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Hsp70 gene polymorphisms in farmed marine shrimp *Litopenaeus vannamei* populations exposed to white spot disease and infectious myonecrosis

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ABSTRACT. Single nucleotide polymorphisms (SNPs) are the best genetic markers for associative studies of the immune system in invertebrates. In the marine shrimp *Litopenaeus vannamei*, SNPs linked to disease resistance have been reported for some genes, such as hemocyanin, anti-lipopolysaccharide factor, and heat-shock protein 70 (Hsp70). In the present study, polymorphisms in the *Hsp70* gene were investigated among three commercial *L. vannamei* populations bred

in Northeast and South Brazil. The first population withstood a strong white spot disease outbreak; the second population suffered extended exposure to infectious myonecrosis; the third population was a high health population, which was experimentally infected with white spot syndrome virus (WSSV) in the present study. All five previously known SNPs (C661A, T712C, C782T, C892T, and C1090T) were detected in the coding region of Hsp70, by Sanger sequencing of 119 shrimp. Significant differences in genetic and genotype frequencies among populations were observed for C661A, C892T, and C1090T. In the population submitted to WSSV challenge, no frequency differences were found between dead and surviving shrimp groups. These results indicate that the *Hsp70* polymorphisms described here cannot be associated with WSSV tolerance. However, significant frequency differences were observed for the population exposed to infectious myonecrosis virus. This is the first time that *L. vannamei Hsp70* gene polymorphisms were studied in correlation with these important shrimp viruses.

Key words: SNP; Chaperone; Shrimp; WSSV; IMNV

INTRODUCTION

Heat-shock proteins (HSPs) are chaperones known to be induced particularly under environmental stress when they actively refold and prevent denaturation of other proteins (Shekhar and Ponniah, 2015). HSPs may exceed 15% of total intracellular proteins in animals subjected to stress, such as temperature shifts, heavy metals, hypoxia, and infection (Srivastava, 2002). One of the most studied HSPs is the Hsp70, for its ubiquity and essential role in the cell (Morano, 2007). In *Litopenaeus vannamei* the transcription of the *Hsp70* gene is induced at 32°C during white spot syndrome virus (WSSV) infection, suggesting a functional role of HSPs in suppressing virus replication and reducing mortality in WSSV-infected shrimps (Lin et al., 2011).

In the past thirty years, viral diseases, such as Taura syndrome, white spot disease, and infectious myonecrosis, caused great impact on shrimp farming worldwide (Lightner et al., 2012). WSSV alone is considered responsible for 10 billion dollar losses in Asia (Karunasagar and Ababouch, 2012). WSSV caused the collapse of Southern Brazil shrimp farming in 2005 and has now reached high production areas in the Northeast (Santos et al., 2013). Infectious myonecrosis virus (IMNV) caused a sharp production break in Brazil, during 2004, and spread to Asia, since then (Senapin et al., 2007). One of the strategies adopted by the shrimp industry to counter the threat posed by viral diseases was to develop shrimp populations with specific characteristics, such as specific pathogen-free and specific pathogen-resistant (Huang et al., 2012; Moss et al., 2012). Quantitative trait loci, development and marker-assisted selection are likely to boost these strategies in the next few years, for farmed penaeids (Robinson et al., 2014; Yu et al., 2014).

Single nucleotide polymorphisms (SNPs) hold the highest potential for the development of animal quantitative trait loci, due to its abundance and ubiquity (Vignal et al., 2002; Ciobanu et al., 2010). SNPs are single-base-pair changes in the genomic DNA sequence where the frequency of the most abundant allele is lower than 99% (Brookes, 1999). In *L. vannamei*, some SNPs were found to be associated with resistance to pathogens, such as those observed

in the C-terminus of hemocyanin (Zhao et al., 2012), in the anti-lipopolysaccharide factor (*nLvALF1*; Liu et al., 2014), and in *Hsp70* (Zeng et al., 2008). Synonym base substitutions in the *L. vannamei Hsp70* gene seem to be correlated with Taura syndrome virus (TSV) resistance (Zeng et al., 2008). In the present study, we examine *Hsp70* SNPs in Brazilian commercial shrimp populations historically exposed to severe natural infection by WSSV and IMNV. This is the first time that *Hsp70* gene polymorphisms are studied in correlation with these important shrimp viruses.

MATERIAL AND METHODS

Sampling and origin of the genetic material

Three *L. vannamei* populations bred in Brazil were sampled. The first population (identified henceforth as Lv-A) was from southern Brazil (Imbituba, Santa Catarina, 28°13'43"S, 48°39'58"W), formed by animals which survived a severe WSSV outbreak in the region (Santos et al., 2013). The second population (Lv-B) was from southern Brazil as well but was formed by animals originating from specific pathogen-free populations introduced in Santa Catarina State for commercial purposes. Population Lv-B was certified as free from WSSV and was kept under high-biosafety laboratory conditions at Laboratório de Camarões Marinhos (LCM), Universidade Federal de Santa Catarina (UFSC). The third population (Lv-C) was formed by animals from a commercial breeding program with strong selection pressure from infectious myonecrosis, located in Northeastern Brazil (Mello et al., 2011). Population Lv-C was confirmed as negative for WSSV and IHHNV (infectious hypodermal and hematopoietic necrosis virus) after the first spawning of each annual reproduction cycle. In all instances, muscle samples were taken from the first abdominal segment of each animal. Samples were individually preserved in 96% ethanol and stored at -20°C until processing.

Viral challenge

The experimental WSSV infection was conducted on 84 juvenile *L. vannamei* (12 ± 2 g) from population Lv-B, obtained at LCM. The animals were initially acclimated for 8 days in 40-L constantly aerated tanks (23° ± 4°C; salinity 32-34‰), at a density of 10 animals per tank. Before infection, each animal was individually tested for the presence of WSSV through nested polymerase chain reaction (PCR) (Lo et al., 1996). The animals were fed pelletized commercial feed provided *ad libitum* once a day. Approximately 30% of the water was renewed daily. Only male and female animals at the intermolt stage were used in the experiments. Experimental infections were carried out by intramuscular injection of 50 µL standardized WSSV inoculum (500 viral particles in total), into the shrimp dorsal region (Goncalves et al., 2014). Mortality was checked three times a day, over 15 days of the viral challenge. Muscle samples were taken from animals found dead, and from all surviving animals at the end of the experiment. Samples were stored in 70% ethanol at -20°C until processing.

Genomic DNA extraction and amplification of the *Hsp70* gene

Genomic DNA was extracted from approximately 20 mg muscle tissue from 119 animals, using a commercial kit (Axygen Biosciences; Norgen Biotek Corp.) according to

manufacturer's specifications. The 1119-bp fragment (position 475 to 1594) of the *Hsp70* gene (Figure 1) was amplified using primers 5'-TCT CGG GTC TGA ATG TGC-3' and 5'-GAA ATA CGG TCC CTC TGC-3' (Zeng et al., 2008). PCRs were carried out in a final volume of 10 µL containing 50 ng genomic DNA; 1.5 mM $MgCl_2$; 200 µM each dNTP; 0.6 µM each primer; 2.5 U GoTaq® G2 Hot Start Polymerase (Promega, USA) and 1X Promega proprietary buffer. PCRs were performed on a Swift MaxPro (ESCO) thermal cycler and comprised an initial denaturation step of 95.0°C for 2 min; followed by 35 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 1 min; followed by a final elongation step of 72.0°C for 10 min.

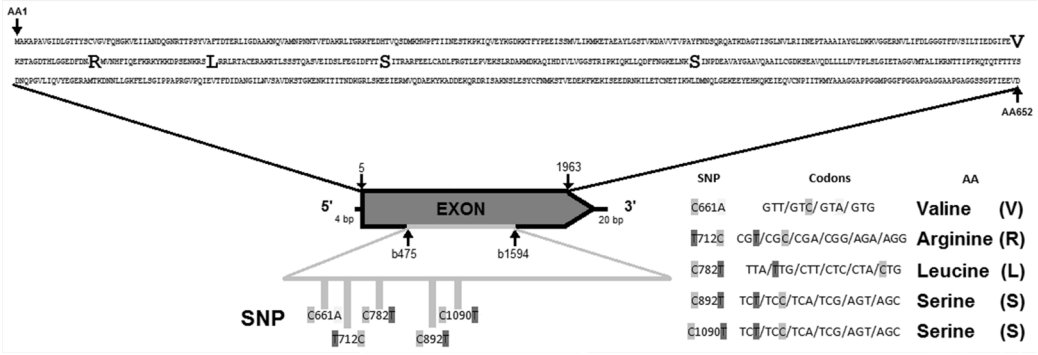

Figure 1. SNP distribution in the *Hsp70* gene from *Litopenaeus vannamei*. The light gray line represents the PCR amplicon; number near arrows indicate base or amino acid position relative to the reference sequence AY645906; amino acids corresponding to the synonymous SNPs are emphasized.

SNP genotyping and analysis

The identification of SNPs was performed by direct Sanger sequencing of PCR amplicons using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems™), following the manufacturer's instructions. Sequencing reactions were purified by precipitation in isopropanol/ethanol (Sambrook et al., 1989), denatured at 95°C for 5 min in 10 µL formamide and read on a 3500 Genetic Analyzer (Applied Biosystems™). Each amplicon was sequenced in both directions. Sequences were checked manually using Sequencing Analysis v. 5.4 (Applied Biosystems™). For SNP genotyping, only polymorphisms with quality values of 10 or higher, where flanking bases presented quality values of 30 or higher, were considered. Genotypes were checked against the complete *L. vannamei Hsp70* gene sequence (GenBank accession No. AY645906). Allele and genotype frequencies, exact tests for Hardy-Weinberg and linkage disequilibrium, F_{IS} and F_{ST} and G_{ST} were calculated using GENEPOP Online v. 4.2 (Rousset, 2008) and GenAlEx 6.5 (Peakall and Smouse, 2012). Allele frequencies were compared by χ^2 pairwise tests, using STATISTICA 10.0 (StatSoft Ltd., 2012). Genetic distances (Nei, 1977) were calculated using the GenAlEx 6.5 program (Peakall and Smouse, 2012).

RESULTS

All five previously reported SNPs (C661A, T712C, C782T, C892T, and C1090T) were identified among sequences of 119 shrimp. Significant frequency differences were detected in

three loci for population Lv-C (Table 1). Linkage disequilibrium was observed only between C892T and C1090T ($P < 0.01$).

Table 1. Allele frequencies for five SNPs at the *Hsp70* gene in three *Litopenaeus vannamei* populations bred in Brazil.

SNP	Allele	Allele frequencies (N)		
		Lv-A	Lv-B	Lv-C
C661A	C	0.903	0.944	0.984*
	A	0.097	0.056	0.016*
		(36)	(45)	(32)
T712C	T	0.051	0.089	0.062
	C	0.949	0.911	0.938
		(39)	(45)	(32)
C782T	C	0.975	0.977	1.000
	T	0.025	0.023	0.000
		(40)	(44)	(32)
C892T	C	0.695	0.733	0.591**
	T	0.305	0.267	0.409**
		(41)	(45)	(33)
C1090T	C	0.829	0.944*	0.636**
	T	0.171	0.056*	0.364**
		(38)	(45)	(33)

Significant differences in allele frequencies: *P < 0.05; **P < 0.01. N = sample size.

The average observed heterozygosity was lower in populations Lv-A (H_O = 0.127) and Lv-B (H_O = 0.062) when compared with Lv-C (H_O = 0.243). The average expected heterozygosity ranged from 16.1% (± 1.3) to 21.9% (± 2.4). These mean heterozygosity estimates were inflated by the linkage between C892T and C1090T. However, observed heterozygosity values for Lv-C were higher for each of the individual loci (Table 2). Southern populations (Lv-A and Lv-B) were not in Hardy-Weinberg equilibrium ($P < 0.05$) in 60 and 100% of the tests, respectively (Table 2). F_{IS} values for population Lv-A and Lv-B were high, which might indicate intense inbreeding.

Table 2. Genetic diversity of SNPs presented in the *Hsp70* gene from three *Litopenaeus vannamei* populations bred in Brazil.

Population (N)		Locus				
		C661A	T712C	C782T	C892T	C1090T
Lv-A (41)	H_O	0.028	0.000	0.000	0.317	0.289
	H_E	0.176	0.097	0.049	0.424	0.284
	F_{IS}	0.842	1.000	1.000	0.252	-0.021
	HWE	0.000	0.000	0.000	0.107	0.898
	N	36	39	40	41	38
Lv-B (45)	H_O	0.022	0.044	0.000	0.178	0.067
	H_E	0.105	0.162	0.044	0.391	0.105
	F_{IS}	0.788	0.726	1.000	0.545	0.365
	HWE	0.000	0.000	0.000	0.000	0.014
	N	45	45	44	45	45
Lv-C (33)	H_O	0.031	0.125	0.000	0.697	0.364
	H_E	0.031	0.117	0.000	0.483	0.463
	F_{IS}	-0.016	-0.067	1.000	-0.442	0.214
	HWE	0.928	0.706	-	0.011	0.218
	N	32	32	32	33	33

H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding index; HWE, Hardy-Weinberg equilibrium probability; N, sample size.

Pairwise F_{ST} and G_{ST} estimates per loci varied widely (0.000 to 0.258) reflecting the distribution of allele frequencies among populations. The average values observed (F_{ST} = 0.042; G_{ST} = 0.031) were strongly influenced by the frequencies observed at loci C892T and C1090T, and the linkage between them. Considering this observation, F_{ST} and G_{ST} estimates should be taken only as an indication of relative genetic differentiation between the studied populations (Table 3). Likewise, the genetic distance (*D*) observed between populations was small, and suggested closer proximity between the Lv-A and Lv-B Southern populations when compared with Lv-C.

Table 3. Pairwise indexes of population differentiation (F_{ST}, G_{ST}) and genetic distance (*D*) among the studied populations.

Populations		F_{ST}	G_{ST}	*D*
Lv-A	Lv-B	**0.028**	0.001	0.004
Lv-A	Lv-C	**0.032**	**0.034**	0.014
Lv-B	Lv-C	**0.091**	**0.096**	0.029

Numbers in bold indicate F_{ST} and G_{ST} values significantly different from zero ($P < 0.05$).

The viral challenge resulted in 84.5% mortality after 15 days. However, no significant differences were observed in gene and genotype frequencies between the surviving and dead groups of shrimp, in any of the five scored SNPs ($P > 0.05$). No significant differences were observed between either of these groups and Lv-B, as well ($P > 0.05$).

DISCUSSION

Genetic diversity is important for shrimp breeding programs because it preserves the opportunity for genetic improvement. Nevertheless, variability loss in captive shrimp populations is frequently observed due to founder and inbreeding effects (Benzie, 2009; Cook et al., 2009; Andriantahina et al., 2013). The populations evaluated in this study showed evidence of different levels of genetic diversity, as revealed by *Hsp70* gene polymorphisms. Lower observed heterozygosity among SNPs from populations Lv-A and Lv-B (Southern Brazil) might be associated with higher inbreeding. On the other hand, the higher observed heterozygosity found in population Lv-C (Northeastern Brazil) was likely the result of a genetic program with controlled crossings. This genetic improvement program has pedigree control and focuses on IMNV resistance, which may have preserved genetic variability after successive generations in population Lv-C (Mello et al., 2011). Differences in founder pool and germplasm management were likely reasons for the significant allele frequency differences observed among the populations surveyed here. However, population Lv-C was under strong selective pressure for IMNV resistance; therefore, a possibility remains that some of the studied SNPs were correlated to this attribute.

The inbreeding index (F_{IS}) showed higher values in populations Lv-A and Lv-B as compared to population Lv-C. A moderate to high level of inbreeding seems to be common in Brazilian shrimp farming. Using microsatellites, Lima et al. (2008) reported F_{IS} ranging from 013 to 0.39 in *L. vannamei* from Bahia State (Northeastern Brazil). Other studies conducted with *L. vannamei* populations from Brazilian post-larvae production laboratories showed F_{IS} ranging from 0.00 to 0.38 (Luvesuto et al., 2007; Lima et al., 2010; Maggioni et al., 2013). Considering the history of geographical isolation, the higher F_{IS} values observed in populations Lv-A and Lv-B from Southern Brazil are likely related to inbreeding.

The index of population differentiation (F_{ST}) is an estimator of genetic differentiation in structured populations (Nei, 1977). Reliable F_{ST} estimates require at least random mating and allele frequency equilibrium at subpopulation level, conditions seldom met in aquaculture conditions. Nevertheless, F_{ST} has been widely used to evaluate genetic relationships among cultivated populations. For instance, low genetic differentiation (F_{ST} = 0.015) was reported among six post-larvae producers from Mexico (Perez-Enriquez et al., 2009). In Brazil, little (F_{ST} = 0.000) to moderate (F_{ST} = 0.175) differentiation was observed among nine post-larvae laboratories (Maggioni et al., 2013). In the present study, F_{ST} and G_{ST} values represented only an estimate of relative differentiation among populations. They suggested that populations Lv-A and Lv-B were genetically closer. This relationship was indicated by the values of genetic distance (*D*) as well. These results agreed with the history of the studied populations. Southern populations (Lv-A and Lv-B) are geographically far away from Lv-C. Populations Lv-A and Lv-B likely descend from a single founder stock, which was split during a severe WSSV outbreak. For at least 10 years, population Lv-A remained exposed to WSSV, while Lv-B was kept clean from this virus. Such scenario seems sufficient to explain the small genetic differences found and its distribution among the studied populations.

Association between *Hsp70* gene polymorphisms and resistance to TSV among Asian commercial *L. vannamei* populations has been reported (Zeng et al., 2008). In addition to that, induction of *Hsp70* gene transcription at 32°C during WSSV infection suggests a connection between this gene and WSSV resistance in shrimp (Lin et al., 2011). In the present study, we have challenged a virus-free population with WSSV, and no significant genetic differences were found between the dead and surviving groups. Therefore, no evidence was found here of any association between *Hsp70* SNPs and WSSV resistance. However, the significant differences found in population Lv-C, exposed to IMNV, remain to be explored.

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