 3. Products used into reaction volume for DNA-amplification

	Products	Per samples (µl)	per 100 samples (µl)
PCR-mix	PCR buffer	1	100
	$MgCl_2$	1	100
	dNTP (10mM)	0.5	50
	dH_2O	1.9	190
Primers (labelling colours)*	Red (*Pvan1815*)	0.3	30
	Blue (*Pvan1758*)	0.2	20
	Green (M1)	1.5	150
	Black (*Pvan0040*)	1.5	150
Taq-polymerase		0.1	10
DNA-sample		2	-

*Colour associated to each primer in order to obtain differential display on the computer-screen during sequencing process.