



Genetic distance between broodstocks of the marine shrimp *Litopenaeus vannamei* (Decapoda, Penaeidae) by mtDNA analyses

Ana Karina de Francisco and Pedro Manoel Galetti Junior

Universidade Federal de São Carlos, Departamento de Genética e Evolução, São Carlos, SP, Brazil.

Abstract

In the constantly growing Brazilian shrimp industry the evaluation of genetic relationships between broodstocks is an useful tool for shrimp culture management programs. We established the genetic relationships between five broodstocks of the white marine shrimp *Litopenaeus vannamei* (Penaeidae) based on the sequencing of the mtDNA 16S rRNA and cytochrome oxidase I (COI) regions. Although no divergence was found between the broodstocks for the highly conservative 16S rRNA gene, we did find an 8.2% distance between *L. vannamei* and the *Farfantepenaeus subtilis*. Analyses of the COI region showed genetic distances of only 0.2 to 1% between the broodstocks, which contrasted with the 10.9% mean distance found between *L. vannamei* and *F. subtilis*. The small genetic distance values obtained may be related to genetic drift or a founder effect that occurred during broodstocks establishment. The mtDNA analysis was able to characterize the genetic divergence between the broodstocks studied and could be helpful for defining better management strategies of these crustacea.

Key words: shrimp, *Litopenaeus vannamei*, genetic variation, mtDNA.

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Introduction

The white marine shrimp *Litopenaeus vannamei* is an important aquaculture species reared several American countries, including Brazil. This species is native to the Pacific Ocean with a geographic distribution ranging from the southern coast of Mexico to the southern coast of Peru (Nunes, 2001). Specimens of *L. vannamei* from different geographical regions were used to compose the Brazilian broodstocks, possibly producing some genetic differentiation among them as resulted from founder effects (Primack, 2002). It is possible that some breeder management practices may be critical to the maintenance of Brazilian captive populations, especially those practices related to the reduction of genetic variability caused by inbreeding and random genetic drift effects (Freitas and Galetti Jr, 2002).

The study of the genetic characteristics of shrimp species and the analysis of genetic relationships between broodstocks can be an useful tool for their management because genetic identification and discrimination of broodstocks are essential to successful rearing programs (Allegrucci *et al.*, 1998; Sekino *et al.*, 2002). Mitochondrial DNA (mtDNA) shows a high level of polymorphism and an evolutionary rate 10 times faster than nuclear genomes and

is subject to maternal and asexual inheritance (Awadalla *et al.*, 1999), characteristics which make mtDNA very useful for genetic studies in aquaculture (Allegrucci *et al.*, 1998). Sequence analyses of mtDNA have been widely used in phylogenetic studies of natural shrimp populations (Bouchon *et al.*, 1994; Baldwin *et al.*, 1998; Klinbunga *et al.*, 1999; Gusmão *et al.*, 2000; Maggioni *et al.*, 2001) and to evaluate genetic divergence between captive populations (Iguchi *et al.*, 1999; Sekino *et al.*, 2002). The 16S rRNA gene is found in the conserved portion of the mtDNA and has been extensively used in phylogenetic studies while the cytochrome oxidase I (COI) gene, which seems to be less conserved than the 16S rRNA gene, has been frequently used in evolutionary studies (Clary and Wolstenholme, 1985; Beard *et al.*, 1993).

The work described in this paper used mtDNA sequencing of the 16S rRNA and COI genes to analyze the genetic interrelationships between *L. vannamei* broodstocks, detailed knowledge of the genetic features of broodstocks being crucial to the development of captive rearing programs.

Material and Methods

Sample collection and DNA extraction

Specimens of the white marine shrimp *Litopenaeus vannamei* (Penaeidae) were obtained from five broodstocks

Send correspondence to Ana Karina de Francisco. Universidade Federal de São Carlos, Departamento de Genética e Evolução, Caixa Postal 676, 13656-905 São Carlos, SP, Brazil. E-mail: pakarina@iris.ufscar.br.

(Aqua-044, Aqua-045, Aqua-046, Aqua-051 and Aqua-ven) owned by the Aquatec hatchery situated in the northern Brazilian state of Rio Grande do Norte. The Aqua-044 broodstock is an F₃ generation and Aqua-045 an F₄ generation, both mainly founded using wild *L. vannamei* imported from Panama but with some specimens from other Brazilian shrimp producers. The Aqua-046 and Aqua-051 broodstocks are both F₁ generations, founded mostly using *L. vannamei* from Secom Farm in the Brazilian state of Ceará but with some specimens from Panama and Ecuador which had been previously reared at the Aquatec hatchery. The Aqua-ven broodstock was founded from an F₁₇ *L. vannamei* imported from a Venezuelan.

The 16S rRNA analyses was made using two specimens from each broodstock while the COI gene sequencing used five specimens from each broodstock with the exception of the Aqua-045 broodstock for which only four specimens were used. Pleopod samples from all specimens were collected and stored in 1 mL of absolute ethanol at -20 °C. Genomic DNA was extracted according to the procedure described by Sambrook *et al.* (1989).

PCR, sequencing and data analyses

The 16S rRNA region was polymerase chain reaction (PCR) amplified using the primers 16Sar (5'-GCCTGTTT AACAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAACT CAGATCATGT-3') (Simon *et al.*, 1991). Amplification was carried out in a 50 µL final volume of 1x buffer containing 5 mM MgCl₂, 200 mM dNTPs, 4 units of *Taq* DNA polymerase, 50 ng of genomic DNA and 50 ng of each primer using 5 min of initial denaturation at 93 °C, followed by 30 cycles of 30 s at 93 °C, 30 s at 50 °C, 45 s at 72 °C and a final extension 5 min at 72 °C. The COI region was PCR amplified using the primers CO9 (5'-TTCGGTTC A(T/C)CCAGAAGT(C/A)TAT-3') and CO10 (5'-TAA GCGTCTGGGTAGTCTGA(A/G)TA(T/G)CG-3') (Baldwin *et al.*, 1998). Amplification was carried out in a 25 µL final volume of 1x buffer containing 1.5 mM MgCl₂, 200 mM dNTPs, 200 nM of each primer, 3 units of *Taq* DNA polymerase and 10 ng of genomic DNA using 3 min of initial denaturation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min at 72 °C and final extension of 5 min at 72 °C. The amplification products were purified using the Wizard PCR Preps DNA Purification System kit (Promega), and then coupled to the pGEM-T vector (pGEM-T Easy System II kit - Promega) for further transformation in competent DH5α *Escherichia coli* cells. The FlexiPrep kit (Amersham Pharmacia Biotech) was adapted to perform minipreparations of plasmids from the recombinant clones bearing the selected fragments.

The sequencing of the selected fragments was performed according to Sanger *et al.* (1977) in an automatic ABI 377 sequencer (Applied Biosystems Inc.). The sequences obtained from three clones of each sample analyzed were aligned using the Multalin site (Copert, 1988) to

find a consensus sequence, confirmed by visual inspection, for each specimen. The consensus sequences generated for each specimen were aligned to obtain a single consensus sequence per broodstock and these sequences used to evaluate the genetic distance by the *p* distance method contained in the Mega 2.1 program (Kumar *et al.*, 1994). Two wild *Farfantepenaeus subtilis* specimens were used for comparing the magnitude of interspecific divergence.

Results

The partial 16S rRNA gene sequences consisted of 529bp fragments with a mean nucleotide composition of adenine (A) = 32%, cytosine (C) = 14.6%, guanine (G) = 21.5% and thymine (T) = 32%. There were only two 16S rRNA haplotypes in the six broodstocks, one haplotype being shared by all five *L. vannamei* broodstocks while the other haplotype occurred only in *F. subtilis*. The consensus sequences of all the specimens studied were deposited in GenBank (access numbers: AY344183 to AY344192 for *L. vannamei* and AY344193 and AY344194 for *F. subtilis*). The total nucleotide diversity and genetic divergence obtained for the *L. vannamei* broodstocks were equal to zero, contrasting with the 41 divergent sites and 8.2% genetic distance found between *L. vannamei* and *F. subtilis*.

The nucleotide composition of a 595bp fragment from the COI gene (GenBank access numbers: AY344200 to AY344223 for *L. vannamei* and AY344195 to AY344199 for *F. subtilis*) was A = 26.8%, C = 17.9%, G = 16.6%, and T = 38.8%. The total nucleotide diversity within *L. vannamei* was 0.041. Eight divergent sites were detected for the *L. vannamei* broodstocks while 70 were observed between *L. vannamei* and *F. subtilis*. Five haplotypes were discriminated within *L. vannamei* (one for each broodstock) plus one for *F. subtilis*. The genetic divergence for the *L. vannamei* broodstocks ranged from 0.002 to 0.01, contrasting with a mean distance of 0.1094 between *L. vannamei* and *F. subtilis* (Table 1).

Discussion

For both shrimp species there was a bias towards A + T in the base-pair composition of both the 16S rRNA and COI gene sequences, which is commonly the case in most penaeid species (Baldwin *et al.*, 1998; Maggioni *et al.*, 2001). Although 16S rRNA analysis failed to discriminate genetic differences between the *L. vannamei* broodstocks it was able to distinguish haplotypes and 41 divergent sites between *L. vannamei* and *F. subtilis*. The 16S rRNA region is known to be conserved and have a low rate of evolution (Meyer, 1994) which means that it is more accurate in discriminating between species than within species. In shrimp, 16S rRNA sequencing has been found to be able to characterize a morphotype usually identified as *F. subtilis* but which displays a different genetic pattern and could be regarded as a new species (Maggioni *et al.*, 2001). However,

Table 1 - Genetic distance (lower diagonal) and standard deviation values (upper diagonal) obtained from COI gene sequence analysis.

Broodstocks	Aqua-ven	Aqua-051	Aqua-046	Aqua-044	Aqua-045	<i>F. subtilis</i>
Aqua-ven	-	0.002	0.002	0.003	0.004	0.013
Aqua-051	0.003	-	0.002	0.003	0.004	0.013
Aqua-046	0.003	0.003	-	0.002	0.004	0.013
Aqua-044	0.005	0.005	0.002	-	0.004	0.013
Aqua-045	0.010	0.010	0.010	0.008	-	0.013
<i>F. subtilis</i>	0.108	0.111	0.108	0.106	0.114	-

the low evolutionary rate of the 16S rRNA gene means that there is little or no differentiation between sequences from specimens belonging to the same species, the homogeneity of the *L. vannamei* broodstocks seen in our study probably being related to the conserved nature of this gene.

In contrast to the situation with the 16S rRNA gene, the five *L. vannamei* broodstocks showed genetic differentiation in the COI gene analysis. The genetic divergences detected by COI gene analysis were consistently higher than those revealed by analysis of the 16S rRNA region. The COI region sequences exhibited sufficient nucleotide diversity to be able to determine the occurrence of one haplotype per broodstock and another for *F. subtilis*, with 8 divergent sites being detected among the five broodstocks and 70 between *L. vannamei* and *F. subtilis*. The substitution rate presented by the COI gene was somewhat higher, allowing the detection of genetic differences between different populations of the same species (Conn, 1998; Lee, 2000; Gusmão *et al.*, 2000). Although the genetic distances for the five *L. vannamei* broodstocks were close to zero (0.2 to 1%) the values were similar to those published by Gusmão *et al.* (2000) for some penaeid species investigated using COI gene sequencing. In addition, in our study the mean genetic distance between *L. vannamei* and *F. subtilis* was 10.94%, which was close to the 8.1% genetic distance obtained by Gusmão *et al.* (2000) for *Farfantepenaeus paulensis* and *F. subtilis*.

Nevertheless, it is probable that the small genetic distance values between the five broodstocks studied by us were not due to mutations that occurred at the time of their foundation but may instead be related to random genetic drift or a founder effects that occurred during the establishment of the broodstocks. Since the mitochondrial genome is maternally inherited without recombination it is to be expected that variation in this genome is highly susceptible to stochastic events such as genetic drift (Bembo *et al.*, 1995). The broodstocks investigated by us may also have been formed by specimens collected from different localities such as Panama and Ecuador and such a divergence, even if only small, may be reflecting the genetic variation present in the natural populations.

However, it is well established that adult *L. vannamei* mate off-shore where their planktonic larvae are submitted to the random effects of winds and tides (Benzie, 2000) and

a large genetically homogeneous and panmictic population is to be expected. Moreover, there is no available information on population substructuring in wild population of *L. vannamei*. In addition, microsatellites analyses have not been able to find isolation by distance in populations of *Litopenaeus schmitti* from eight different localities on the Brazilian coast (Maggioni *et al.*, 2003). Similar results have been obtained by Gusmão *et al.* (2000) for *Farfantepenaeus brasiliensis* populations using allozyme analysis.

It thus appears that the genetic divergence observed by us in the five *L. vannamei* broodstocks studied is the result of random genetic drift or founder effects. It appears that mtDNA analysis allowed us to identify a certain degree of genetic divergence among the broodstocks studied and that this type of analysis could be an important tool for better defining the use of these broodstocks in genetic improvement programs.

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