Journal of Shellfish Research, Vol. 26, No. 4, 1247–1258, 2007.

CHARACTERIZATION AND MAPPING OF EXPRESSED SEQUENCE TAGS ISOLATED FROM A SUBTRACTED CDNA LIBRARY OF *LITOPENAEUS VANNAMEI* INJECTED WITH WHITE SPOT SYNDROME VIRUS

ACACIA ALCIVAR-WARREN,[1]* LINSHENG SONG,[2] DAWN MEEHAN-MEOLA,[1] ZHENKANG XU,[1,†] JIAN-HAI XIANG[2] AND WILLIAM WARREN[1]

[1]*Environmental and Comparative Genomics Section, Department of Environmental and Population Health, Cummings School of Veterinary Medicine at Tufts University, North Grafton, Massachusetts 01536;* [2]*Institute of Oceanology, Experimental Marine Biology Laboratory, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao, 266071, China*

ABSTRACT A large number of polymorphic simple sequence repeats (SSRs) or microsatellites are needed to develop a genetic map for shrimp. However, developing an SSR map is very time-consuming, expensive, and most SSRs are not specifically linked to gene loci of immediate interest. We report here on our strategy to develop polymorphic markers using expressed sequence tags (ESTs) by designing primers flanking single or multiple SSRs with three or more repeats. A subtracted cDNA library was prepared using RNA from specific pathogen-free (SPF) *Litopenaeus vannamei* juveniles (~1 g) collected before (0) and after (48 h) inoculation with the China isolate of white spot syndrome virus (WSSV). A total of 224 clones were sequenced, 194 of which were useful for homology comparisons against annotated genes in NCBI nonredundant (nr) and protein databases, providing 179 sequences encoded by nuclear DNA, 4 mitochondrial DNA, and 11 were similar to portions of WSSV genome. The nuclear sequences clustered in 43 groups, 11 of which were homologous to various ESTs of unknown function, 4 had no homology to any sequence, and 28 showed similarities to known genes of invertebrates and vertebrates, representatives of cellular metabolic processes such as calcium ion balance, cytoskeleton mRNAs, and protein synthesis. A few sequences were homologous to immune system-related (allergens) genes and two were similar to motifs of the sex-lethal gene of Drosophila. A large number of EST sequences were similar to domains of the EF-hand superfamily (Ca^{2+} binding motif and FRQ protein domain of myosin light chains). Single or multiple SSRs with three or more repeats were found in approximately 61% of the 179 nuclear sequences. Primer sets were designed from 28 sequences representing 19 known or putative genes and tested for polymorphism (EST-SSR marker) in a small test panel containing 16 individuals. Ten (53%) of the 19 putative or unknown function genes were polymorphic, 4 monomorphic, and 3 either failed to satisfactorily amplify genomic DNA or the allele amplification conditions need to be further optimized. Five polymorphic ESTs were genotyped with the entire reference mapping family, two of them (actin, accession #CX535973 and shrimp allergen arginine kinase, accession #CX535999) did not amplify with all offspring of the IRMF panel suggesting presence of null alleles, and three of them amplified in most of the IRMF offspring and were used for linkage analysis. EF-hand motif of myosin light chain (accession #CX535935) was placed in *ShrimpMap*'s linkage group 7, whereas ribosomal protein S5 (accession #CX535957) and troponin I (accession #CX535976) remained unassigned. Results indicate that (a) a large number of ESTs isolated from this cDNA library are similar to cytoskeleton mRNAs and may reflect a normal pathway of the cellular response after im infection with WSSV, and (b) primers flanking single or multiple SSRs with three or more repeats from shrimp ESTs could be an efficient approach to develop polymorphic markers useful for linkage mapping. Work is underway to map additional SSR-containing ESTs from this and other cDNA libraries as a plausible strategy to increase marker density in *ShrimpMap*.

KEY WORDS: *Litopenaeus vannamei,* White Spot Syndrome Virus, WSSV, linkage mapping, expressed sequence tags, ESTs, *ShrimpMap*, myosin light chain, E-F hand motif, actin, ribosomal proteins

INTRODUCTION

White spot syndrome (WSS) disease, caused by white spot syndrome virus (WSSV), affects most commercially important shrimp species and threatens the shrimp farming industry worldwide causing widespread morbidity and mortality in postlarval, juvenile, and adult stages of many penaeid species common to aquaculture (Lightner 1996, Flegel et al. 1997, OIE 2007). WSSV is a double-stranded DNA nonoccluded virus that belongs to a new family of viruses (*Nimaviridae*) infecting crustaceans (Lightner 1996, van Hulten et al. 2001, Yang et al. 2001, Marks et al. 2003). WSSV often causes mortality in the infected animals within 48 h under laboratory conditions and within a few days under field conditions and different WSSV isolates have been reported (Cesar et al. 1998, Wongteerasupaya et al. 2003). Infected animals in commercial ponds show a reddish discoloration and lethargy and occasionally, white spots in the exoskeleton (Rodríguez et al. 2003). As there is no treatment for WSS, efforts focus on understanding the response of the shrimp immune system to laboratory challenges with WSSV and examine the mechanisms of resistance to and pathogenesis of WSSV (Rojtinnakorn et al. 2002, Astrofsky et al. 2002, Leu et al. 2007). A maternal component for resistance to WSSV has been postulated (Huang & Song 1999) and resistant (''immune'') *P. japonicus* has been reported after subsequent challenge with WSSV (Wu et al. 2002, Wu & Muroga 2004). Protection of *P. monodon* against WSSV has also been reported by oral vaccination with food pellets coated with inactivated bacteria over expressing WSSV envelope protein VP28 (Witteveldt et al. 2004). An antiviral *P. monodon* gene, *PmAV*, has also been identified encoding for a 170 amino acid peptide

*Corresponding author. E-mail: acacia.warren@tufts.edu

†Current address: Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204

with a C-type lectin-like domain (CTLD) (Luo et al. 2003). The above results indicate that both innate and adaptive immunity play a critical role in WSSV infection and suggests that selection for WSSV-resistance is possible. To date however, no heritability data for resistance to WSSV has been published.

In 1995, efforts to develop WSSV-resistant specific pathogen-free (SPF) stocks were initiated at the breeding program maintained by the United States Marine Shrimp Farming Program (USMSFP) at the Oceanic Institute in Oahu, HI. These efforts, however, have been mostly unsuccessful. As disease resistance traits are generally of low heritability, and are expensive to measure, it may be more efficient to breed for these traits with marker-assisted selection (MAS) than through traditional selective breeding. Breeding for WSSV resistance would be more efficient with MAS or through marker-assisted breeding. However, specific markers for WSSV resistance have yet to be identified. In shrimp, efforts have been made to find associations between randomly amplified polymorphic DNA (RAPD) markers and susceptibility to viral diseases (Alcivar-Warren et al. 1997, Alcivar-Warren et al. 2002 and references therein) but dominant RAPD markers are not useful for pedigree tracing purposes. A better approach to understand the genetic mechanisms of WSSV resistance, viral pathogenesis, host immune response, host-virus interactions and epigenetic control of gene expression, would be to identify candidate genes or quantitative trait loci (QTL) responsible for this trait. This could be done using a combination of genomics tools such as linkage mapping, cDNA and/or protein microarrays and appropriate resource mapping families. Some differentially expressed genes have already been identified in *P. stylirostris* infected with WSSV by cDNA microarrays (Dhar et al. 2003) and WSSV-infected *Penaeus monodon* (Tong et al. 2002, Leu et al. 2007). In addition, antiviral substances that bind both DNA and RNA viruses have been isolated in *P. setiferus* shrimp (Pan et al. 2000, Pan et al. 2005, He et al. 2004) and a B-1-3-glucan-binding protein has been proposed as an inducible acute phase protein that regulates the activation and/or activity of the prophenoloxidase cascade (Roux et al. 2002).

To identify candidate QTL(s) for resistance to diseases, candidate genes and/or a highly saturated linkage map for shrimp are needed. Linkage maps based on dominant Type II markers such as amplified length polymorphism (AFLP) technique have been reported for *P. japonicus* (Moore et al. 1999), *P. monodon* (Wilson et al. 2002) and *L. vannamei* (Perez et al. 2005a, Zhang et al. 2007), but AFLP maps are not transferables to other species or even families of the same species. The *L. vannamei* map of Zhang et al. (2007) also included some microsatellites, most of which were developed at our laboratory (Meehan et al. 2003). A large number of highly polymorphic, codominant markers such as simple sequence repeats (SSRs) or microsatellites are needed to develop a highly saturated genetic map for shrimp. Our initial effort to construct a linkage map for SPF *L. vannamei* (*ShrimpMap*) focused on microsatellites isolated from genomic libraries (Alcivar-Warren et al. 2002, Meehan et al. 2003, Alcivar-Warren et al. 2006, Garcia & Alcivar-Warren 2007). However, marker development is very time-consuming, inefficient (only ~15% of sequences are polymorphic), expensive, some of them may correspond to the same transposable elements (Alcivar-Warren et al. 2006, Alcivar-Warren et al. 2007), and most markers are not specifically linked to gene loci of immediate economic interest to the shrimp aquaculture community such as resistance to pathogens, high survival and growth and cold tolerance. To increase marker density of *ShrimpMap*, a pilot project was initiated to determine usability of ESTs to develop gene-associated polymorphic markers from SPF *L. vannamei*. The specific aims of the study were (1) to isolate and characterize ESTs from a subtracted cDNA library of control and experimental shrimp injected with the China isolate of WSSV, (2) to identify polymorphic ESTs by designing primers flanking single or multiple SSRs with three or more repeats, and (3) to determine usability of the ESTs in linkage mapping. We report here our strategy to develop polymorphic ESTs that could increase density of *ShrimpMap* based on a strategy that relies on designing primers flanking single or multiple SSRs with three or more repeats.

MATERIALS AND METHODS

Experimental Animals

Cultured SPF *L. vannamei* from the USMSFP's breeding program maintained at the Oceanic Institute (OI) in Oahu, HI were used in this study (Carr et al. 1994, Carr et al. 1997, Lotz 1997). Juveniles from each of 60 *L. vannamei* family crosses (Batch #2) were used to examine the possibility of developing WSSV-resistant stocks. Shrimp were shipped from OI to the quarantine facility at the University of Arizona (UAZ, Dr. Donald Lightner's laboratory) where they were acclimated before challenging by injection with the China isolate of WSSV. Approximately 590 juveniles (~10 per cross) were inoculated with WSSV and 600 (~10 per cross) were noninjected controls. Juveniles (~2.5–3.8 g) were inoculated with WSSV (day 0) and checked three times daily for eight days until termination of the challenge study. Dead and moribund animals were collected during these observation periods, animals were typed as to family cross, weighed, and ~0.5–1.0 g tail muscle was collected and placed into tubes containing 100% cold, molecular biology grade, ethyl alcohol. Because many animals died between observation periods, most of the infected animals were dead on collection. Only animals that could be typed as to family cross were collected. The WSSV-challenged tissue samples were maintained frozen at –20°C until shipped to Tufts' laboratory. The uninjected siblings were collected on days 7 and 8 of the challenge study. All control animals were alive at collection and the whole animal was stored frozen in whirl pack bags and maintained at –70°C. All samples were shipped to Tufts laboratory in dry ice and stored at –70°C until total RNA was isolated. Histological analysis to confirm presence of WSSV in selected shrimp was performed at UAZ following established protocols (Lightner 1996).

RNA Isolation and Cloning of the cDNA Library

Total RNA was isolated following a modified guanidine isothiocyanate-based protocol (Garcia et al. 1996, Alcivar-Warren et al. 1997). Pooled tissues from six animals of a family cross (Cross #30) with the largest number of dead shrimp (7/10) 48 h post WSSV inoculation, were used for RNA isolation. Good quality RNA from these six WSSV-injected (''tester'') and six uninjected control siblings (''driver'') were used for mRNA purification and cDNA library construction. Poly(A)$^+$ RNA was purified using oligo(dT) cellulose columns (Gibco, BRL). The CLONTECH PCR-Select cDNA subtraction kit

(K1804-1, CLONTECH, Palo Alto, CA) was used for constructing the subtracted cDNA library. Approximately 300 μg total RNA was obtained from each the WSSV-injected juveniles (''tester'') and uninjected siblings (''driver''), from which ~3.1 μg and ~2.5 μg of poly(A)$^+$ mRNA was obtained from tester and driver shrimp RNA, respectively, using GenElute mRNA Miniprep kit (Sigma, St. Louis, MO). Two μg mRNA were used as template for cDNA synthesis and subtractive hybridization following the kit manufacturer's instructions. Amplified cDNA fragments were ligated to PCR-Trap vector and transformation performed using GH-competent cells. Transformed cells were plated onto agar plates containing LB-tetracycline and X-gal. Positive recombinants were placed in colony lysis buffer, spun for 2 min at room temperature and supernatant used for PCR reaction using Lgh and Rgh primers following manufacturer's instructions. PCR products were run in a 2% agarose gel to check for clone sizes before sequencing.

DNA Sequencing and BLAST Analysis

Recombinant clones were sequenced at Tufts University DNA Sequencing facility in Boston, MA. Sequence comparisons were performed by querying them against the National Center of Biotechnology Information (NCBI) nonredundant (nr) and EST databases of Blastn and Blastx. Output files were created for easy access to individual sequence information and automatic programming established in our shrimp EST database (*ShrimpESTbase*) so that it will continuously update new homologous genes and other known sequences. The statistical significance of DNA sequence homologies was based on reported cut-off values of E value [E()] < 0.001 (Pearson 2000). All motifs with 3 or more repeats were counted as SSRs (Meehan et al. 2003). These researchers reported amplification of 51 out of 93 polymorphic SSRs that contained single or multiple motifs of less than 6 repeats each, greatly increasing the number of useful markers.

Primer Design, Microsatellite Amplification and Scoring, Polymorphism Analysis

The Primer3 program (Rozen & Skaletsky 2002), as well as visual editing, was used to design primer sets flanking one or more motifs within a clone. Primer sets chosen were based on the uniqueness of sequences and percentage of GC content. Oligonucleotide primers were synthesized by Integrated DNA Technology Inc. (IDT, Coraville, IA) and used to amplify alleles with DNA (100 ng) from a small test panel of 16 SPF *L. vannamei* individuals from the reference and resource mapping families of *ShrimpMap*. Initially, 32 SSR-containing sequences were selected for primer design (many with no similarities to any sequence in the GenBank database) and all primers were tested. However, after bioinformatics and updated search capability was established, some of these primers were found to be part of the same sequence cluster and four were deleted from further analysis. The remaining 28 primers were designed from 24 EST sequences representing 19 putative or unknown function genes (Table 1).

Polymerase chain reaction (PCR) was performed in a 25-μL reaction volume containing 100 ng DNA, 7.5 ng of γ-^{32}P-ATP labeled reverse primer, 50 ng of forward primer, 2.0 mM Mg Cl_2, 0.2 mM of dNTPs, 2.5 units of *Taq* polymerase (Promega, WI), and 1X buffer. The Thermal cycler (PTC-100, MJ Research, MA) profile was: 94°C for 3 min, followed by 21 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. (Wolfus et al. 1997, Meehan et al. 2003). Polyacrylamide gel electrophoresis of amplified products was performed using standard laboratory procedures. Samples were run next to a known sequence (*B20* locus) (Garcia et al. 1996) to provide estimates of allele sizes. Some primer sets did not amplify DNA well at the chosen 52°C annealing temperature and their amplification conditions were optimized by varying the annealing temperature conditions at 44°C, 48°C, 52°C, and 56°C. A microsatellite was regarded as polymorphic when the frequency of the most common allele was equal to or less than 0.99 (Nei 1987).

Linkage Mapping

Once polymorphic ESTs were identified using the small test panel, they were then used for genotyping with the entire reference mapping panel following a standard laboratory protocol, modified from the user's manual of ABI PRISM 377 DNA sequencer. PCR mixture consisted of 0.3 μM (20 ng) template DNA, 0.333 μM reverse primer (fluorescently labeled with 6-FAM, TET or HEX), 0.333 μM forward primer, 0.125 mM dNTPs, 0.04 U/μL Taq polymerase (Promega), 2.5 mM MgCl2, and × 1 buffer in a total of volume of 15 μL. The PCR profile using a MJ Research thermocycler PTC-100 was: 95°C for 12 min. followed by 30 cycles of 94°C for 1 min annealing temperature for 1 min 72°C for 2 min and ending with 72°C for 30 min. The amplified products were then multiplexed by combining in a total volume of HEX-, TET-, and 6-FAM-labeled PCR products in different ratios for best amplification results. Three microliters of loading mix (ABI Prism 377 DNA sequencing manual) and 1 μL of the multiplexed PCR product were combined and used in loading the gel. After the ABI run was completed, GeneScan Analysis Software processed the gel image. This was also manually checked to make sure all lanes used in the gel line up properly and the size standard being used is applied appropriately. Once the gel image was processed, the information was exported into GenoTyper Software, which assigns allele sizes (base pairs, bp) to the amplified product in each lane based on the size standard used. To avoid inaccuracy in scoring among different gels, a control DNA sample provided by ABI of known genotype was included in each set of samples for each gel. All genotypic results were compiled in an excel sheet and checked manually for different types of genotyping errors. The allele sizes were reviewed by two researchers, and the allele data obtained from the entire reference mapping panel was analyzed with CRIMAP software using a LOD score of 3.0 to identify linkage groups and determine marker order.

RESULTS AND DISCUSSION

Survival of L. vannamei *after WSSV Injection*

WSSV-injected animals succumbed to infection starting day 2 postinjection. All but 2 of the 590 shrimp injected with WSSV were dead 8 days post inoculation (B. Poulus & D. Lightner, pers. obser.). None of the uninjected control animals died during the 8 days of the challenge study. Histology results from selected WSSV-injected shrimp confirmed WSSV infection as

TABLE 1.

Simple sequence repeats (SSRs) identified in expressed sequence tags (ESTs) isolated from a subtracted cDNA library using RNA from White Spot Syndrome Virus (WSSV)-injected and control SPF *Litopenaeus vannamei*.

Cellular Metabolic Process /putative or Known Genes	# of Clones[a]	SSR Motifs[b]; (*n* = # of clones)
Cytoskeleton mRNAs		
Actin	30	$(CAC)_3$, $(AAGG)_4$, $(TG/AC)_3$, $(CT/AG)_3$, $(AAGG)_3$, $(AGC)_3$, $(GC)_3$, […$(GC)_3$…$(CA)_3$…], […$(CAC)_3$…$(CA)_3$..], [..$(TG)_3$…$(TG)_3$..$(AAGG)_3$…$(AGC)_3$…], […$(AAGG)_3$…$(AGC)_3$…$(GGT)_3$…]; ($n = 22$)
Tropomyosin (shrimp allergen, involved in actin-binding and muscle contraction)	2	[…$(CT)_3$…$(CT)_3$…], […$(TC)_3$…$(AT)_3$…$(AT)_3$…]; ($n = 2$)
Troponin C	1	$(GA)_6G(GA)_5$…(AG)3… ($n = 1$)
Troponin I (similar to Drosophila wupA, has actin-binding activity)	12	$(TA)_3$, […$(TA)_3$…$(GAC)_3$…$(TCT)_3$…$(CTT)_3$…], […$(CTT)_3$…$(CTT)_3$…$(CTT)_3$…], […$(TCT)_3$…$(CTT)_3$…]; ($n = 11$)
Myosin heavy chain – fast	4	ND
Spectrin repeats[c] – Muscle specific protein 300 (actin-binding activity)	2	[$(TG)_3$…$(CTT)_3$..], ($n = 1$)
Spectrin repeats, Actinin alpha (flightless-A, F-actin cross-linking protein)	1	…$(CTT)_3$..], ($n = 1$)
Calcium ion balance (also cytoskeleton mRNAs)		
Partial homology to putative orthologs of Myosin light chains (MLC1, MLC2, myosin regulatory light chain) – some include EF-hand Ca2 + binding motif.[d]	3	$(GA)_3$, […$(AG)_3$…$(TCT)_3$…], […$(TGA)_3$ATG$(GA)_3$…$(GA)_4$AGTA$(AGC)_3$…$(GCT)_3$…]; ($n = 3$)
ESTs similar to SAL096′3 – with similarities to portion of EF-hand calcium binding motif of MLC2	17	$(TG)_3$, $(GTCA)_4$, […$(AC)_3$…$(GTCA)_4$…], […$(AC)_3$…$(GTCA)_4$…]; ($n = 15$)
Similar to TUASPvWSu233 and 45 other ESTs – some containing the FRQ1 protein domain (EF-hand superfamily) of MLC2.[e]	46	$(TCA)_3$, [$(TC)_3$…$(TCA)_3$…], […$(TGA)_3$ATG$(GA)_3$…], […$(TGC)_3$TTACT$(TC)_3$ …$(TCA)_3$…], [$(TGA)_3$ATG$(GA)_3$…$(GA)_4$AGTA$(AGC)_3$…]; ($n = 11$)
Sarco/endoplasmic reticulum calcium-transporting ATPase	2	[…$(CT/AG)_3$…$(AC/GT)_3$..], ($n = 2$)
Sarcoplasmic calcium-binding protein (SCP1) – contains 4 EF-hand domains	4	$(ATA)_3$, ($n = 1$)
Protein synthesis		
28s rRNA	1	ND
60S ribosomal protein L27	4	(CAC)3; ($n = 4$)
60S ribosomal protein L32	1	ND
60S ribosomal protein L10 (Wilms tumor suppression homolog)	1	ND
60S acidic ribosomal protein P2	1	$(TGG)_5$; ($n = 1$)
40S ribosomal protein S4	1	ND
40S ribosomal protein S5	1	$(TC)_3$; ($n = 1$)
40S ribosomal protein S10	5	$(CT)_3$, $(AGT)_3$; ($n = 3$)
40S ribosomal protein S19	1	ND
40S ribosomal protein S20	1	ND
40S ribosomal protein S23	3	ND
40S ribosomal protein S24 (gene is mutated in Dimond-Blackfan anemia)	3	$(AAG)_4$, […$(CTT)_4$…$(GT)_3$..]; ($n = 3$)
Immune-related		
Arginine kinase (allergen, a putative actin-binding domain, with energy regulatory and transport properties)	7	$(TA)_3$, $(TC)_3$, […$(GC)_3$…$(GA)_3$…], […$(AGG)_3$…$(TG)_3$…], […$(TA)_3$…$(CA)_3$…$(CA)_3$…], […$(TA)_3$…$(CA)_3$…$(CCT)_3$…]; ($n = 6$)
Heat shock protein 27	1	[…$(GGA)_3$…$(GA)_3$…$(ACA)_3$…]; ($n = 1$)
Nucleoside diphosphate kinase (putative oncoprotein nm23)	1	[…$(CT)_3$…$(GA)_3$…$(CA)_3$…]; ($n = 1$)
Metabolism		
Pyruvate kinase	1	$(CA)_3$; ($n = 1$)

continued on next page

TABLE 1.

continued

Cellular Metabolic Process /putative or Known Genes	# of Clones[a]	SSR Motifs[b]; (*n* = # of clones)
Aspartic rich protein aspolin1–2	3	[..ATA)$_3$...(TCA)$_4$...(TCA)$_3$C(CAT)$_3$...(TCA)$_4$C(CAT)$_3$...], [...(TGA)$_5$C(GAT)$_6$(GAC)$_3$AT(TGA)$_3$...(TGA)$_3$...], [...(GTC)$_3$...(GTC)$_6$...(CGT)$_3$(ATC)$_5$ ...(TCA)$_3$CCA(TCA)$_3$AT(GTC)$_3$(ATC)$_6$G(TCA)$_5$...]; (*n* = 3)
Homologous to motifs of sex-lethal gene	2	[...(GGT)$_3$...(GCG)$_3$...], [...(GGT)$_3$...(GCG)$_3$...(GGC)$_3$...]; (*n* = 2)
Other ESTs of unknown function	15	(CT/AG)$_4$, (AT)$_3$, (GT/AC)$_3$, (CG)$_3$, [(CG)$_3$...(GA)$_3$...(TGA)$_3$...], [...(TC)$_3$CT(TC)$_3$TTC(CT)$_3$...(TG)$_4$...(AC)$_5$...], [...(GA)$_5$...(GA)$_3$...(AG)$_5$...(AG)$_6$...], [...(CT)$_6$...(CT)$_5$...(TC)$_3$...(TTC)$_3$...]; (*n* = 12)
No significant homology to any gene	3	(GCC)$_3$, [...(AG)$_3$...(TC)$_4$...], [...(AG)$_4$...(TCT)$_3$...]; (*n* = 3)
Total number of clones	179	(*n* = 111)

[a] There are nucleotide differences among some clones and may be candidate single nucleotide polymorphisms (SNPs). ND = not present.
[b] Examples of repeat motifs present in ESTs. Some of the repeat motifs were present singly () or in combination [...] within a sequence.
[c] Spectrin repeats are found in several proteins involved in cytoskeletal structure, family members include spectrin, alpha-actinin and dystrophin.
[d] The EF-hand superfamily represents a family of calcium sensors & calcium signal modulators involved in signal transduction mechanisms, cytoskeleton, and cell division.
[e] TUASPvWSu233 is similar to other shrimp ESTs such as est_l_vannamei10501 (CV468710), est_l_vannamei1730 CK768263) and others in the GenBank database. Su233 is similar to EF-hand Ca2 + binding motif (includes. TUASPvWSu233 is TUASPvWSu073, which is similar to EF-hand calcium-binding motif of myosin light chain.

evidenced by lesions in the gills, appendages, stomach, antennal gland, heart, and lymphoid organs. The results suggested that the initial founder stock (Batch #2) of SPF *L. vannamei* is highly susceptible to WSSV, and that WSSV-resistant stocks could not be developed with the allele diversity present in the germplasm of the USMSFP's breeding program. It is possible that the allele diversity of the founder stocks and subsequent selective pressure to breed for high growth (Wyban et al. 1993, Carr et al. 1994, Carr et al. 1997) before selecting for TSV-resistance (Moss et al. 1999, Argue et al. 2002, Argue & Alcivar-Warren 1999), may have contributed to the inability to develop SPF *L. vannamei* resistant to WSSV and suggest that additional allele diversity should be incorporated in the germplasm of the USMSFP breeding program to develop WSSV-resistant lines.

Characterization of Expressed Sequence Tags (ESTs) from a Subtracted cDNA Library

Out of the 818 plasmids isolated from the subtracted library, 216 clones were selected for sequencing based on the estimated length of the clones (~250–580 bp), and 194 of these (GenBank accession numbers CX535808-CX536001) were useful for homology comparisons against annotated genes in NCBI non-redundant (nr) and protein databases. The remaining 22 sequences were either too short (<50 nucleotides), contained too many 'N's or had no insert.

Homology searches of the 194 sequences identified 179 nuclear-encoded sequences, 4 mitochondrial DNA-encoded (three 16s rRNA and one cytochrome oxidase I clone), and 11 corresponded to portions of WSSV genome. Most ($n = 145$) of the 179 nuclear DNA-encoded sequences were representative of cellular metabolic processes such as cytoskeleton mRNAs ($n = 52$), calcium ion balance ($n = 70$), and protein synthesis ($n = 23$). The remaining ESTs were similar to immune-related genes ($n = 9$), metabolic genes ($n = 4$; 1 pyruvate kinase and 3 aspartic rich protein aspolin 1–2), other ESTs of unknown function ($n = 18$; 10 from shrimp ESTs, 5 from other species ESTs, 3 from hypothetical or unknown proteins), homologs of sex lethal gene motifs ($n = 2$), and no homology to any sequence in the database ($n = 2$). Similar results on cellular metabolic processes likely to be affected after WSSV infection of *P. monodon* included oxidative phosphorylation, protein synthesis, glucolytic pathway and calcium ion balance (Leu et al. 2007), but a large number of immune-related genes in *L. vannamei* were reported by Zhao et al. (2007).

ESTs Similar to Cytoskeleton mRNAs

Of the 52 cytoskeleton mRNAs, 30 were similar to actin genes, 12 troponin I, 1 troponin C, 2 tropomyosin, 4 myosin heavy chain, 2 spectrin repeats of muscle specific protein 300, and 1 spectrin repeats of actinin alpha gene). The large number of ESTs with homology to cytoskeleton mRNAs identified here may reflect a normal pathway of the cellular response after infection with WSSV and other viruses such as TSV (Alcivar-Warren & Warren 2005, Cevallos & Sarnow 2005). In other species, infection with poliovirus -but not with vesicular stomatitis virus- elicited a cellular response of shutting-off host protein synthesis by a unique mechanism that lead to the release of host mRNAs (e.g., actin) from the cytoskeletal framework and inhibition of their translation (Bonneau et al. 1985). It is

possible that WSSV also causes cells to inhibit host protein synthesis and release cytoplasmic structural genes such as actin and EF-hand binding motifs of myosin light chains, among others. In addition, genes coding for proteins of the sarcomere (beta- and alpha-myosin heavy chain, troponin C, alpha tropomyosin, myosin binding proteins), which have important regulatory functions in animal biology and familial hyperthrophic cardiomyopathy in human (Ramirez & Padron 2004), may also have important regulatory functions in shrimp biology. Alpha and beta actins were also identified among eight genes (out of 4,133 genes examined in a DNA microarray) that are differentially expressed between normal uterine and ectopic endometrium (Eyster et al. 2002).

Of the 72 ESTs associated with calcium ion balance, 3 showed partial homology to putative orthologs of Myosin light chains (MLC1, MLC2, myosin regulatory chain), 46 were similar to clone TUASPvWSu233 (accession # CX535935) -some of which contained the FRQ1 protein domain (EF-hand superfamily) of myosin light chain 2 (MLC2), 17 were similar to SAL096′3 –with similarities to portion of EF-hand calcium binding motif of MLC2, 2 were similar to sarco/endoplasmic reticulum calcium-transporting ATPase, and 4 were similar to sarcoplasmic calcium binding protein 1 (SCP1) which contains four EF-hand domains. The abundance of transcripts associated with the EF-hand calcium-binding motif of myosin light chains may relate to the tissue's site of WSSV injection. Myosins are involved in muscle contraction, and calcium signaling, one of the most widespread signaling mechanisms in cells, is generally carried out by EF-hand proteins. These proteins are characterized by a helix-loop-helix motif paired in functional domains, and are considered molecular switches activated by calcium concentration transients (Capozzi et al. 2006).

The 179 nuclear DNA-encoded sequences represented various clusters of genes including structural genes involved in cytoskeletal cell structure and motility, protein synthesis, and allergens. The category of the genes identified in this study may reflect either the specificity of the major tissue (muscle) used to prepare the library, route of inoculation (intramuscular injection), age/weight of shrimp, or time of sample collection (48 h after WSSV inoculation). Some of our sequences are similar to those reported from WSSV-challenged *P. stylirostris* using mRNA differential display technique (Astrofsky et al. 2002). These researchers found that 6 of 32 unique differentially expressed had similarities to shrimp arginine kinase, mitochondrial ATPase, other shrimp ESTs of unknown function and WSSV ORF116.

ESTs with Homology to Genes Involved in Protein Synthesis

A total of 23 EST sequences with similarity to genes involved in the protein synthesis pathway were isolated from this cDNA library, including 28s rRNA, translation initiation factor, seven subunits of 40S ribosomal proteins (S4, S5, S10, S19, S20, S23, S24), three subunits of 60S ribosomal proteins [L10, L27, L32], and acidic ribosomal protein P2. The biological significance of these ribosomal genes in WSSV-infected shrimp is unknown. In addition to being identified in healthy shrimp, ribosomal proteins 28S rRNA and 40S ribosomal protein SA have been reported in WSSV-infected *P. stylirostris* (Dhar et al. 2003). Ribosomal proteins S10 and S24 were also reported in WSSV-infected *P. monodon* (Phongdara & Rattanahirankul, GenBank database) and *P. japonicus* (Rojtinnakorn et al. 2002).

Though ribosomal proteins are known to be involved in protein synthesis and are highly conserved, they are also involved in other unique biological functions. For instance, S3 functions as both a ribosomal protein and as endonuclease in mammals and *Drosophila* (Kenmochi et al. 1998); S5 interacts with the internal ribosomal entry site of hepatitis C virus (Fukushi et al. 2001); S5 and other ribosomal proteins (S2, S3, S6, S13, L9, L14, L19) are involved in the *Minute* phenotype, and a correlation between S3 mRNA levels and the severity of the *Minute* phenotype was identified in which faulty differentiation of somatic tissues and arrest of gametogenesis represented the extreme case (Sæbøe-Larssen et al. 1998). S5 RNA expression also changes in differentiation and cell death (apoptosis) of murine erythroleukemia cells (Vizirianakis et al. 1999); S6 functions as a tumor suppressor in the hematopoietic system; S10 functions as a minor allergen; S23 is differentially expressed in uterine and ectopic endometrium (Eyster et al. 2002); S18, L3, and L8 have been identified as ovarian tumor antigens (Luo et al. 2002); and S18 and S26 are involved in the immune response of mosquito *Anopheles gambiae* after treatment with bacterial lipopolysaccharide (Oduol et al. 2000). In addition, L3 is both a tumor antigen (Luo et al. 2002) and a positive regulator of cell division (Popescu & Tumer 2004); L10 (similar to QM protein) is a putative tumor suppressor (Chavez-Rios et al. 2003); and expression of L10, L32 and S16 has been correlated with progression of human prostate cancer by cDNA microarrays (Karan et al. 2002). Considering that the entire repertoire of ribosomal proteins in eukaryotes consists of approximately 80 protein genes (Kenmochi et al. 1998), we hypothesize that the few ribosomal proteins identified in this study could have an important role in regulating the translational machinery in shrimp after WSSV infection. Also, it is possible that mutations in ribosomal proteins could directly perturb diverse cellular functions in shrimp, without affecting translation, and thereby produce disease.

ESTs with Homology to Immune-related Genes

Very few known immune-related genes were found in this cDNA library, and 7 of them were similar to arginine kinase, a shrimp allergens, 1 was similar to heat shock protein 27 and 1 similar to nuleoside diphosphate kinase. This low number of immune-related genes may relate to the tissue (mostly muscle) used to prepare the cDNA library, the site of virus injection (intramuscular), and genetic background of the host (SPF shrimp originated from the WSSV-susceptible, Kona Line of the USMSFP). The large number of immune response genes reported in other shrimp studies originated from cDNA libraries prepared after *per os* challenge with WSSV and isolated from hemocytes of adult *M. japonicus* (Rojtinnakorn et al. 2002, He et al. 2004) and *P. monodon* (Supungul et al. 2002), hepatopancreas of WSSV-challenged *P. stylirostris* (Dhar et al. 2003) and WSSV-challenged *M. japonicus* (Pan et al. 2005), and gills (Claveros-Salas et al. 2007) and hepatopancreas (Zhao et al. 2007) of WSSV-injected *L. vannamei*. Hemocytes and hepatopancreas are the tissues involved in the shrimp humoral defense response (Rodriguez et al. 1995, van de Braak et al. 2002) and are expected to contain large number of immune-related genes in response to WSSV infection, as reported in hemocytes of

WSSV-resistant *M. japonicus* (He et al. 2004) and hepatopancreas of WSSV-resistant *L. vannamei* (Zhao et al. 2007). Robalino et al. (2006) also reported up-regulation in the hepatopancreas of *L. vannamei* of genes encoding known and potential antimicrobial effectors, whereas some genes involved in protection from oxidative stress were found to be down-regulated by the virus.

ESTs Similar to Allergen Genes

Crustaceans are a major cause of seafood allergy in humans. In this study, we identified two major shrimp allergens (tropomyosin and arginine kinase) and two minor human allergens (60S acidic ribosomal protein P1/P2 and ribosomal protein S10). The biological significance of these allergens on shrimp pathology is unknown at this time, but may reflect changes in levels of expression of these genes in shrimp muscle development.

Two EST sequences similar to muscle protein tropomyosin were found in this library. Tropomyosin has been identified as the major seafood allergen in shrimp (Shanti et al. 1993), American lobster (Leung et al. 1998) and other crustaceans, and has been recognized by IgE from patients with crustacean allergens.

The seven arginine kinase sequences identified in this library were very similar to the arginine kinase of *P. monodon* (Pen m 2), an allergen with a putative actin-binding domain (Yu et al. 2003). Arginine kinase Pen m2 is similar to other food allergens such as plant profilings, which are involved in actin binding; the iron transport protein allergen from egg white; animal serum albumins; the fish parvalbumin allergen, which has calcium binding properties; and shellfish tropomyosin, which is involved in actin binding and muscle contraction (Yu et al. 2003, and references therein). Arginine kinase catalyzes the reversible transfer of the high-energy phosphoryl group from ATP to arginine, yielding ADP and *N*-phosphoarginine (Yu et al. 2003). Phosphoarginine is commonly referred to as a phosphagen and represents an intermediate storage and transport form of energy in a wide variety of invertebrates. Therefore, arginine kinase is a major food allergen with potential regulatory and/or transport properties in shrimp, but is unclear what the relationship is, if any, with WSSV infection. Arginine kinase has also been reported in WSSV-injected *P. stylirostris* (Astrofsky et al. 2002) and a large number of arginine kinase sequences were also identified in SPF shrimp of TSV-R and TSV-S (Kona) lines challenged both *per os* and in a waterborne assay with Taura Syndrome Virus (Alcivar-Warren et al. unpublished).

There was a single EST sequence for acidic ribosomal protein P2, which is also considered a minor allergen (Francoeur et al. 1985). The acidic phosphoproteins of the large ribosomal subunit, designated P1/P2(L40/L41), function in the elongation step of protein synthesis along with two small rRNP antigens (Francoeur et al. 1985). The human protein has been identified as an autoantigen, with ~20% of patients with systemic lupus erythematosus and with antiSm autoantibodies synthesizing autoantibodies called antirRNP, to components of the ribosome (Hasegawa et al. 1999). P2 is also considered a potential molecular target for antisense therapy of human malignancies (Gardner-Thorpe et al. 2003). Antibody activity against ribosomal protein S10 was also reported in antiSm sera from patients with systemic lupus.

The ESTs reported here add to the large number of ESTs being generated for *L. vannamei* (Gross et al. 2001, Claveros-Salas et al. 2007, Zhao et al. 2007), *P. monodon* (Lehnert et al. 1999, Whan et al. 2000, Tong et al. 2002, de la Vega et al. 2007), *P. setiferus* (Gross et al. 2001), *P. stylirostris* (Dhar et al. 2003, de Lorgeril et al. 2005), *Marsupenaeus japonicus* (Rojtinnakorn et al. 2002, He et al. 2004, Pan et al. 2005, Yamano & Unuma 2006), and *Fenneropenaeus chinensis* (Wang et al. 2005).

Simple Sequence Repeats in Shrimp ESTs

Approximately 61% ($n = 111$) of the 179 nuclear ESTs contained single or multiple SSRs with three or more repeat motifs with enough flanking sequences to design primers. This percent cannot be compared with the 3.8% of repeat motifs found in the evaluated ESTs of Perez et al. (2005b) because many of our motifs may correspond to the same known or putative genes identified in this library (see section below). There was also variability in the motif sequences, number and length of motifs within a sequence and number of clones with SSR motifs from each gene (Table 1). For instance, 22 of the 30 ESTs with homology to actin gene contained single or multiple SSRs with three or more repeats, with individual sequences containing either single repeat motifs of variable length such as $(CAC)_3$, $(AAGG)_4$, $(TG/AC)_3$, $(CT/AG)_3$, $(AAGG)_3$, $(AGC)_3$, $(GC)_3$ or a combination of repeats within a sequence such as $[\ldots(GC)_3\ldots(CA)_3\ldots]$, $[\ldots(CAC)_3\ldots(CA)_3..]$, $[..(TG)_3\ldots(TG)_3..(AAGG)_3\ldots(AGC)_3\ldots]$, $[\ldots(AAGG)_3\ldots(AGC)_3\ldots(GGT)_3\ldots]$, among others (Table 1). This diversity in SSR arrays for a single gene may reflect the genetic variation within the pooled SPF juveniles used in the virus challenge and used to construct the library.

Polymorphism Analysis

Oligonucleotide primer sets were designed from 28 sequences representing 19 putative genes or unknown function genes, and tested for polymorphism in the small test panel of 16 *L. vannamei* individuals our reference and resource mapping families (Table 2). Two primer sets were designed from some of these sequences (Table 2). Results showed that 21 (75%) out of the primer sets amplified products. Ten (36%) of the 28 primers were polymorphic, 11 monomorphic and 7 either failed to satisfactorily amplify genomic DNA or the allele amplification conditions need to be further optimized. This percentage is similar to the ~30% reported for *P. monodon* ESTs (Tong et al. 2002). The ten polymorphic ESTs included: a putative EF-hand calcium binding domain and the FRQ1 protein domain of myosin light chain (TUASPvWSu233), ribosomal proteins S10 and S5, actin, troponin I, nucleoside diphosphate kinase, arginine kinase, and 3 ESTs of unknown function (Table 2). Five polymorphic EST-SSRs (actin, 40S ribosomal protein S5, troponin I, arginine kinase and the putative EF-hand calcium binding domain of myosin light chain) were further genotyped with the entire reference mapping family for *L. vannamei* and their alleles were found to segregate after expected Mendelian inheritance. Additional primer sets are being designed from the remaining ESTs to determine their utility for linkage mapping.

Linkage Mapping of EST-SSRs

Five polymorphic ESTs were genotyped with the entire IRMF panel and two of them (actin, accession #CX535973

TABLE 2.

Polymorphism analysis of SSR-containing ESTs isolated from a subtracted cDNA library of WSSV-injected *Litopenaeus vannamei*.*.

Clone ID	Accession #	Clone Size (bp)	Microsatellite Arrays[a,b]	Primers 5′-3′	Expected PCR (bp)	Annealing Temp (°C)	P[d]	Linkage Group # in[d] *ShrimpMap*	Putative Gene
TUASPvWSu233[f]	CX535935	572	**…(TGA)$_3$ATG(GA)$_3$…**(GA)$_4$AGTA(AGC)$_3$…	F:CCCGACTTGGCTTTTAGTTG	251	44	P	7*	EF-hand motif of myosin light chain
TUASPvWSu647	CX535989	747	**…(TGA)$_3$ATG(GA)$_3$…(GA)$_4$AGTA(AGC)$_3$…**	F:CCCTGCACTTAACCTGCTTG	206	52	M	*	EF-hand motif of myosin light chain
TUASPvWSu356	CX535958	394	**…(CA)$_3$…(GTCA)$_3$…**	F:AGGGGGAGTGTCACAGTCAG	160	52	NA	*	Unknown function
TUASPvWSu789	CX536001	276	**…(GTCA)$_4$…**	F:TCACAAGGCCACATGTCA	180	52	M	*	Unknown function
TUASPvWSu272	CX535941	579	**…(AGT)$_3$…**	F:TGTTGGCTTCTTCAGTGTCG	222	52	P[g]	*	40S ribosomal protein S10 (minor allergen)
TUASPvWSu352	CX535957	690	**…(TC)$_3$…**	F:TAACACGACGCAGTGGAGAC	161	52	P	U**	40S ribosomal protein S5
TUASPvWSu323	CX535950	257	**(AC)$_3$…(AAG)$_4$…**	F:GGAAAGGCAAGCTCTCATTG	150	48	NA	*	40S ribosomal protein S24
TUASPvWSu298–1	CX535945	778	…(CT)$_6$…**(CT)$_5$…(TC)$_3$…(TTC)$_3$…(CT)$_7$**	F:TGGCTTCAACCCACACTTTC	130	52	P[g]		Shrimp EST of Unknown function
TUASPvWSu298–2	CX535945	778	**…(CT)$_6$…**(CT)$_5$…(TC)$_3$…(TTC)$_3$……(CT)$_7$	F:GCCAACGCGAGTGCACGTCT	215	52	M		Shrimp EST of unknown function
TUASPvWSu319	CX535948	677	**(CG)$_3$…(GA)$_3$…(TGA)$_3$**	F:CAGCTCGTTGAGTTTCTCC	195	52	NA	*	Shrimp EST of Unknown function
TUASPvWSu347–1	CX535956	660	**…(TG)$_3$…**(AAGG)$_3$…(AGC)$_3$…(GGT)$_3$…	F:ACTGTGTTGATAACGTTG	235	52	M		Actin 2
TUASPvWSu347–2	CX535956	660	…(TG)$_3$**…(AAGG)$_3$…(AGC)$_3$…(GGT)$_3$…**	F:ATGGAGTTGTGGACGGTTTC	265	44–52	M		Actin 2
TUASPvWSu479–1	CX535973	646	**…(TG)$_3$…(TG)$_3$**…(AAGG)$_3$…(AGC)$_3$…	F:CAGTACATCATATTGGTTGG	280	52	P	U*	Actin 2
TUASPvWSu479–2	CX535973	646	…(TG)$_3$…(TG)$_3$…**(AAGG)$_3$…(AGC)$_3$…**	F:GTTGGCAAACAGATCCTTCC	210	52	M		Actin 2
TUASPvWSu378[e]	CX535960	610	**…(TG)$_3$…(CTT)$_3$…**	F:TTTCAGTTCGTCCTAACCGC	246	52–60	P	*	Spectrin repeats - muscle-specific protein 300
TUASPvWSu411	CX535965	184	**…(ATA)$_3$…**	F:CATTTCAACGTTTATTGG	90	52	M	*	Unknown function
TUASPvWSu428	CX535966	382	**…(TGG)$_5$…**	F:TGCCGAAGGTATGGAGAAAC	160	52	M	*	60S acidic ribosomal protein P2
TUASPvWSu591	CX535977	329	**…(CT)$_4$…**	F:TTCCTGAGGGCATTGTTAGG	230	52	M	*	Unknown function
TUASPvWSu508	CX535976	594	**…(AGA)$_3$…(GTC)$_5$**…(TA)$_3$…	F:CACCTACAGGAGAAGCAGCC	158	52	P	U*	Troponin I
TUASPvWSu759	CX535996	626	…(TA)$_3$**…(GAC)$_5$…(TCT)$_3$…(CTT)$_3$**	F:AAGAATTACAGGGGACGCAG	250	52	NA		Troponin I
TUASPvWSu598	CX535978	499	**…(TG)$_3$…(AG)$_3$**...	F:ACCACCTTGCCAACATTCTC	217	48	M	*	Sarcoplasmic reticulum Ca2+-ATPase
TUASPvWSu754–1	CX535995	723	**…(CT)$_3$…(GA)$_3$**…(CA)$_3$…	F:GTTATTGCACGTCGCCTCAC	350	52	P[g]	*	Nucleoside diphosphate kinase
TUASPvWSu754–2	CX535995	723	…(CT)$_3$…(GA)$_3$…**(CA)$_3$…**	F:TGGACACAGACCAACGAGAG	191	52	M		Nucleoside diphosphate kinase
TUASPvWSu769	CX535997	457	**…(AT)$_3$…**	F:AAAGACACCGCAAGGTCATC	230	52	P[g]		Shrimp EST of Unknown function
TUASPvWSu771	CX535998	549	**…(GGT)$_3$…(GCG)$_3$…(GGC)$_3$…**	F:AACTACAACAACCGAGGGGG	250	52	NA		Homolog to motifs of sex-lethal gene
TUASPvWSu779	CX535999	557	**…(GC)$_3$…(GA)$_3$…**	F:CAGAAAGTGAGGAAGCCCAG	292	56	P	U*	Arginine kinase (major allergen)
TUASPvWSu722	CX535991	375	**…(AGG)$_3$…(TG)$_3$…**	F:TACAGTCATTCCCGGCTCTC	217	52	NA		Arginine kinase (major allergen)
TUASPvWSu785	CX535600	610	**…(AG)$_3$**…(TC)$_4$…	F:AGCGTGTTCTATCAGGCTGG	239	52	NA		No homology to any sequence

[a] Repeat sequences in bold indicate they were flanked by the primers ordered.

[b] Motifs with out (…) indicate there was no flanking sequence to design a primer.

[c] Primers were first amplifed at 52°C. If primers did not amplify the annealing temperature was optimized at 44°C, 48°C, 52°C, 56°C, and 60°C. 7/07/05

[d] P = Polymorphic; M = Monomorphic; NA = did not amplify. U = unlinked, not yet assigned to a linkage group after genotyping with the entire reference mapping family. Potential single nucleotide polymorphism (SNPs) among penaeid species.

[e] These primers amplified polymorphic alleles at high molecular weight using ^{32}P assay.

[f] Markers in bold were polymorphic ESTs tested with the entire mapping panel.

[g] These markers are being repeated with the entire reference mapping family.

and shrimp allergen arginine kinase, accession #CX535999) did not amplify with all offspring of the IRMF panel suggesting presence of null alleles. Three of these ESTs amplified in most of the IRMF offspring and were used for linkage analysis, the EF-hand motif of myosin light chain (accession #CX535935) was placed in *ShrimpMap*'s linkage group 7, whereas ribosomal protein S5 (accession #CX535957) and troponin I (accession #CX535976) remained unassigned. Genotyping of additional EST-SSR markers should place these unlinked markers in other linkage groups. The mapped EST-SSR adds to the existing microsatellite loci genotyped with the SPF *L. vannamei* mapping panel. Work is underway to develop additional EST-SSRs from this and other cDNA libraries to increase density of *ShrimpMap*. ESTs from *L. vannamei* were also found polymorphic and potentially useful for mapping (Perez et al. 2005b) but have not yet been placed in their linkage map (Perez et al. 2005a). This is the first report of an EST mapped to any of the *L. vannamei* linkage maps. Tong et al. (2002) reported that ~30% of ESTs from *P. monodon* were polymorphic in a test panel and were included in the *P. monodon* linkage map (Maneeruttanarungroj et al. 2006).

Single Nucleotide Polymorphism (SNP) in EST Sequences of L. vannamei *Challenged with WSSV*

Sequence alignments of the ESTs listed in Table 2 revealed that in addition to showing length polymorphisms (''P''), potential SNPs (*) were also identified among penaeid species. This was the case for some of our monomorphic ESTs such as 60S acidic ribosomal protein P2 (a minor allergen), sarcoplasmic CA2+-ATPase, and unknown function genes, and polymorphic ESTs such as actin, AK and ESTs that did not amplify (NA) (Table 1). Only two of the ESTs listed do not show potential for SNP discovery. For actin gene, in addition to length polymorphisms identified in the 30 sequences reported here, potential single nucleotide polymorphism (SNPs) were also found not only among SPF *L. vannamei* lines of the USMSFP's breeding program but also among penaeid species (*P. monodon, P. stylirostris, P. setiferus, P. japonicus, L. vannamei*) and between invertebrates and vertebrate genomes (Alcivar-Warren et al. unpublished). Work is underway to study the evolutionary relationship of actin genes among invertebrates (Alcivar-Warren, unpublished). Insect muscle actins differ distinctly from invertebrate and vertebrate cytoplasmic actins (Mounier et al. 1992). For AK gene, our finding that this gene is polymorphic in parental broodstock used to develop our *ShrimpMap* families indicates that there is possibility for the USMSFP to develop a niche market to produce hypoallergenic lines of SPF shrimp for human consumption. In addition to allele length polymorphisms, the AK sequences also identified SNPs within SPF *L. vannamei* of the USMSFP, among penaeid shrimp species and between crustaceans and other invertebrate and vertebrate species (not shown).

TABLE 3.

Simple sequence repeats (SSRs) in partial sequences of WSSV isolated from a subtracted cDNA library of WSSV-injected *Litopenaeus vannamei*.

Clone ID	Clone Size (bp)	Simple Sequence Repeats[a]	Blastx (protein query) Homologous Sequences	Identity %	E Value	GenBank Access. #
TUASPvWSu011	516	…$(TA)_4$…$(GT)_3$…	ORF94	114/114 (100%)	1.00E-64	AAW71793
TUASPvWSu023	361		WSSV270; WSSV269, VP15, VP14	94/94 (100%)	4.00E-51	AAL89138
TUASPvWSu031	394	…$(TGT)_3$…$(GA)_6$…	WSSV126, WSSV069; ORF55	111/111 (100%)	1.00E-60	AAL88994
TUASPvWSu036	404	…$(TGT)_3$…$(TG)_4$…	WSSV524; WSSV465; ORF16, WSSV240	104/104 (100%)	1.00E-58	AAL89392
TUASPvWSu051	522	…$(TA)_4$…	ORF94	109/109 (98%)	2.00E-61	AAW71793
TUASPvWSu093	412	…$(AAT)_3$…	VO216; WSSV304; wsv249; ORF125; WSV306	112/112 (100%)	3.00E-58	AAM12822
TUASPvWSu095	522	…$(GAC)_3$…	unknown (WSSV); WSSV234, ORF94; WSV178	114/116 (98%)	7.00E-58	NP_477701
TUASPvWSu245	188		WSSV235; wsv179	25/43 (58%)	1.00E-06	AAL89103
TUASPvWSu317[b,c]	505	**…$(CA)_3$…$(CGT)_3$**	ORF19; WSV108, WSSV051; ORF44	104/108 (96%)	2.00E-57	AAM73709
TUASPvWSu344[b,d]	211	**…$(CTT)_3$…**	WSSV009; WSV482	45/47 (99%)	1.00E-19	AAL88877
TUASPvWSu400[e]	443	…$(CTA)_3$..	WSSV sequence: 160729–160393	313/340 (92%)	1.00E-129	AF440570

[a] Have enough flanking sequences to design primers. Clones in bold were used to design primers for viral amplification. Letters in bold indicate the SSRs flanked by the primers.

[b] P = Primers did not amplify in DNA of specific pathogen-free *L. vannamei* from the USMSFP using published procedures (Reville et al. 2005) with annealing temperature at 52°C.

[c] Used the following primers: forward: 5′-AGCTAAAGTTGTTGCGACGG-3′ and reverse: 5′-CGACCCATCAGAAAATCCAC-3′.

[d] Used the following primers: forward 5′-CGACCCATCAGAAAATCCAC-3′ and reverse 5′-CCGAGGTCAGTTACTGTGAA-3′.

[e] Based on comparisons against the Blastn (nucleotide-nucleotide) database.

EST Sequences Homologous to White Spot Syndrome Virus

Eleven sequences from the library reported here showed homology to portions of the WSSV genome. WSSV has also been detected in tail muscle of *P. stylirostris* juveniles as early as 16 h and 32 h after injection with WSSV (Dhar et al. 2003). Nine of the 11 cDNAs contained single or multiple SSRs with three or more repeat motifs and had enough flanking sequences to design primers (Table 3). Primer sets for two clones were used to amplify WSSV in SPF shrimp using a ^{32}P-based assay. No PCR products were amplified in SPF shrimp of the USMSFP, confirming that SPF shrimp from the USMSFP are free of WSSV. This result differ from another study using a microarray assay indicating that SPF shrimp from a private company (BIOTEC, Bangkok, Thailand) had latency-related WSSV genes (ORFs 151, 366, and 427) expressed in these shrimp (Khadijah et al. 2003). It remains to be confirmed if the latency-related ORFs identified in the SPF shrimp of Asia are present in SPF shrimp of the USMSPF under same assay conditions. Alternatively, we suggest that the SPF shrimp used by Khadijah et al. (2003) may have been asymptomatic carriers of the virus.

In summary, various EST sequences representatives of calcium, glycolytic, and proteins synthesis pathways were identified, primers were designed from ESTs regions flanking single or multiple SSRs with three or more repeats, and so far, the gene encoding the EF-hand calcium-binding domain of myosin light chain has been mapped to linkage group 7. Results show that EST-SSRs are an efficient approach to develop polymorphic markers useful for linkage mapping. The ESTs can also serve as genetic markers for genetic diversity and pedigree tracing, population differentiation and evolutionary studies of shrimp. Mapped ESTs of virus-challenged shrimp will add to the shrimp linkage map and provide valuable comparative genomic links between *L. vannamei* and other penaeid species as well as invertebrate and vertebrate genomes. Work is underway to map the remaining SSR- or SNP-ESTs from this and other cDNA libraries of viral challenged shrimp as a plausible strategy to increase marker density of *ShrimpMap*. Immediate efforts focus on mapping all shrimp ribosomal genes from a BAC library by exploiting known strategies to identify PCR-detectable sequence-tagged sites at introns to distinguish from potential processed pseudogenes (Kenmochi et al. 1998).

The potential Type I markers reported here would add to the current microsatellite-based linkage map (Alcivar-Warren et al. 2007) and facilitate development of a transcript-based, medium-density linkage map for *L. vannamei*. Availability of a highly saturated linkage map will facilitate not only integration of shrimp physical and linkage maps but also detection of candidate genes and mutations responsible for variation in economically important quantitative traits. EST-SSR markers would directly sample variation in the transcribed regions of the shrimp genome and enhance their utility in marker-assisted selection, comparative genetic analysis and exploitation of shrimp genetic resources.

ACKNOWLEDGMENTS

The authors thank Mr. Jim Sweeney, Dr. William Carr and the staff at The Oceanic Institute at Makapuu Point, Oahu, HI for access to SPF shrimp from the USMSFP's breeding program, and Ms. Bonnie Poulos and Dr. Donald Lightner at the University of Arizona for access to samples from their WSSV experiments. This research was supported in part by grant #98-38808-1424 from the US Department of Agriculture to the US Marine Shrimp Farming Program (A-W and DL), NOAA International Program, US Department of Commerce (A-W), the Department of Environmental and Population Health at Tufts University School of Veterinary Medicine (A-W), and Chinese Academy of Sciences, Institute of Oceanology (LS and JX).

LITERATURE CITED

Alcivar-Warren, A., R. Overstreet, A. K. Dhar, K. Astrofsky, W. Carr, J. Sweeney & J. Lotz. 1997. Genetic susceptibility of cultured shrimp (*Penaeus vannamei*) to Infectious Hypodermal and Hematopoetic Necrosis Virus and *Baculovirus penaeid*: possible relationship with growth status and metabolic gene expression. *J. Inv. Pathol.* 79:190–197.

Alcivar Warren, A., Z. Xu, D. Meehan, Y. Fan & L. Song. 2002. Shrimp genomics: development of a genetic map to identify QTLs responsible for economically important traits in *Litopenaeus vannamei*. In: Nobuyoshi Shimizu, Takashi Aoki, Ikuo Hirono & Fumio Takashima, editors. Aquatic genomics: steps toward a great future. Tokyo, Japan: Springer-Verlag. 450 pp.

Alcivar-Warren, A. & W. Warren. 2005. Taura Syndrome Virus affects the protein synthesis machinery and mRNA associated with cytoplasmic cytoskeletal structure: a preliminary hypothesis. Book of abstracts. World Aquaculture Society, Bali, Indonesia, May 8–13, 2005.

Alcivar-Warren, A., D. Meehan-Meola, Y. Wang, X. Guo, L. Zhou, J. Xiang, S. Moss, S. Arce, W. Warren, Z. Xu & K. Bell. 2006. Isolation and mapping of telomeric pentanucleotide $(TAACC)_n$ repeats of the Pacific whiteleg shrimp, *Penaeus vannamei*, using fluorescent in situ hybridization. *Mar. Biotechnol.* (NY) 8:467–480.

Alcivar-Warren, A., D. Meehan-Meola, Z. Xu, S. W. Park, M. Delaney & G. Zuniga. 2007. *ShrimpMap*: a low-density, microsatellite-based linkage map of the Pacific whiteleg shrimp, *Litopenaeus vannamei*: identification of sex-linked markers in linkage group 4. *J. Shellfish Res.* (This issue).

Argue, B. J. & A. Alcivar-Warren. 1999. Genetics and breeding applied to the penaeid shrimp farming industry. In: R. A. Bullis & G. D. Pruder, editors. Controlled and biosecure production systems. Proceedings of a Special Session on Evolution and Integration of Shrimp and Chicken Models. pp. 29–54.

Argue, B. J., S. M. Arce, J. M. Lotz & S. M. Moss. 2002. Selective breeding of Pacific White Shrimp (*Litopenaeus vannamei*) for growth and resistance to Taura Syndrome Virus. *Aquaculture* 204:447–460.

Astrofsky, K. M., M. M. Roux, K. R. Klimpel, J. G. Fox & A. K. Dhar. 2002. Isolation of differentially expressed genes from white spot virus (WSV) infected Pacific blue shrimp (*Penaeus stylirostris*). *Arch. Virol.* 147:1799–1812.

Bonneau, A. M., A. Darveau & N. J. Sonembr. 1985. Effect of viral infection on host protein synthesis and mRNA association with the cytoplasmic cytoskeletal structure. *J. Cell Biol.* 100:1209–1218.

Capozzi, F., F. Casadel & C. Luchinat. 2006. EF-hand protein dynamics and evolution of calcium signal transduction: an NMR view. *J. Biol. Inorg. Chem.* 11:949–962.

Carr, W., J. N. Sweeney & J. Swingle. 1994. The oceanic institute's specific pathogen free (SPF) shrimp breeding program: preparations and infrastructure. USMSFP (US Marine Shrimp Farming Program) 10th Anniversary Review. *GCRL Special Publication No.* 1:47–54.

Carr, W. H., K. T. Fjalestad, D. Godin, J. Swingle, J. N. Sweeney & T. Gjedrem. 1997. Genetic variation in weight and survival in a population of specific pathogen-free shrimp, *Litopenaeus vannamei*. In:

T. W. Flegel & I. H. MacRae, editors. Diseases in Asian aquaculture III, fish health section, Asian Fisheries Society, Manila. pp. 265–271.

Cesar, E., B. Nadala, Jr. & P. C. Loh. 1998. A comparative study of three different isolates of white spot virus. *Dis. Aquat. Org.* 33:231–234.

Cevallos, R. C. & P. Sarnow. 2005. Factor-independent assembly of elongation-competent ribosomes by an internal ribosome entry site located in an RNA virus that infects penaeid shrimp. *J. Virol.* 79:677–683.

Chavez-Rios, R., L. E. Arias-Romero, M. J. Almaraz-Barrera, R. Hernandez-Rivas, N. Guillén & M. Vargas. 2003. L10 ribosomal protein from *Entamoeba histolytica* share structural and functional homologies with QM/Jif-1: proteins with extraribosomal functions. *Mol. Biochem. Parasitol.* 127:151–160.

Clavero-Salas, A., R. R. Sotelo-Mundo, T. Gollas-Galvan, J. Hernandez-Lopez, A. B. Peregrino-Uriarte, A. Muhlia-Almazan & G. Yepiz-Plascencia. 2007. Transcriptome analysis of gills from the white shrimp *Litopenaeus vannamei* infected with White Spot Síndrome Virus. *Fish Shellfish Immunol.* 23:459–472.

de la Vega, E., B. M. Degnan, M. R. Hall & K. J. Wilson. 2007. Differential expression of immune-related genes and transposable elements in black tiger shrimp (*Penaeus monodon*) exposed to a range of environmental stressors. *Fish Shellfish Immunol.* [May 13 Epub ahead of print].

de Lorgeril, J., D. Saulnier, M. G. Janech, Y. Gueguen & E. Bachere. 2005. Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with *Vibrio penaeicida*. *Physiol. Genomics* (February 22, 2005), doi: 10.1152/physiolgenomics.00281.2004.

Dhar, A. K., A. Dettori, M. M. Roux, K. R. Klimpel & B. Read. 2003. Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White spot syndrome virus by cDNA microarrays. *Arch. Virol.* 148:2381–2396.

Eyster, K. M., A. L. Boles, J. D. Brannian & K. A. Hansen. 2002. DNA microarray analysis of gene expression markers in endometriosis. *Fertil. Steril.* 77:38–42.

Flegel, T. W., S. Boonyaratpalin & B. Withyachumnarnkul. 1997. Progress in research on yellow-head virus and white spot virus in Thailand. In: T. W. Flegel & I. H. MacRae, editors. Diseases in Asian Aquaculture III. Manila: Fish Health Section, Asian Fisheries Society 285–295.

Francoeur, A. M., C. L. Peebles, K. J. Heckman, J. C. Lee & E. M. Tan. 1985. Identification of ribosomal protein autoantigens. *J. Immunol.* 135:2378–2384.

Fukushi, S., M. Okada, J. Stah, T. Kagemaya, F. B. Hoshino & K. Katayama. 2001. Ribosomal protein S5 interacts with the internal ribosomal entry site of hepatitis C virus. *J. Biol. Chem.* 276:20824–20826.

Garcia, D. K. & A. Alcivar-Warren. 2007. Characterization of 35 new microsatellite genetic markers for the Pacific whiteleg shrimp, Litopenaeus vannamei: Their usefulness for studying genetic diversity of wild and cultured stocks, tracing pedigree in breeding programs, and linkage mapping. *J. Shellfish Res.* (This issue).

Garcia, D. K., A. K. Dhar & A. Alcivar-Warren. 1996. Molecular analysis of a RAPD markers (B20) reveals two microsatellites and differential mRNA expression in Litopenaeus vannamei. *Mol. Mar. Biol. Biotechnol.* 5:71–83.

Gardner-Thorpe, J., H. Ito, S. W. Ashley & E. E. Whang. 2003. Ribosomal protein P2: a potential molecular target for antisense therapy of human malignancies. *Anticancer Res.* 23:4549–4560.

Gross, P. S., T. C. Bartlett, C. L. Browdy, R. W. Chapman & G. W. Warr. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. *Dev. Comp. Immunol.* 25:565–577.

Hasegawa, H., T. Uchiumi, T. Sato, Y. Ofuchi, S. Murakami, S. Honda, S. Hirose, S. Ito, M. Nakano, M. Arakawa & F. Gejvo. 1999. High frequency of antibody activity against ribosomal protein S10 in anti-Sm sera from patients with systemic lupus erythematosus. *Lupus* 8:439–443.

He, N., Q. Qin & X. Xu. 2004. Differential profile of genes expressed in hemocytes of White Spot Syndrome Virus-resistant shrimp (*Penaeus japonicus*) by combining suppression subtractive hybridization and differential hybridization. *Antiviral Res.* 66:39–45.

Huang, C. C. & Y. L. Song. 1999. Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Dev. Comp. Immunol.* 23:545–552.

Karan, D., D. L. Kelly, A. Rizzino, M. F. Lin & S. K. Batra. 2002. Expression profile of differentially-regulated genes during progression of androgen-independent growth in human prostate cancer cells. *Carcinogenesis* 23:967–975.

Kenmochi, N., T. Kawaguchi, S. Rozen, E. Davis, N. Goodman, T. Hudson, T. Tanaka & C. Page. 1998. A map of 75 human ribosomal protein genes. *Genome Res.* 8:509–523.

Khadijah, S., S. Y. Neo, M. S. Hossain, P. D. Miller, S. Mathavan & J. Kwang. 2003. Identification of White Spot Syndrome Virus latency-related genes in specific-pathogen-free shrimps by use of a microarray. *J. Virol.* 77:10162–10167.

Lehnert, S. A., K. J. Wilson, K. Byrne & S. S. Moore. 1999. Tissue-specific expressed sequence tags from the black tiger shrimp *Penaeus monodon*. *Mar. Biotechnol.* 1:465–476.

Leu, J.-H., C.-C. Chang, J.-L. Wu, C.-W. Hsu, I. Hirono, T. Aoki, H.-F. Juan, C.-F. Lo, G.-H. Kou & H.-C. Huang. 2007. Comparative analysis of differentially expressed genes in normal and white spot syndrome virus infected *Penaeus monodon*. *BMC Genomics* 8:120–134.

Leung, P. S., Y. C. Chen, D. L. Mykles, W. K. Chow, C. P. Li & K. H. Chu. 1998. Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Mol. Mar. Biol. Biotechnol.* 7:12–20.

Lightner, D. V. 1996. White spot syndrome baculovirus complex (WSBV). In: *A Handbook of Pathology and Diagnostic Procedures for Diseases of Penaeid Shrimp*. Baton Rouge: World Aquaculture Society. 304 pp.

Lotz, J. M. 1997. Special topic review: Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. *World J. Microbiol. Biotechnol.* 13:405–413.

Luo, L. Y., I. Herrera, A. Soosaipillai & E. P. Diamandis. 2002. Identification of heat shock protein 90 and other proteins as tumor antigens by serological screening of an ovarian carcinoma expression library. *Br. J. Cancer* 87:339–343.

Luo, T., X. Zhang, Z. Shao & X. Xu. 2003. *Pm*AV, a novel gene involved in virus resistance of shrimp, Penaeus monodon. *FEBS Lett.* 11;551:53–57.

Marks, H., M. Mennens, J. M. Vlak & M. C. van Hulten. 2003. Transcriptional analysis of the white spot syndrome virus major virion protein genes. *J. Gen. Virol.* 84:1517–1523.

Meehan, D., Z. Xu, G. Zuniga & A. Alcivar-Warren. 2003. High frequency and large number of polymorphic microsatellites in cultured shrimp *Penaeus* (*Litopenaeus*) *vannamei*, (Crustacea:Decapoda). *Mar. Biotechnol.* (NY) 5:311–330.

Maneeruttanarungroj, C., S. Pongsonboon, S. Wuthisuthimethavee, S. Klinbunga, K. J. Wilson, J. Swan, Y. Li, V. Whan, K. H. Chu, C. P. Li, J. Tong, K. Glenn, M. Rothschild, D. Jerry & A. Tassanakajon. 2006. Development of polymorphic expressed sequence tag-derived microsatellites for the extension of the genetic linkage map of the black tiger shrimp (Penaeus monodon). *Anim. Genet.* 37:363–368.

Mounier, N., M. Gouy, D. Mouchiroud & J. C. Prudhomme. 1992. Insect muscle actins differ distinctly from invertebrate and vertebrate cytoplasmic actins. *J. Mol. Evol.* 34:406–415.

Moore, S. S., V. A. Whan, G. P. Davis, K. Byrne, D. J. S. Hetzel & N. Preston. 1999. The development and application of genetic markers for the kuruma prawn *Penaeus japonicus*. *Aquaculture* 173:19–32.

Moss, S. M., B. J. Argue & S. M. Arce. 1999. Genetic improvement of the Pacific White Shrimp, Litopenaeus vannamei, at the Oceanic Institute. *Global Aquaculture Advocate* 2:41–43.

Nei, M. 1987. Molecular evolutionary genetics. Columbia New York: University Press. pp. 176–207.

OIE. Office International des Epizooties. 2007. OIE classification and disease description. <http://www.oie.int/eng/maladies/en_fiches.htm> Updated February 12, 2007.

Oduol, F., J. Xu, O. Niari, R. Natarajan & K. D. Vernick. 2000. Genes identified by an expression screen of the vector mosquito Anopheles gambiae display differential molecular immune response to malaria parasites and bacteria. *Proc. Natl. Acad. Sci. USA* 97:11397–11402.

Pan, J., A. Kurosky, B. Xu, A. K. Chopra, D. H. Coppenhaver, I. P. Singh & S. Baron. 2000. Broad antiviral activity in tissues of crustaceans. *Antiviral Res.* 48:39–47.

Pan, D., N. He, Z. Yang, H. Liu & X. Xu. 2005. Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridization. *Dev. Comp. Immunol.* 29:103–112.

Pearson, W. R. 2000. Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol. Biol.* 132:185–219.

Perez, F., C. Erazo, M. Zhinaula, F. Volckaert & J. Calderon. 2005a. A sex-specific linkage map of the white shrimp Penaeus vannamei based on AFLP markers. *Aquaculture* 242:105–118.

Perez, F., J. Ortiz, M. Zhinaula, C. Gonzabay, J. Calderon & F. A. Volckaert. 2005b. Development of EST-SSR markers by data mining in three species of shrimp: *Litopenaeus vannamei, Litopenaeus stylirostris*, and *Trachypenaeus birdy*. *Mar. Biotechnol.* 7:554–569.

Popescu, S. C. & N. E. Tumer. 2004. Silencing of ribosomal protein L3 genes in *N. tabacum* reveals coordinate expression and significant alterations in plant growth, development and ribosomal biogenesis. *Plant J.* 39:29–44.

Ramirez, C. D. & R. Padron. 2004. Familial hypertrophic cardiomyopathy: genes, mutations and animal models. *Invest. Clin.* 45:69–99.

Reville, C., J. Al-Beik, D. Meehan-Meola, Z. Xu, M. L. Goldsmith, W. Rand & A. Alcívar-Warren. 2005. White Spot Síndrome Virus in frozen shrimp sold at Massachusetts supermarkets. *J. Shellfish Res.* 24:285–290.

Robalino, J., J. S. Almeida, D. McKillen, J. Colglazier, H. F. Trent Iii, Y. A. Chen, M. E. Peck, C. L. Browdy, R. W. Chapman, G. W. Warr & P. S. Gross. 2006. Insights into the immune transcriptote of the shrimp *Litopenaeus vannamei*: tissue-specific expresión profiles and transcriptomic responses to immune challenge.

Rodríguez, J., B. Bayot, Y. Amano, F. Panchana, I. de Blas, V. Alday & J. Calderon. 2003. White spot syndrome virus infection in cultured *Litopenaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and ultrastructure. *J. Fish Dis.* 26:439–450.

Rodriguez, J., V. Boulo, E. Mialhe & E. Bachere. 1995. Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. *J. Cell Sci.* 108(Pt 3):1043–1050.

Rojtinnakorn, J., I. Hirono, T. Itami, Y. Takahashi & T. Aoki. 2002. Gene expression in haemocytes of kuruma prawn, *Penaeus japonicus*, in response to infection with WSSV by EST approach. *Fish Shellfish Immunol.* 13:69–83.

Roux, M. M., A. Pain, K. R. Klimpel & A. K. Dhar. 2002. The lipopolysaccharide and beta-1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp (*Penaeus stylirostris*). *J. Virol.* 76:7140–7149.

Rozen, S. & H. J. Skaletsky. 2002. Primer 3 on the WWW for general users and for biologist programmers. In: S. Krawtz & S. Misener, editors. Bioinformatics methods and protocols: methods in molecular biology. Totowa, NJ: Humana Press. pp. 365–386. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Sæbøe-Larssen, S., M. Lyamouri, J. Merriam, M. P. Oksvold & A. Lambertsson. 1998. Ribosomal protein insufficiency and the *Minute* syndrome in Drosophila: a dose-response relationship. *Genetics* 148:1215–1224.

Shanti, K. N., B. M. Martin, S. Nagpal, D. D. Metcalfe & P. V. Rao. 1993. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J. Immunol.* 151:5354–5363.

Supungul, P., S. Klinbunga, R. Pichyangkura, S. Jitrapakdee, I. Hirono, T. Aoki & A. Tassanakajon. 2002. Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). *Mar. Biotechnol.* (NY) 4:487–494.

Tong, J., S. A. Lehnert, K. Byrne, H. S. Kwan & K. H. Chu. 2002. Development of polymorphic EST markers in *Penaeus monodon*: applications in penaeid genetics. *Aquaculture* 208:69–79.

van Hulten, M. C. W., J. Witteveldt, S. Peters, et al. 2001. The white spot syndrome virus DNA genome sequence. *Virology* 286:7–22.

van de Braak, C. B., M. H. Botterblom, E. A. Huisman, J. H. Rombout & W. P. van der Knaap. 2002. Preliminary study on haemocyte response to white spot syndrome virus infection in black tiger shrimp *Penaeus monodon*. *Dis. Aquat. Organ.* 51:149–155.

Vizirianakis, I. S., I. S. Pappas, D. Gougoumas & A. S. Tsiftsoglou. 1999. Expression of ribosomal protein S5 cloned during differentiation and apoptosis in murine erythroleukemia (MEL) cells. *Oncol. Res.* 11:409–419.

Wang, H., X. Li Fuhua & X. Jianhai. 2005. Polymorphic EST-SSR markers and their mode of inheritance in *Fenneropenaeus chinensis*. *Aquaculture* 249:107–114.

Whan, V. A., K. J. Wilson & S. S. Moore. 2000. Two polymorphic microsatellite markers from novel *Penaeus monodon* ESTs. *Anim. Genet.* 31:143–144.

Wilson, K., Y. Li, V. Whan, S. Lehnert, K. Byrne, S. Moore, S. Pongsomboon, A. Tassanakajon, G. Rosenberg, E. Ballment, Z. Fayazi, J. Swan, M. Kenway & J. Benzie. 2002. Genetic mapping of the black shrimp *Penaeus monodon* with amplified fragment length polymorphism. *Aquaculture* 204:297–309.

Witteveldt, J., J. M. Vlak & M. C. van Hulten. 2004. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. *Fish Shellfish Immunol.* 16:571–579.

Wolfus, G. M., D. K. Garcia & A. Alcivar-Warren. 1997. Application of the Microsatellites technique for analyzing genetic diversity in shrimp breeding programs. *Aquaculture* 152:35–47.

Wongteerasupaya, C., P. Pungchai, B. Withyachumnarnkul, V. Boosaeng, S. Panyim, T. W. Flegel & P. J. Walker. 2003. High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. *Dis. Aquat. Organ.* 24:253–257.

Wu, J. L., T. Nishioka, K. Mori, T. Nishizawa & K. Muroga. 2002. A time-course study on the resistance of *Penaeus japonicus* induced by artificial infection with white spot syndrome virus. *Fish Shellfish Immunol.* 13:391–403.

Wu, J. P. & K. Muroga. 2004. Apoptosis does not play an important role in the resistance of 'immune' *Penaeus japonicus* against white spot syndrome virus. *J. Fish Dis.* 27:15–21.

Wyban, J. A., J. S. Swingle, J. N. Sweeney & G. D. Pruder. 1993. Specific pathogen-free *Litopenaeus vannamei*: commercial trials indicate the progeny of SPF shrimp can dramatically outperform the progeny of wild shrimp in cultured systems. *World Aquacult.* 24:39–45.

Yamano, K. & T. Unuma. 2006. Expressed sequence tags from eyestalk of kuruma prawn, Marsupaneus japonicus. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 143:155–161.

Yang, F., J. He, X. Lin, Q. Li, D. Pan, X. Zhang & X. Xu. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. *J. Virol.* 75:11811–11820.

Yu, C. J., Y. F. Lin, B. L. Chiang & L. P. Chow. 2003. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J. Immunol.* 170:445–453.

Zhang, L., C. Yang, T. Zhang, L. Li, X. Zhang, Q. Zhang & J. Xiang. 2007. A genetic linkage map of Pacific white shrimp (*Litopenaeus vannamei*): sex-linked microsatellite markers and high recombination rates. *Genetica* 131:37–49.

Zhao, Z. Y., Z. X. Yin, S. P. Weng, H. J. Guan, S. D. Li, K. Xing, S. M. Chan & J. G. He. 2007. Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridization. *Fish Shellfish Immunol.* 22:520–534.