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Genotyping of White Spot Syndrome Virus in Chinese Cultured Shrimp during 1998-1999*

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Abstract: Recent studies showed that white spot syndrome virus (WSSV) isolates from different geographic locations share a high genetic similarity except the variable regions in ORF23/24 and ORF14/15, and variable number of tandem repeats (VNTR) within ORF94. In this study, genotyping was performed according to these three variable regions among WSSV isolates collected during 1998/1999 from Southern China. These WSSV isolates contain a deletion of 1168, 5657, 5898, 9316 and 11093 bp, respectively in the variable region ORF23/24 compared with WSSV-TW, and a deletion of 4749 or 5622 bp in the variable region ORF14/15 relative to TH-96-II. Four types of repeat units (RUs) (6, 8, 9 and 13 RUs) in ORF94 were detected in these isolates, with the shortest 6 RUs as the most prevalent type. Our results provide important information for a better understanding of the spatio-temporal transmission mode and the WSSV genetic evolution lineage.

Key words: WSSV; Genotyping; Variable region ORF23/24; ORF14/15; ORF94, China

White spot syndrome virus (WSSV), the unique member within the virus family *Nimaviridae* [10, 17], has been the most devastating aquatic virus infecting shrimp and other crustaceans. It can cause up to 100% cumulative mortality within 3-7 days in infected shrimps and leads to enormous losses to the shrimp farming industry worldwide [7]. Since WSSV was first reported in Fujian province in China in 1991, it has been found in almost all shrimp farming areas all over the world [4].

The WSSV particle is rod-shaped about 275 nm in length and 120 nm in width with a tail-like appendage at one end [3]. WSSV contains a circular, supercoiled, double-stranded DNA genome, estimated to be about 300 kilobase pairs (kb). Three WSSV isolates have been sequenced so far, WSSV-TW [18], WSSV-CN [20] and WSSV-TH[16]. Various geographical WSSV isolates share similar genomic and proteomic characteristics. However, there are still several genetic variations along WSSV genome which were widely used for genotyping the WSSV isolates [2, 8, 9, 13]. These

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variable regions include hot deletion regions in ORF23/24 and ORF14/15 (corresponding to WSSV-TH), and variations in repeat units (RUs) numbers within homologous regions (*hrs*) [8]. In this study, these variable regions were analyzed in WSSV isolates collected from cultured shrimp in China during 1998-99.

MATERIALS AND METHODS

Shrimp sampling

The WSSV-infected shrimp specimens in this study were collected from WSSV outbreak farms in three cities of two provinces along East China Sea and South China Sea: Ning-Bo (NB), Shen-Zhen (SZ) and Guang-Zhou (GZ). The origin of shrimp was documented in Table 1. All samples were stored at -80℃ until further analysis.

DNA extration

Genomic DNA was extracted from the shrimp gill tissue using the standard Phenol/Chloroform DNA extraction protocol. Briefly, 0.1 mg of frozen gill tissue was homogenized using 300 μL of lysis buffer containing 0.4 mol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid (EDTA) and 10 mmol/L Tris-HCL (pH7.5) in a 1.5 mL tube and centrifuged at 6000 ×*g* for 10 min. Supernatant was collected and supplemented with sodium dodecyl sulphate and Proteinase K to a final concentration of 2% and 200 μg /mL, respectively. Samples were incubated at 56 ℃ for 3 h and nucleic acid was separated from the proteinaceous components using phenol-chloroform extraction. Purified DNA was precipitated with cold ethanol or isopropanol and then washed with 75% cold ethanol. Extracted DNA was stored in TE buffer (10mmol/L Tris-HCl, 1mmol/L EDTA, pH 8.0). The quality and quantity of each sample was determined photometrically using a Biophotometer (Eppendorf). All DNA extractions were stored at -20 ℃until use.

PCR analysis of WSSV variable regions

The shrimp samples were screened for WSSV with a pair of primer designed from WSSV VP26 gene [2] (Table 2), using *Taq* polymerase (BioStar). PCR amplification was conducted to pinpoint the 3 variable regions in the WSSV positive samples. Primers VR23/24-3, VR23/24-6 and VR14/15-1F [14] were used to further characterize variable region ORF23/24 and ORF14/15, respectively. VNTR in ORF94 was analyzed with PCR primers described by Dieu *et al* [2]. All primers used in this study, PCR conditions and the sizes of the expected PCR products are list in Table 2.

Sequencing and analysis

Amplified PCR products were purified using the PCR purification kit (Omega) and bi-directionally sequenced (Invitrogen) using the respective forward and reverse primers described above. The sequence data were analyzed using the DNASTAR 4.2

Table 1. Sample information of WSSV isolates

WSSV isolate	Host	Place	Province	Date of collection
WSSV98NB1	*Penaeus monodon*	Ning-Bo	Zhe-Jiang	May 1998
WSSV98NB2	*Penaeus chinensis*	Ning-Bo	Zhe-Jiang	May 1998
WSSV98SZ1	*Penaeus chinensis*	Shen-Zhen	Guang-Dong	June 1998
WSSV98SZ2	*Penaeus vannamei*	Shen-Zhen	Guang-Dong	June 1998
WSSV98SZ3	*Penaeus monodon*	Shen-Zhen	Guang-Dong	June 1998
WSSV98SZ4	*Penaeus chinensis*	Shen-Zhen	Guang-Dong	June 1998
WSSV99GZ	*Penaeus monodon*	Guang-Zhou	Guang-Dong	June 1999

Table 2. Primer sequences and PCR conditions used during PCR analysis

Primer Pair	Sequence (5'-3')	Annealing (℃) / Elongation time (s)	Viral sequence coordinate	Size of PCR product (bp) and References
WSSV screening				
VP26F	ATGGAATTTGGCAACCTAACAAACCTG	52 / 30	213603-213629[a]	304[a]
VP26R	GGGCTGTGACGGTAGAGATGAC		213347-213326[a]	[16]
ORF23/24				
VR23/24-southF	CTACAACGGCCAAGTCAT	52 / 240	2126-2143[a]	13047[a]
VR23/24-1R	ATGATTGTATTCGTCGAAGG		15191-15172[a]	[2]
VR23/24-3F	CTTCTTCTGGCTCCCTCTT	51 / 30	283267-283285[b]	546[b]
VR23/24-3R	ATTAAACCATCGACATCCC		283812-283794[b]	[14]
VR23/24-6F	AAAGGCAAACGAAACGTAC	52 / 120	280082-280100[b]	6400[b]
VR23/24-6R	TTCACAGGCATGTGGAGGC		286481-286463[b]	[14]
ORF14/15				
VR14/15-1F	CAAGGCACCTTCAAGGCTGT	51 / 60	22658-22677[c]	This study
VR14/15-screenF	GAGATGCGAACCACTAAAAG		22904-22923[c]	1254[c]
VR14/15-screenR	ATGGAGGCGAGACTTGC		24157-24141[c]	[2]
ORF94				
ORF94-F	TCAATGAGAGCTTGGTCC	51/80	142656-142672[c]	682[c]
ORF94-R	AAGTAGACAGCCGCGCTT		143337-143319[c]	[2]

[a] According to WSSV-TW sequence, GenBank accession NO. AF440570. [b] According to WSSV-CN sequence, GenBank accession NO. AF332093. [c] According to WSSV-TH sequence, GenBank accession NO. AF369029.

(DNASTAR Inc.) software package. Alignment of nucleotide sequences was conducted using software ClustalW, version 1.83 software[15]. The number of tandem repeat within ORF94 of each WSSV isolate was determined by using Tandem Repeats Finder (TRF) program [1]. All sequences were compared with three complete WSSV genomes, which are obtained from the NCBI database (http://www.ncbi.nlm. nih.gov/). GenBank accession numbers of the WSSV on the NCBI server are as follows WSSV-TW (AF440570), WSSV-CN (AF332093) and WSSV-TH (AF369029).

RESULTS

All shrimps in this study were chosen randomly from samples showing obvious signs of white spots disease. Samples were confirmed as WSSV positive by PCR using primer pair VP26 [2], and all sequences are identical to the published VP26 gene sequences (data not shown). One shrimp from each place was chosen randomly as the representative geographical isolate. Three categories of variable genetic loci were chosen as the genetic markers to analyze genomic variations among different WSSV isolates.

Variable region ORF23/24

Variable region ORF23/24, a hot deletion region, is within ORF23 and ORF24 coding frames of WSSV-TH genome. Th-96-II and WSSV-TW isolates contain the longest fragment in this region (Fig. 1). Other WSSV isolates identified from various geographical locations all contain deletions with varying sizes [2, 6, 8, 13, 14]. In this study, with the primer pair VR23/24-southF and VR23/24-1R [2] WSSV-98NB2 and WSSV98SZ3 isolates gave an amplicon of 1980 bp, and WSSV99GZ of 3756 bp. These results indicated that WSSV98NB2 and WSSV98SZ3 contain a deletion of 11093 bp and the WSSV99GZ of 9316 bp, compared with WSSV-TW (Fig.1). Surprisingly, with the primer pair VR23/24-3 [14], a 546 bp PCR

amplicon was obtained from WSSV98SZ3. Sequence of this PCR product revealed that the WSSV98SZ3 sample contains a second isolate with a deletion of 1168 bp in ORF23/24 and has a similar genomic structure to WSSV-CN, which was isolated in 1996 in Fujian province, China (Fig. 1). With the extended flanking primer pair VR23/24-6 designed in this study (Table 2), a 1910 bp fragment was amplified from WSSV98NB1 and WSSV98NB2 , a 1669 bp fragment from WSSV98SZ1, WSSV98SZ2, and WSSV98SZ4 isolates, indicating that WSSV98NB1 and WSSV 98NB2 contain a deletion of 5657 bp, and WSSV 98SZ1, WSSV98SZ2, and WSSV98SZ4 contain a deletion of 5898 bp compared with WSSV-TW (Fig. 2).

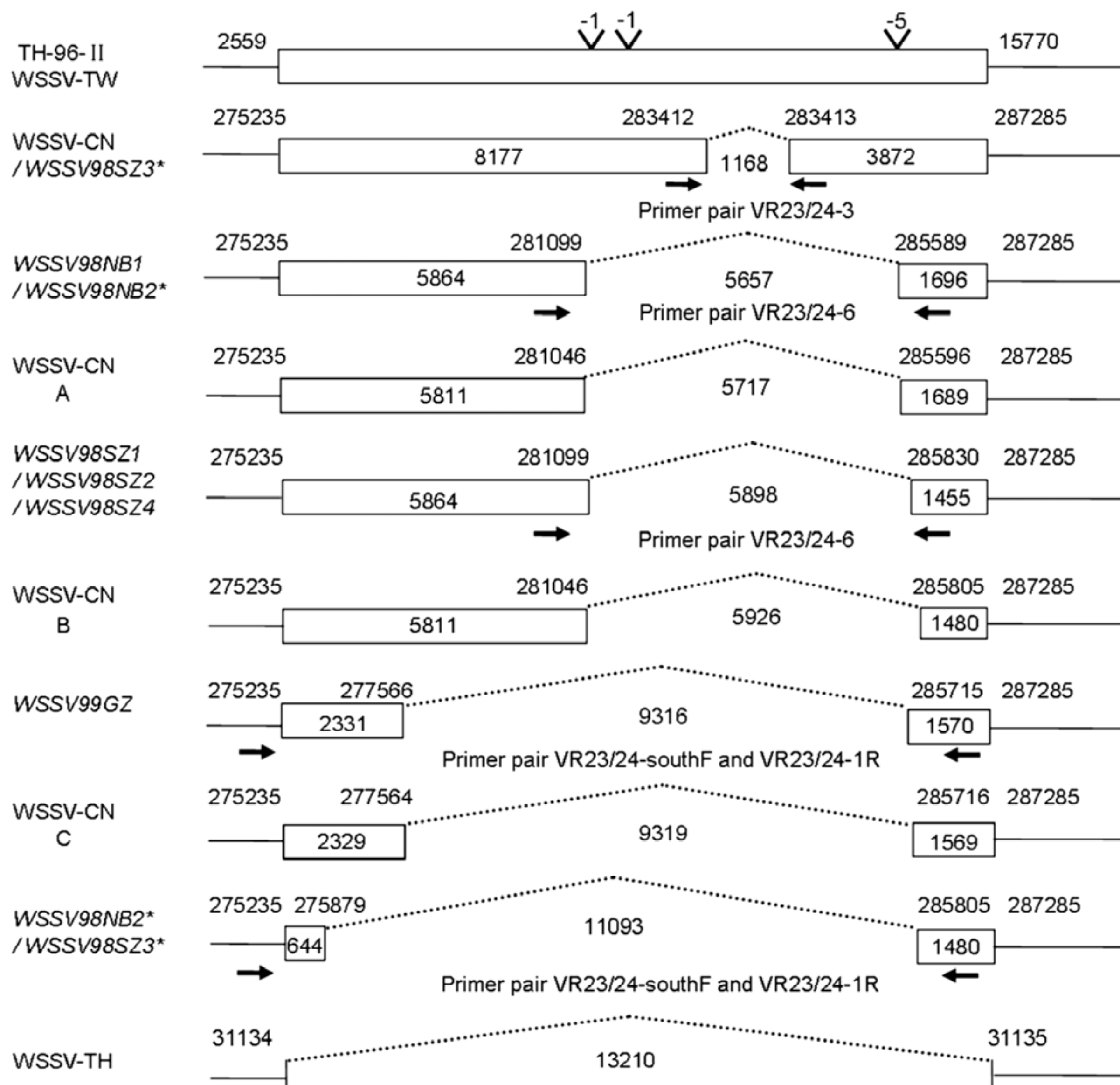

Fig.1. Schematic representation of the variable region ORF23/24. The WSSV isolates in this study are shown in italics. Nucleotide positions are denoted according to the respective WSSV isolates in GenBank database. Nucleotide coordinates of the Chinese WSSV isolates are assigned according to the annotation for the WSSV-CN isolate. The length of the flanking nucleotide sequence is indicated in the boxes. WSSV strains marked by asterisks indicate the coinfection with multiple genotypes in one individual shrimp. Arrows indicate the annealing location of each primer.

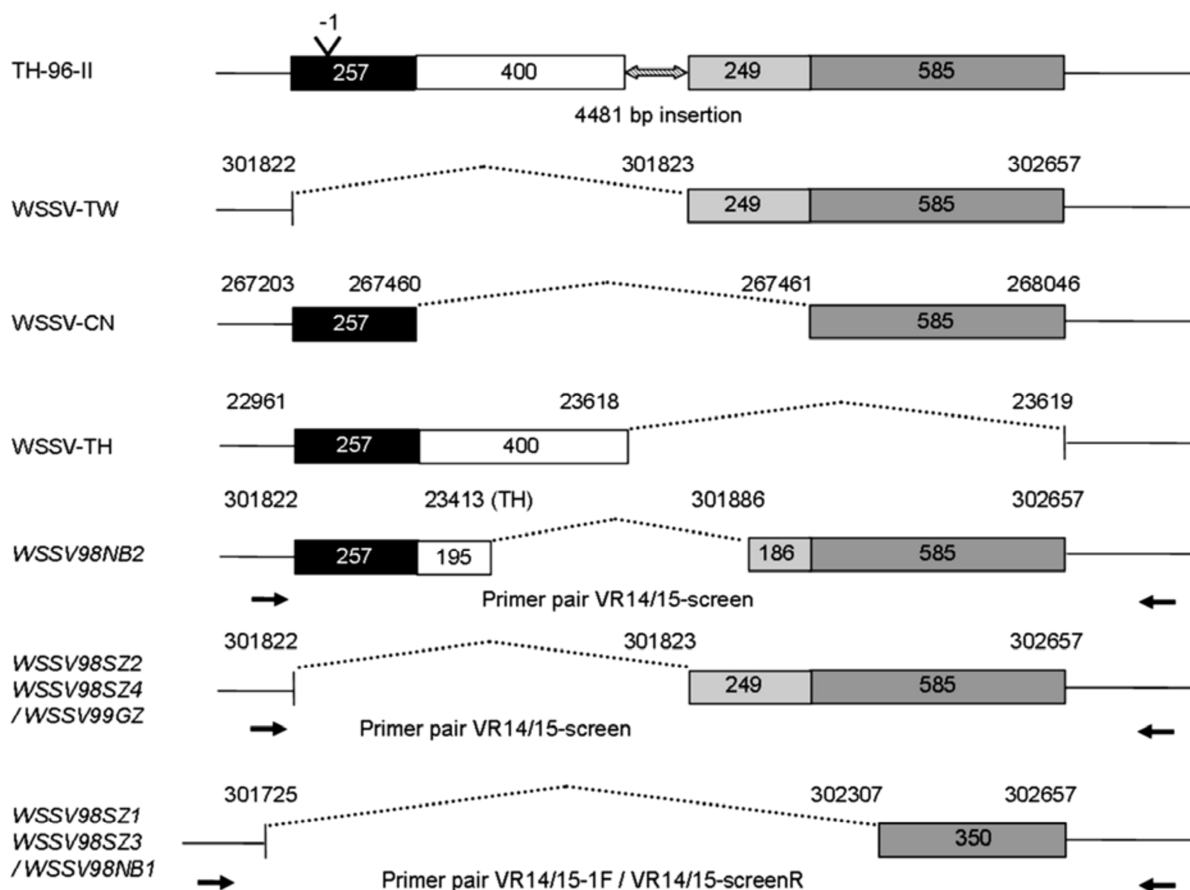

Fig. 2. Schematic representation of the variable region ORF14/15. The WSSV isolates in this study are shown in italics. Nucleotide positions are denoted according to the respective WSSV isolates in GenBank database. Nucleotide coordinates of the Chinese WSSV isolates assigned according to the annotation for the WSSV-TW isolate. The length of the flanking nucleotide sequence is indicated in the boxes. The bidirectional arrow indicates insertion of 4481 bp of TH-96-II in variable region ORF14/15. Short arrows indicate annealing location of each primer.

The WSSV98NB2 were coinfected with two isolates, one with a deletion of 11093 and another with 5657 bp in ORF23/24 region. All the flanking sequences of the deletion showed 100% identity to the counterpart of WSSV-TW and WSSV-CN.

Compared with other published WSSV isolates, WSSV98NB1, WSSV98SZ1, WSSV98SZ2, and WSSV98SZ4 contain deletions of similar sizes with WSSV-CN-A and WSSV-CN-B which contain deletions of 5717 and 5926 bp, respectively, and WSSV99GZ has a deletion fragment as WSSV-CN-C [6]. The larger deletion of 11093 bp in WSSV98SZ3 and WSSV98NB2 is similar to that of WSSV-VN (Tv) which contains a deletion of 11450 bp (Fig. 2) [2].

Variable region ORF14/15

Variable region ORF14/15 locates within the WSSV-TH segment coding for ORF14 and ORF15, and has been confirmed to be prone to recombination [2, 8, 13]. Up to now, it was found that the TH-96-II isolate contains the largest fragment in this region[9]. Using the same mapping strategy as used for variable region ORF23/24, we pinpointed the variation within ORF14/15 among different WSSV isolates. Primer pair VR14/15-screen [2] successfully produced a 1819 bp amplicon in the WSSV98SZ2 isolate, and a 1431 bp amplicons in WSSV98SZ2, WSSV98SZ4, and

WSSV99GZ isolates, but not in other samples. Sequencing result showed that WSSV98NB2 contains a 4749 bp deletion compared to TH-96-II, and a 381 bp insertion compared to WSSV-CN. The WSSV-98SZ2, WSSV98SZ4 and WSSV99GZ isolates have identical structures relative to WSSV-TW, which contain a 5140 bp deletion compared to TH-96-II. Subsequently, by extending the forward primer, VR14/15-1F/VR14/15-screenR produced a 1095 bp amplicon in WSSV98SZ1, WSSV98SZ3 and WSSV-98NB1, indicating that these isolates contain a 5719 bp deletion compared to TH-96-II, and part of 585 bp in the corresponding region of WSSV-TW (Fig. 2).

Variable number tandem repeat (VNTR) in ORF94

The ORF94 (corresponding to WSSV-TH) locates between genes encoding the large (RR1) and small (RR2) subunits of ribonucleotide reductase. In this ORF, various WSSV isolates show different numbers of 54 bp of Repeat Unit (RU), furthermore, the position 48 of each RU shows a single nucleotide polymorphism (SNP) (guanine or thymine) [2, 5, 11, 12, 19]. After PCR amplification with the flanking primer pair ORF94 F/R [2] of this region and sequences analysis, 4 different repeat types ranging from 6 to 14 RUs were obtained among the analyzed WSSV isolates, with the 6 RUs as predominant type (Table 3). Consistently, three categories of tandem repeat sequences were obtained from WSSV98NB2: 14, 9 and 6 RUs, further confirmed that WSSV98NB2 was coinfected with two or three WSSV isolates. Nucleotide polymorphism analysis at position 48 of each RU indicated that thymine is more common than guanine.

DISCUSSION

Chinese shrimp farming industry has been suffering from WSSV rampant spread since its first discovery in China, however, little information has been reported concerning the molecular epidemiology of WSSV [6]. In this study, we focused on the genomic variation of WSSV using molecular tools, which not only enhance our understanding of the quick geographical spread of the virus, but also help to identify virulence-related genes on the WSSV genome.

WSSV genomic variation has been seen as a departure to address fundamental questions concerning how WSSV evolved overt time and space as well as the regulation of gene expression of WSSV in this process. Since WSSV was firstly discovered in the early 1990s, various WSSV isolates have been identified in different regions which displayed important sequence variations [2, 8, 13, 14]. Our results showed that the prevalent WSSV isolates in China during 1998-1999 displayed great genetic diversity in

Table 3. Variable number of tandem repeats within ORF94 in analyzed WSSV isolates from shrimp samples

WSSV isolate	Amplification size (bp)	No. of RU	Genotypes (Position 48)
WSSV98NB1	790	8	GTGTGTTT
	1157	14	GTGGTTTTTTTTTT
WSSV98NB2	962	9	GTGGTTTTT
	682	6	GTGTTT
WSSV98SZ1	682	6	GTGTTT
WSSV98SZ2	682	6	GTGTTT
WSSV98SZ3	682	6	GTGTTT
WSSV98SZ4	808	9	TTTGTGTTT
WSSV99GZ	682	6	GTGTTT

two variable regions, ORF23/24 and ORF14/15. ORF23/24 contained 5 different deletions ranging from 1168 to 11093 bp, compared to WSSV-TW. WSSV isolates with intermediate or large size of deletions in the ORF23/24 region are predominant in this study, which is consistent with our previous results [14].

According to ORF14/15 region, all the WSSV isolates from our samples have three genotypes. Compared to WSSV-TH-96-II, which has the largest fragment in ORF14/15 region, a 4749 bp deletion was detected in WSSV98NB2, a 5719 bp deletion in WSSV98SZ1, WSSV98SZ3 and WSSV98NB1, and a 5138 bp deletion in WSSV98SZ2, WSSV98SZ4, and WSSV99GZ. The finding that the deletions in the WSSV genomes are larger than the ones reported previously suggests that the WSSV genome has been suffering a progressive deletion in this region.

Due to the variable number of tandem repeats (VNTR), ORF94 is believed to be a better marker than ORF75 and ORF125 for general epidemiological studies of WSSV [12]. In previous studies, a wide range of RU number (2 RUs to 20 RUs) in ORF94 have been identified from geographically different isolates [2, 11, 12, 19]. But in this study, 6 RUs was found to be the predominant genotype. However, WSSV98NB2 contained three genotypes (6, 9 and 14 RUs), suggesting a co-infection of multiple WSSV genotypes. Together with the two genotypes of deletion in ORF23/24 for WSSV98NB2, a co-infection with various genotypes has occurred among this sample.

According to the increasing deletions in the variable regions ORF23/24 and ORF14/15 among various WSSV isolates in China, Thailand and Vietnam, Dieu *et al* proposed that the ancestral WSSV isolates spread from either side of Taiwan Strait into Vietnam by multiple pathways [2]. However, TH-96-II, a putative ancestral of the WSSV isolate which contained the largest genome until now (~312 kb) was obtained from *Penaeus monodon* in Thailand in 1996 [9], not in Chinese South-east coast where WSSV was firstly discovered. Recently, Pradeep *et al* reported that Indian strains were more closely related to Thailand, suggesting WSSV spreading from Thailand to India and other regions of the world, but not southern regions of China [13]. We suppose that WSSV has existed in the wild shrimp grown within the aquatic regions of Southeast Asia prior to the first severe outbreak of white spot syndrome (WSS) in China. Therefore, it is essential to obtain more data on molecular epidemiology of WSSV among cultured and wild shrimp samples in Southeast Asia to better understand the origin, evolution, and transmission route of this virus in this region.

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References

1. **Benson G.** 1999. Tandem repeats finder:a program to analyze DNA sequences. **Nucleic Acids Res,** 27: 573-580.
2. **Dieu B T, Marks H, Siebenga J J,** *et al*. 2004. Molecular epidemiology of white spot syndrome virus within Vietnam. **J Gen Virol**, 85: 3607-3618.
3. **Durand S, Lightner D V, Redman R M,** *et al*. 1997. Ultrastructure and morphogenesis of White spot syndrome baculovirus (WSSV). **Dis Aquat Organ,** 28: 205-211.
4. **Escobedo-Bonilla C M, Alday-Sanz V, Wille M,** *et al*. 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot

syndrome virus. **J Fish Dis,** 31: 1-18.

5. **Hoa T T T, Hodgson R A J, Oanh D T H,** *et al*. 2005. Genotypic variations in tandem repeat DNA segments between ribonucleotide reductase subunit genes of white spot syndrome virus (WSSV) isolates from Vietnam. In: **Diseases in Asian Aquaculture V, Fish Health Section (**Walker P, Lester R, Bondad-Reantaso, ed.), Asian Fisheries Society, Manila (2005): 339-351.
6. **Lan Y, Lu W, Xu X.** 2002. Genomic instability of prawn white spot bacilliform virus (WSBV) and its association to virus virulence. **Virus Res,** 90: 269-274.
7. **Lightner DV**. 1996. **A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp**. In Special publication of the World Aquaculture Society LA: Baton Rouge
8. **Marks H, Goldbach R W, Vlak J M,** *et al*. 2004. Genetic variation among isolates of White spot syndrome virus. **Arch Virol,** 149: 673-697.
9. **Marks H, van Duijse J J, Zuidema D,** *et al*. 2005. Fitness and virulence of an ancestral White Spot Syndrome Virus isolate from shrimp. **Virus Res,** 110: 9-20.
10. **Mayo M A**. 2002. A summary of taxonomic changes recently approved by ICTV. **Arch Virol,** 147: 1655-1663.
11. **Musthaqa S S, Sudhakarana R, Ishaq Ahmeda V P,** *et al*. 2006. Variability in the tandem repetitive DNA sequences of white spot syndrome virus (WSSV) genome and suitability of VP28 gene to detect different isolates of WSSV from India. **Aquaculture,** 256: 34-41.
12. **Pradeep B, Shekar M, Gudkovs N,** *et al*. 2008. Genotyping of white spot syndrome virus prevalent in shrimp farms of India. **Dis Aquat Organ,** 78: 189-198.
13. **Pradeep B, Shekar M, Karunasagar I,** *et al*. 2008. Characterization of variable genomic regions of Indian white spot syndrome virus. **Virology,** 376: 24-30.
14. **Tan Y, Xing Y, Zhang H,** *et al*. 2009. Molecular detection of three shrimp viruses and genetic variation of white spot syndrome virus in Hainan Province, China, in 2007. **J Fish Dis,** 32: 777-784.
15. **Thompson J D, Higgins D G, Gibson T J**. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Res,** 22: 4673-4680.
16. **van Hulten M C, Witteveldt J, Peters S,** *et al*. 2001. The white spot syndrome virus DNA genome sequence. **Virology,** 286: 7-22.
17. **Vlak J M, Bonami J R, Flegel T W,** *et al*. 2005. Nimaviridae. In VIIIth Report of the International Committee on Taxonomy of Viruses. Edited by C M Fauquet, M A Mayo, J Maniloff, U Desselberger & L A Ball: Elsevier pp: 187-192.
18. **Wang C H, Lo C F, Leu J H,** *et al*. 1995. Purification and genomic analysis of baculovirus associated with White spot syndrome (WSBV) of Penaeus monodon. **Dis Aquat Org,** 23: 239-242.
19. **Wongteerasupaya C, Pungchai P, Withyachumnarnkul B,** *et al*. 2003. High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. **Dis Aquat Organ,** 54: 253-257.
20. **Yang F, He J, Lin X,** *et al*. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. **J Virol**, 75: 11811-11820.