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Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and Southern blot hybridization

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ABSTRACT: White spot syndrome virus (WSSV) is widely distributed in most of the Asian countries where penaeid shrimp are cultured, as well as in some regions of the USA. Six geographic isolates of WSSV—1 each from penaeid shrimp from China, India, Thailand, and the US states of Texas and South Carolina, and 1 isolated from crayfish at the National Zoological Park in Washington, DC—were compared by combining the methods of restriction analysis and Southern blot hybridization. DNA was extracted from purified viruses and then digested with selected endonucleases: *Acc*I, *Bgl*II, *Cla*I, *Bam*HI, *Eco*RI, *Hin*dII, *Hae*I, *Sac*I and *Xho*I. The blots were detected with digoxigenin-11-dUTP-labeled WSSV genomic probes: LN4, C42 and A6. No distinctive differences among the 5 WSSV isolates from penaeid shrimp were detected; however, differences in the WSSV isolate from crayfish were observed. A 2.8 kb DNA fragment originating from the crayfish isolate and encompassing the LN4 region was subcloned into pBluescript and sequenced for comparison with the LN4 fragment from the Thailand WSSV isolate. The results indicate that some genomic components of WSSV from different geographic regions share a high degree of homology. This method can be used to distinguish between the WSSV isolate from crayfish and the WSSV isolates from penaeid shrimp.

KEY WORDS: White spot syndrome virus · WSSV · Penaeid shrimp · Restriction analysis · Southern blot hybridization · Genomic variation

INTRODUCTION

White spot syndrome disease of penaeid shrimp emerged during the early 1990s and has caused a serious ongoing epizootic in the shrimp growing countries of Asia, including China, India, Thailand, Japan, Taiwan, Korea, Indonesia, Malaysia, and Vietnam (Inouye et al. 1994, 1996, Momoyama et al. 1994, Nakano et al. 1994, Takahashi et al. 1994, Chen 1995, Flegel et al. 1995, Huang et al. 1995a, Wang et al. 1995, Wongteerasupaya et al. 1995, Kimura et al. 1996, Mohan et al. 1998). Several disastrous outbreaks of white spot syndrome disease have also occurred in the Gulf of Mexico and on the Southeastern coast of the United States (Lightner et al. 1997, Lo et al. 1999, Wang et al. 1999).

Almost all the species of penaeid shrimp are susceptible to white spot syndrome virus (WSSV) infection. The major species naturally infected by the virus in Asian countries include *Penaeus monodon*, *P. chinensis*, *P. indicus*, *P. penicillatus*, and *P. japonicus* (Inouye et al. 1994, 1996, Nakano et al. 1994, Takahashi et al. 1994, Chou et al. 1995, 1998, Flegel et al. 1995, Huang et al. 1995a, Wang et al. 1995, Wongteerasupaya et al. 1995, Chang et al. 1996, Kimura et al. 1996, Lo et al. 1996a, Kasornchandra et al. 1998,

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Mohan et al. 1998, Nunan et al. 1998, Park et al. 1998). Severe WSSV-induced mortalities have been observed in *P. setiferus* stocks from the states of Texas and South Carolina in the USA (Lightner et al. 1997, Lo et al. 1999, Wang et al. 1999). Additional penaeid species infected by WSSV include *Metapenaeus ensis*, *P. aztecus*, *P. duorarum*, *P. merguiensis*, *P. semisulcatus*, *P. stylirostris*, *P. vannamei*, and *Trachypenaeus curvirostris* (Cai et al. 1995, Lightner et al. 1997, 1998, Nunan & Lightner 1997, Tapay et al. 1997, Chang et al. 1998, Nunan et al. 1998, Wang et al. 1998, Wang et al. 1999). Among these penaeid species, the cumulative mortality caused by this disease can reach as high as 100%. Severe mortalities among several non-penaeid species, including *Exopalaemon orientalis*, *Macrobrachium rosenbergii* (caridean shrimp), *Orconectes punctimanus* and *Procambarus* sp. (crayfish), have also been reported (Richman et al. 1997, Chang et al. 1998, Peng et al. 1998, Wang et al. 1998). WSSV has also been detected by polymerase chain reaction (PCR), *in situ* hybridization or monoclonal antibody assays in wild crabs (*Calappa lophos*, *Portunus sanguinolentus*, *Charybdis* sp., *Helice tridens*), wild lobsters (*Panulirus* sp.), palaemonidian pest shrimp, copepoda plankton, *Artemia* spp. and pupae of an ephydridian insect (Huang et al. 1995b, Lo et al. 1996b, Chang et al. 1998, Kanchanaphum et al. 1998, Wang et al. 1998).

Since WSSV has a wide geographic distribution and host range, efforts have been directed to compare morphology, virulence, genomic composition and protein composition among WSSV isolates (Wongteerasupaya et al. 1996, Kasornchandra et al. 1998, Nadala Jr et al. 1998a,b, Park et al. 1998, Lo et al. 1999, Wang et al. 1999). The results have demonstrated that WSSV isolates from different locations are almost identical, although slight differences may exist among some.

The objective of the present study was to compare the genomic composition of 6 geographic isolates of WSSV in order to develop genetic markers that could be used to distinguish different WSSV strains.

MATERIALS AND METHODS

Viruses. The 6 WSSV isolates were derived from shrimp collected from China, India, Thailand, Texas and South Carolina and from crayfish maintained at the US National Zoological Park, Washington, DC (Table 1). The viral quantities were amplified in specific pathogen free (SPF) *Penaeus vannamei* (Wyban et al. 1992) (Oceanic Institute 'Kona' stock, population number UAZ 10-97). Viral extracts were prepared by centrifugation of tissue homogenate of infected shrimp at low speed (500 × *g*). The supernatant fluid was diluted 20 times with 2% saline before injection into SPF shrimp. WSSV infection in the shrimp and crayfish samples and in the experimentally infected SPF shrimp were confirmed by standard procedures using Davidson's alcohol formalin acetic acid (AFA) fixed tissue followed by routine histological processing, and hematoxylin and eosin-phloxine (H & E) staining (Bell & Lightner 1988, Lightner 1996). The severity of infection was rated according to Lightner (1996).

Virus purification. A gradient purification procedure was modified from the method of Bonami et al. (1990). WSSV-infected tissue was collected from the epidermis, stomach, appendages and gills of severely infected shrimp. A Tris-NaCl buffer (TN; 20 mM Tris-Cl, 400 mM NaCl, pH 7.4) was used throughout the purification procedure. The tissue homogenate was first clarified at 500 × *g* and 3000 × *g* sequentially. The viruses in the resultant supernatant fluids were pelleted for 1 h, at 70 000 × *g* (4°C), and then loaded carefully onto the top of 15 to 45% (w/w, in 1 × TN) linear Renografin-76® (Solvay Animal Health, Inc., Mendota Heights, MN) gradients. The gradients were centrifuged at 153 200 × *g* for 1.5 h at 4°C. The visible bands were drawn out with syringes, and then centrifuged at 124 100 × *g* for 1.5 h at 4°C to pellet the viruses. Each viral pellet was then resuspended in 500 µl distilled H_2O.

DNA extraction. The DNA extraction procedure was modified from the method of Lo et al. (1996a,b). Digestion buffer, 500 µl (100 mM NaCl; 10 mM Tris-HCl, pH 8; 25 mM EDTA, pH 8; 0.5% N-lauryl sarcosine;

Table 1. Description of the WSSV samples collected directly from the original geographic locations

WSSV isolate	Identification number	Collection location	Host species	Life stage of host	Wild or cultured	Collection time
WSSV China	UAZ95-116A	Shandong, China	*Penaeus chinensis*	Adult	Cultured	May 1995
WSSV India	UAZ95-314	Visak, India	*Penaeus monodon*	Juvenile	Cultured	28 Aug 1995
WSSV Thailand	UAZ95-38A	Thailand	*Penaeus monodon*	Adult	Cultured	27 Feb 1995
WSSV Texas	UAZ97-85	Texas, Gulf of Mexico	*Penaeus setiferus*	Adult	Wild	13 May 1997
WSSV South Carolina	UAZ97-5	South Carolina	*Penaeus setiferus*	Adult	Captive wild	8 Jan 1997
From crayfish	UAZ96-52	National Zoological Park, Washington, DC	*Orconectes punctimanus*	Subadult	Captive wild	8 Mar 1996

0.5% mg ml^{-1} Proteinase K), was added to a 100 µl viral suspension and incubated for 1 h at 65°C. Then 100 µl of 5 M NaCl and 70 µl of CTAB/NaCl solution (10% N-cetyl-N, N, N-trimethylammonium bromide in 0.7 M NaCl) was added and incubated at 65°C for 10 min. Afterwards, DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved into 30 µl H_2O.

Endonuclease digestion of DNA. Nine endonucleases—*Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, *Hin*dII, *Sac*I and *Xho*I—were used to digest the extracted viral DNA according to the manufacturer's protocol (Boehringer Mannheim, now Roche Molecular Biochemicals, Indianapolis, IN). To ensure complete digestion, each reaction was performed in a 100 µl volume for more than 10 h.

Gel electrophoresis. After endonuclease digestion, DNA was precipitated with ethanol and dissolved in 15 µl H_2O. One µl of 10× RNase Plus™ gel loading buffer (5 Prime - 3 Prime, Inc., Boulder, CO) was added to digest RNA in the samples. Electrophoresis was performed in a 1% agarose gel (containing 0.5 µg ml^{-1} ethidium bromide) in 0.5× TBE (Tris-Borate-EDTA) buffer (Sambrook et al. 1989). A digoxigenin-labeled DNA molecular Marker III (Roche Molecular Biochemicals) was co-electrophoresed to indicate the molecular weight of the DNA samples.

DNA probe labeling. Three DNA inserts—LN4 (Nunan & Lightner 1997), C42 (GenBank accession number AF295124) and A6 (GenBank accession number AF295123)—were labeled with digoxigenin DIG-11-dUTP by PCR using a Genius™ non-radioactive DNA labeling and detection kit (Roche Molecular Biochemicals). The C42 clone was developed using a China WSSV isolate (Table 1), and the LN4 and A6 clones were from a Thailand WSSV isolate (Table 1). The C42 and A6 clones were generated from purified viral DNA of the respective strains by using the standard cloning procedure for endonuclease digestion, ligation to a cloning vector and transformation into competent *E. coli* bacterial strains (Sambrook et al. 1989). The cloning of the LN4 fragment was accomplished by using PCR primers that were designed from sequence information from another penaeid virus of shrimp, *Baculovirus penaei* (BP). This cloning method is discussed in detail in Nunan & Lightner (1997). Three pairs of primers, derived from sequence data of the DNA viral inserts—5′ CTT GGT TCC AGA TGT GGT 3′& 5′ GGA GAT CCT TCG ACG AAT 3′, and 5′ AGG TAT AGT GGC TGT TGC 3′ & 5′ CTG GAG AGG ACA AGA CAT 3′, and 5′ TGT AGC AGC AGA GAA GAG 3′& 5′ ACT GCA CCA AAT TGT CCA CC 3′—were used to generate probes for C42, A6 and LN4 respectively. For each insert, a 100 µl labeling reaction solution containing H_2O, 10 µl 10× PCR Buffer II, 10 pmol of each dNTP, 10 µl DIG label mix, 200 pmol $MgCl_2$ and 1 µl AmpliTag Gold DNA polymerase (PE Applied Biosystem, Foster City, CA), as well as 250 ng DNA template and 250 ng of each primer for C42 and LN4 or 200 ng DNA template and 100 ng of each primer for A6, was reacted in a thermal cycler (PE Applied Biosystem). PCR initiated with a hot start at 94°C for 10 min; continued with 50 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and ended with an extension at 72°C for 7 min. The probes were precipitated with 10 µl 200 mM EDTA (PH 8.0), 11 µl 4 M LiCl, 1 µl 20 mg ml^{-1} glycogen, and 360 µl absolute ethanol and then resuspended in 100 µl distilled H_2O. Using the low molecular mass ladder (Gibco BRL, Grand Island, NY) as a standard, the concentration of probes was adjusted to 50 ng $µl^{-1}$ in H_2O. The specificity of these probes was tested by *in situ* hybridization according to Bruce et al. (1993) and dot blot hybridization according to the application manual from Roche Molecular Biochemicals.

Southern blot hybridization. The Southern blot hybridization procedure was modified from Sambrook et al. (1989). DNA was blotted onto a positively charged nylon membrane (Roche Molecular Biochemicals), then hybridized with DIG-labeled DNA probe (50 ng ml^{-1}) under stringent conditions (wash with 0.1 × SSC/0.1% SDS at 65°C for 15 min, 2 times). A chemofluorescent kit CSPD® (Roche Molecular Biochemicals) was applied to visualize the probe signal on X-ray film. To rehybridize the same membrane with different probes, the probe was denatured from the membrane with alkaline solution (0.2 N NaOH, 1% SDS) at 37°C for 30 min.

Gene cloning and sequencing. The endonuclease *Cla*I was used to digest the viral DNA of the crayfish isolate and the vector pBluescript II SK+ according to the manufacturer's protocol (Roche Molecular Biochemicals). The digested pBluescript plasmids were dephosphorylated using alkaline phosphatase (Roche Molecular Biochemicals) and then ligated with viral restriction fragments using T4 DNA ligase (Roche Molecular Biochemicals). Transformation was performed using JM109 High Efficiency Competent Cells (1×10^8 cfu $µg^{-1}$ DNA, Promega, Madison, WI) following the manufacturer's protocol. Resulting white bacterial clones were streaked for isolation and then grown to a large quantity in Terrific Broth (12 g Bactotrypton, 24 g yeast extract, 4 ml glycerol, 100 ml 0.17 M KH_2PO_4/0.72 M K_2HPO_4 in 1 H_2O). Plasmids were quickly extracted by using Insta-Mini-Prep™ (5 Prime - 3 Prime, Inc.) for initial screening. For preparing plasmids for sequencing, a modified mini alkaline-lysis/polyethylene (PEG) precipitation procedure was adapted from the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit protocol of Applied Bio-

systems, Inc. DNA sequencing was performed on an Applied Biosystem (ABI) 373 DNA sequencer using fluorescently labeled dideoxynucleotides and Taq polymerase at the Division of Biotechnology at the University of Arizona.

RESULTS AND DISCUSSION

All shrimp samples used for viral extraction were confirmed to be heavily infected with WSSV by histological examination and *in situ* hybridization (data not shown). The sizes of labeled probes LN4, C42, and A6 were 750, 450 and 1600 bp, respectively (Fig. 1). All these probes at similar concentration showed similar high specificity in the dot blot and *in situ* hybridization assays (data not shown).

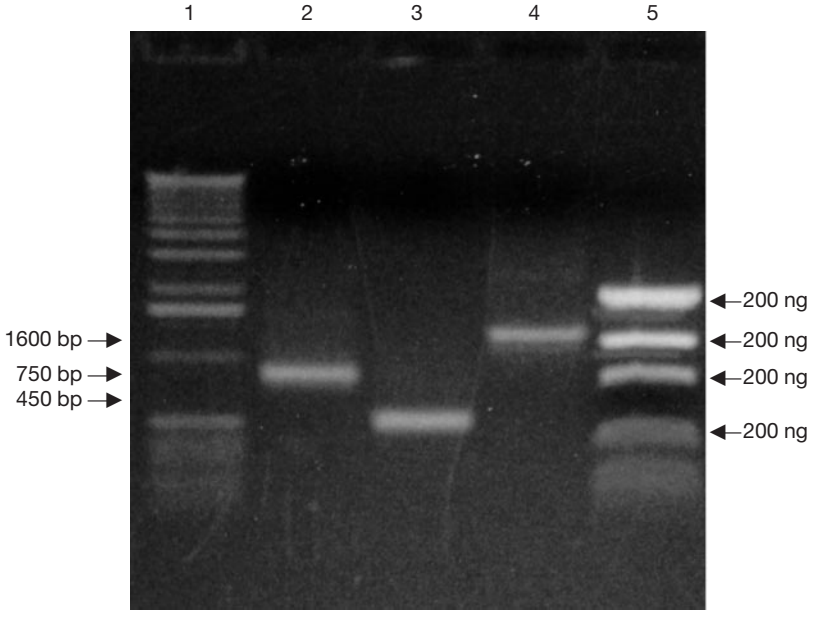

Fig. 1. Electrophoresis of specific WSSV probes LN4, C42 and A6 after the PCR labeling. Lane 1: 1 kb ladder; Lane 2: LN4; Lane 3: C42; Lane 4: A6; and Lane 5: low molecular weight mass ladder

The 5 shrimp isolates and 1 crayfish isolate of WSSV gave strong DNA hybridization signals for the areas of the genome studied. All 3 probes, LN4, C42, and A6, reacted with each of the 6 isolates of WSSV. No apparent differences were detected among the 5 geographic isolates originally obtained from shrimp. These 5 WSSV isolates presented very similar band patterns when their DNA was digested with endonucleases and hybridized with the 3 specific genomic probes. Our findings are consistent with the results reported in a previous publication (Lo et al. 1999) in which several PCR products were analyzed using the restriction fragment length polymorphism (RFLP) assay, with little or no band differences among WSSV infected shrimp from various geographic locations. However, the genomic fragments studied in this paper account for only about 1% of the total viral genome (200–300 kb), and it is possible that genomic differences may occur in other parts of the genome.

Differences in DNA composition between the crayfish isolate of WSSV and other WSSV isolates were detected through several combinations of restriction analysis and Southern blot hybridization, as summarized in Table 2. Of these combinations, *Cla*I for DNA digestion, and LN4 or C42 for hybridization, best dis-

Table 2. Different lengths of viral DNA fragments detected in the crayfish WSSV and other geographic isolates of WSSV by Southern blot analysis when using the following combinations of endonuclease digested DNA (*Bgl*II, *Cla*I, *Eco*RI, *Sac*I, and *Xho*I) and genomic probes (LN4, C42 and A6)

Genomic probe	Fragment lengths (kb) after digestion				
	*Bgl*II	*Cla*I	*Eco*RI	*Sac*I	*Xho*I
Crayfish WSSV					
LN4	6	3, 7	2.2, 3	15	4.5
C42	0.1, 5	7			
A6	15				
Other WSSV					
LN4	15	3, 3.5	2.2, 2.8	>21	15
C42	0.1, 18	5.2			
A6	10				

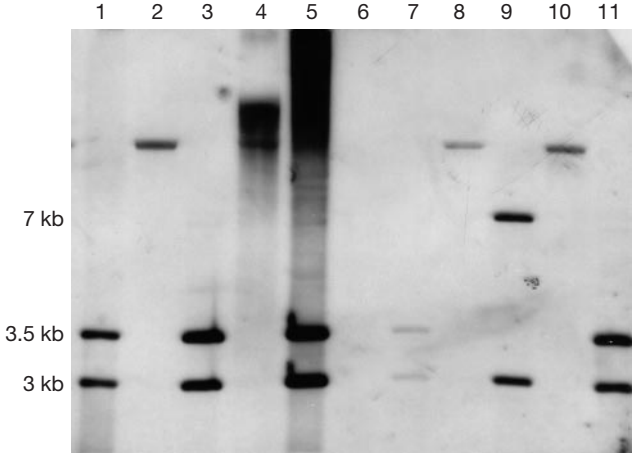

Fig. 2. Southern blot hybridization with the LN4 probe. Lanes 1, 3, 5, 7, 9, and 11 show DNA of WSSV digested with *Cla*I from the following geographic regions: 1, China; 3, India; 5, Thailand; 7, South Carolina; 9, crayfish from Washington, DC; 11, Texas. Lanes 2, 4, 6, 8, and 10 show undigested DNA of WSSV from the following regions: 2, India; 4, Thailand; 6, South Carolina; 8, crayfish from Washington, DC; 10, Texas

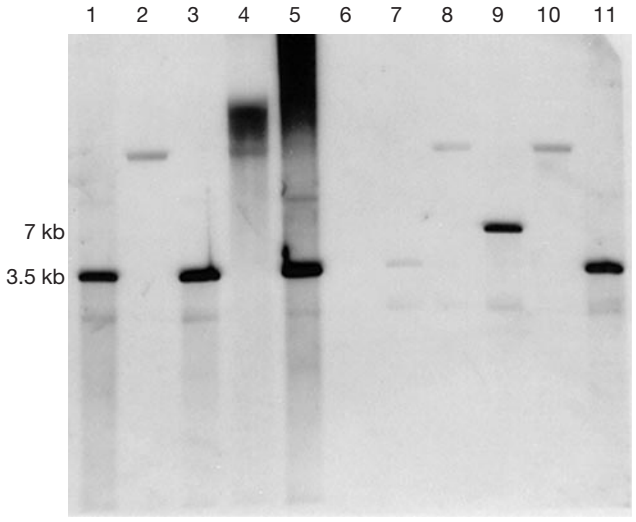

Fig. 3. Southern blot hybridization with the C42 probe. Lanes 1, 3, 5, 7, 9, and 11 show DNA of WSSV digested with *Cla*I from the following geographic regions: 1, China; 3, India; 5, Thailand; 7, South Carolina; 9, crayfish from Washington, DC; 11, Texas. Lanes 2, 4, 6, 8, and 10 show undigested DNA of WSSV from the following regions: 2, India; 4, Thailand; 6, South Carolina; 8, crayfish from Washington, DC; 10, Texas

tinguished the crayfish isolate of WSSV (Fig. 2 & 3). Since differences occurred with several endonucleases, it is not likely that the difference was due to a point mutation randomly arising in a viral population. However, it is possible that a host species may play a role in the selection of a mutant within a viral population. Further studies are needed to conclude whether the differences observed resulted from geographic isolation or from host selection. That the crayfish isolate of WSSV is slightly different from other WSSV isolates is also supported by previous virulence and protein composition studies (Wang et al. 1999, 2000). More research is needed to correlate information on the genomic sequence, protein data and virulence. The method used in this paper, however, can be employed to distinguish crayfish WSSV from the other WSSV isolates. The technique may also be useful for examination of new WSSV isolates.

The Southern blot hybridization results indicate that the regions flanking the C42 and A6 fragments have fewer variations than the region flanking the LN4 fragment. The 2.8 kb *Cla*I fragment from the crayfish isolate of WSSV (GenBank AF178573), which overlaps a portion of the LN4 region, was compared with the LN4 sequence data from the Thailand isolate (Fig. 4). The LN4 fragment itself was almost identical in the 2 isolates, except in the primer region, which was not unexpected since the LN4 primer was originally developed using the sequence data from a different penaeid virus of shrimp, BP (Nunan & Lightner 1997). Mismatched base pairs between the 2 fragments are not unexpected. The LN4 fragment was sequenced from a PCR product, and single base pair substitutions and frame shift errors are inherent in the PCR process (Bell 1989). The Southern blot results (Figs. 2 & 3) and the sequence information from the 2.8 kb *Cla*I and LN4 fragments (Fig. 4) (Nunan & Lightner 1997) de-

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   1  TGTAGCAGCA GAGAAGAGAG GTTAACAACA ACACCAGTAA GGAAAATGAT
2471  AAGAGGAACA GAGAAGAGAG GTTAACAACA ACACCAG-AA GGAAAATGAT

  51  ACAATGGAAA AAAAGACTGA GACGGCTGCA ACAACAGAAA AAGACCCAGA
2521  ACAATGGAAA AAAAGACTGA GACGGCTGCA AC-ACAGAAA AAGACCCAGA

 101  ACCGTCTGTC AGTAAAAGGT CCAGAAATAA AGAACCCAAA ACAACTTCTA
2569  ACCGTCTGTC AGTAAAAGGT CCAGAAATAA AGAACCCAAA ACAACTTCTA

 151  CTGTTTACAC TTCTGTAAAG TGTTACCTTT CTTCCATAAT CAAGAGTGAA
2619  CTGTTTACAC TTCTGTAAAG TGTTACCTTT CTTCCATAAT CAAGAGTGAA

 201  AGTAGTAGAA GTAATGTCAC CTCAACCAAA GAAAGGTTTG AGGAGAGGTG
2669  AGTAGTAGAA GTAATGTCAC CTCAACCAAA GAAAGGTTTG AGGAGAGGTG

 251  TAAATCCGTA AGCAAGATGA TGGTCAAAGG TTCACTTGTT TTTGGGGGTT
2719  TAAATCCGTA AGCAAGATGA TGGTCAAAGG TTCAC-TGTT TTT-GAGGTT

 301  AGTAGTGGAC GAGTGTCTTG AGACGTTACA AACCATCTTA GAAGACGAAA
2769  AGTAGTGGAC GAGTGTC-TG AGACGTTAC- AACCATC-TA GAAGACGAAA

 351  TCGAT
2815  TCGAT
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Fig. 4. Sequence comparison of the overlapping region of the LN4 PCR fragment from the Thailand WSSV isolate (upper sequence) and the 2.8 kb *Cla*I fragment from the crayfish WSSV isolate (lower sequence in **bold**). The underlined region in the LN4 sequence denotes the forward primer. Homologous base pairs are shaded

monstrate that the variation in the crayfish WSSV genome is located about 3.5 kb from the *Cla*I site of the 2.8 kb fragment. The significance of this region to pathogenicity has not yet been determined. When the full sequence of the flanking region of the LN4 fragment is known, a PCR assay may be developed to differentiate different WSSV strains.

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