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Molecular epidemiology of white spot syndrome virus within Vietnam

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White spot syndrome virus (WSSV), the sole member of the virus family *Nimaviridae*, is a large double-stranded DNA virus that infects shrimp and other crustaceans. By alignment of three completely sequenced isolates originating from Taiwan (WSSV-TW), China (WSSV-CN) and Thailand (WSSV-TH), the variable loci in the genome were mapped. The variation suggests the spread of WSSV from a common ancestor originating from either side of the Taiwan Strait to Thailand, but support for this hypothesis through analysis of geographical intermediates is sought. RFLP analysis of eight Vietnamese WSSV isolates, of which six were collected along the central coast (VN-central) and two along the south coast (VN-south), showed apparent sequence variation in the variable loci identified previously. These loci were characterized in detail by PCR amplification, cloning and sequencing. Relative to WSSV-TW, all VN-central isolates showed a ~8·5 kb deletion in the major variable region ORF23/24, whereas the VN-south isolates contain a deletion of ~11·5 or ~12·2 kb, compared to a ~1·2 or ~13·2 kb deletion in WSSV-CN and WSSV-TH, respectively. The minor variable region ORF14/15 showed deletions of various sizes compared with WSSV-TH for all eight VN isolates. The data suggest that the VN isolates and WSSV-TH have a common lineage, which branched off from WSSV-TW and WSSV-CN early on, and that WSSV entered Vietnam by multiple introductions. A model is presented for the spread of WSSV from either side of the Taiwan Strait into Vietnam based on the gradually increasing deletions of both 'variable regions'. The number and order of repeat units within ORF75 and ORF125 appeared to be suitable markers to study regional spread of WSSV.

INTRODUCTION

White spot syndrome virus (WSSV) is a large double-stranded (ds) DNA virus belonging to the family *Nimaviridae* (Mayo, 2002). Since its discovery in China (Fujian) in 1991/1992, the virus spread quickly, causing serious losses to commercial shrimp farming worldwide (Cai *et al.*, 1995; Flegel, 1997). The natural marine ecology is also threatened by WSSV as the virus has a wide host range, including salt and brackish water penaeids, crabs, spiny lobsters and freshwater shrimp and crayfish (Lo *et al.*, 1996; Flegel, 1997; Wang *et al.*, 1998; Chen *et al.*, 2000; Maeda *et al.*, 2000; Hameed *et al.*, 2003). An overt clinical sign of infected shrimp is the presence of white spots on the exoskeleton (Chou *et al.*, 1995).

Electron microscopic analysis showed that WSSV consists of a rod-shaped nucleocapsid with a cross-hatched appearance, surrounded by a trilaminar envelope with a unique tail-like appendix at one end (Wongteerasupaya *et al.*, 1995; Durand *et al.*, 1997; Nadala *et al.*, 1998). The circular dsDNA genome of WSSV has a size of around 300 kb and is one of the largest animal virus genomes that has been entirely sequenced (van Hulten *et al.*, 2001; Yang *et al.*, 2001). Only 6 % of the putative 184 ORFs encoded by the viral genome have homologues in public databases, mainly representing genes encoding enzymes for nucleotide metabolism, DNA replication and protein modification (van Hulten *et al.*, 2001).

In addition to South-East Asia, WSSV has been reported from the United States in 1995 (Rosenberry, 1996) and from Central and South America since early 1999 (Rosenberry, 2000). In 2002, WSSV was also detected in France and Iran (Rosenberry, 2002). The various geographical isolates of WSSV identified so far are very similar in morphology and proteome. Limited differences in RFLP patterns have been reported, suggesting either a high degree of genomic

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stability or a recent emergence (Nadala & Loh, 1998; Lo *et al.*, 1999; Wang *et al.*, 2000a, b; Marks *et al.*, 2004). Preliminary studies indicated that there is also little difference in virulence between various WSSV isolates, although direct comparisons were not made (Wang *et al.*, 1999; Lan *et al.*, 2002). After the complete sequencing of three different WSSV isolates originating from Taiwan (WSSV-TW; Wang *et al.*, 1995), China (WSSV-CN; Yang *et al.*, 2001) and Thailand (WSSV-TH; van Hulten *et al.*, 2001), the major variable loci in the WSSV genome were mapped by alignment of these sequences (Marks *et al.*, 2004). Roughly, the variable loci can be divided into deletions, variable numbers of tandem repeats (VNTRs), single nucleotide indels and single nucleotide polymorphisms (SNPs). The variation within these loci, in particular in the large genomic deletions, suggested a geographical spread from a common ancestor from either side of the Taiwan Strait to Thailand (Marks *et al.*, 2004), but genetic intermediates were missing to support this hypothesis.

The present study focuses on WSSV isolates from Vietnam (VN), from eight different locations along the central and south coast. The variable loci, as identified by Marks *et al.* (2004), were subjected to detailed analysis, including sequencing. Using these newly characterized WSSV-VN genotypes, the value of each of the identified loci as a genetic marker for strain identification as well as epidemiological and ecological studies is evaluated. Furthermore, molecular typing was used to analyse the relationship between the eight WSSV isolates from Vietnam and those from Taiwan, China and Thailand. The genetic changes could be correlated with the spread of WSSV radiating out from either side of the Taiwan Strait to Thailand.

METHODS

Infected shrimp sampling. The origin of the collected WSSV-infected shrimp (*Penaeus monodon*) analysed in this study is shown in Table 1. The shrimp were cleaned with ethanol and transported in liquid nitrogen from the respective ponds to Can Tho University (Vietnam), where they were stored at −80 °C until further processing.

DNA extraction. DNA extracts of collected shrimp were obtained from muscle tissue. A small piece (approx. 50 mg) of the tail of dead shrimp was homogenized using a disposable rod and mixed with 200 µl 5 % (w/v) Chelex X-100 resin (Bio-Rad) and 16 µl proteinase K (20 mg ml^{-1} stock). This mixture was incubated overnight at 56 °C, followed by 10 min at 95 °C to inactivate the proteinase K and centrifugation for 1 min at 18 000 ***g*** to pellet cellular debris. One microlitre of the supernatant was used in PCRs.

PCR analysis of WSSV-infected shrimp. To screen for WSSV, we developed a standardized PCR-based WSSV detection protocol. One microlitre of DNA extract was tested in two similar single-step PCRs with a shrimp 16S rRNA or a WSSV VP26 primer pair (Table 2), using *Taq* DNA polymerase (Promega). The 16S rRNA primer pair amplifies a shrimp mitochondrial DNA fragment encoding the 16S rRNA and is used as a positive control for the presence of host DNA. The VP26 primer pair amplifies part of the WSSV VP26 ORF (van Hulten *et al.*, 2000b) and is used to screen for WSSV-positive shrimp. PCR conditions used and sizes of the PCR products are shown in Table 2.

PCR analysis for WSSV variable loci. PCR on the genomic variable loci of WSSV was performed with 1 µl DNA extracts, using *Taq* DNA polymerase (Promega). The specific primer sets, PCR conditions used and sizes of the PCR products are shown in Table 2.

Cloning of PCR products. PCR products were purified from 1 % agarose gels using a DNA extraction kit (MBI Fermentas). These products were subsequently cloned into *E. coli* DH5α competent cells using the pGEM-T easy vector system I (Promega). Plasmids containing the correct insert, as screened by restriction enzyme analysis and/or by colony PCR, were prepared for sequencing by purification with the High Pure plasmid isolation kit (Roche).

Virus production and purification. The virus isolate WSSV-TH used in this study originated from infected *P. monodon* imported from Thailand in 1996 and was obtained as described before (van Hulten *et al.*, 2000a). The virus WSSV-VN isolate T (Table 1) originated from a single infected *P. monodon*. Tissue of a WSSV-VN-T-infected *P. monodon* was homogenized in 330 mM NaCl. After centrifugation at 1700 ***g*** for 10 min, the supernatant was filtered (0·45 µm filter; Schleicher & Schuell) to obtain the virus. Crayfish *Orconectes limosus* or *Astacus leptodactylus* were injected intramuscularly with a lethal dose of WSSV (WSSV-TH or WSSV-VN-T), using a 26-gauge needle (Microfine B&D). Virus was isolated and processed according to published procedures (van Hulten *et al.*, 2000a).

Table 1. Origin of the Vietnamese WSSV isolates used in this study

Isolate	Pond	Place (district)	Province	Date of collection	Origin of postlarvae*
Central WSSV-VN isolates					
K	Khanh	Hoi An	Da Nang	18 March 2003	Local
T	Thanh	Son Tinh	Quang Ngai	19 March 2003	Da Nang
L	Luong	Qui Nhon	Binh Dinh	20 March 2003	Local
X	Xu	Tuy Hoa	Phu Yen	21 March 2003	Local
S	Suu	Tuy Hoa	Phu Yen	21 March 2003	Local
A	Anh	Ninh Hai	Ninh Thuan	22 March 2003	Local
South WSSV-VN isolates					
Tv	C. Thanh A	Long Hoa	Tra Vinh	10 January 2004	Unknown
Kg	T. Sang	Kien Luong	Kien Giang	4 March 2003	Local

*All ponds obtained their postlarvae from hatcheries; the locations of the hatcheries are given in this column.

Table 2. Primers used during PCR analysis for WSSV screening and for the variable loci of WSSV

Primer pair	Primer	Sequence (5′–3′)	Annealing temperature (°C)/ elongation time (s)	WSSV sequence coordinates	Size (bp) of PCR product
WSSV screening					
16S rRNA	16S-FW	GTGCGAAGGTAGCATAATC	52/50	–	414
	16S-RV	CTGCTGCAACATAAGGATAC		–	
VP26	VP26-FW	ATGGAATTTGGCAACCTAACAA-ACCTG	52/50	228835–228809†	304*†
	VP26-RV	GGGCTGTGACGGTAGAGATGAC		228532–228553†	
WSSV variable loci					
VR23/24-1	Forward	ATGGGCTCTGCTAACTTG	50/360	4359–4376*	10833*
	Reverse	ATGATTGTATTCGTCGAAGG		15191–15172*	
VR23/24-screen	Forward	CACACTTGAAAAATACACCAG	49/65	5503–5523*	9088*
	Reverse	GTAAGTTTATTGCTGAGAAG		14590–14571*	
VR23/24-south	Forward	CTACAACGGCCAAGTCAT	49/100	30701–30718†	1555†
	Reverse	CGCAATTCTCCTCGCAGTT		32255–32237†	
VR14/15-screen	Forward	GAGATGCGAACCACTAAAAG	49/75	22904–22923†	1254†
	Reverse	ATGGAGGCGAGACTTGC		24157–24141†	
Transposase	Forward	GTGGATAATATTCGTCTTCAAC	55/120	253988–254009†	1489* (151†)
	Reverse	CTCAAAGACAACGACATTAG		254138–254119†	
ORF75-flank	Forward	GAAGCAGTATCTCTAACAC	49/80	107875–107893†	868†
	Reverse	CAACAGGTGCGTAAAAGAAG		108742–108723†	
ORF94-flank	Forward	GTGCCGCAGGTCTACTC	51/80	142656–142672†	682†
	Reverse	CATACGACTCTGCTTCTTG		143337–143319†	
ORF125-flank	Forward	CGAAATCTTGATATGTTGTGC	52/100	187791–187811†	652†
	Reverse	CCATATCCATTGCCCTTCTC		188442–188423†	
Polymerase	Forward	CAATATTACACGCCCTTCAG	49/60	35867–35886†	504*†
	Reverse	GCTTGCATGATTTTTCTCC		36370–36352†	

*According to WSSV-TW sequence.
†According to WSSV-TH sequence.

Purification of viral DNA and restriction enzyme analysis. Viral DNA was isolated from purified virions as described by van Hulten *et al.* (2000a). WSSV DNA was digested with *Bam*HI (Invitrogen) and fragments were separated by electrophoresis in a 0·6 % agarose gel at 40 V (1·3 V cm^{-1}) for 20 h. After separation, the gels were stained with ethidium bromide (0·5 μg ml^{-1} in TAE).

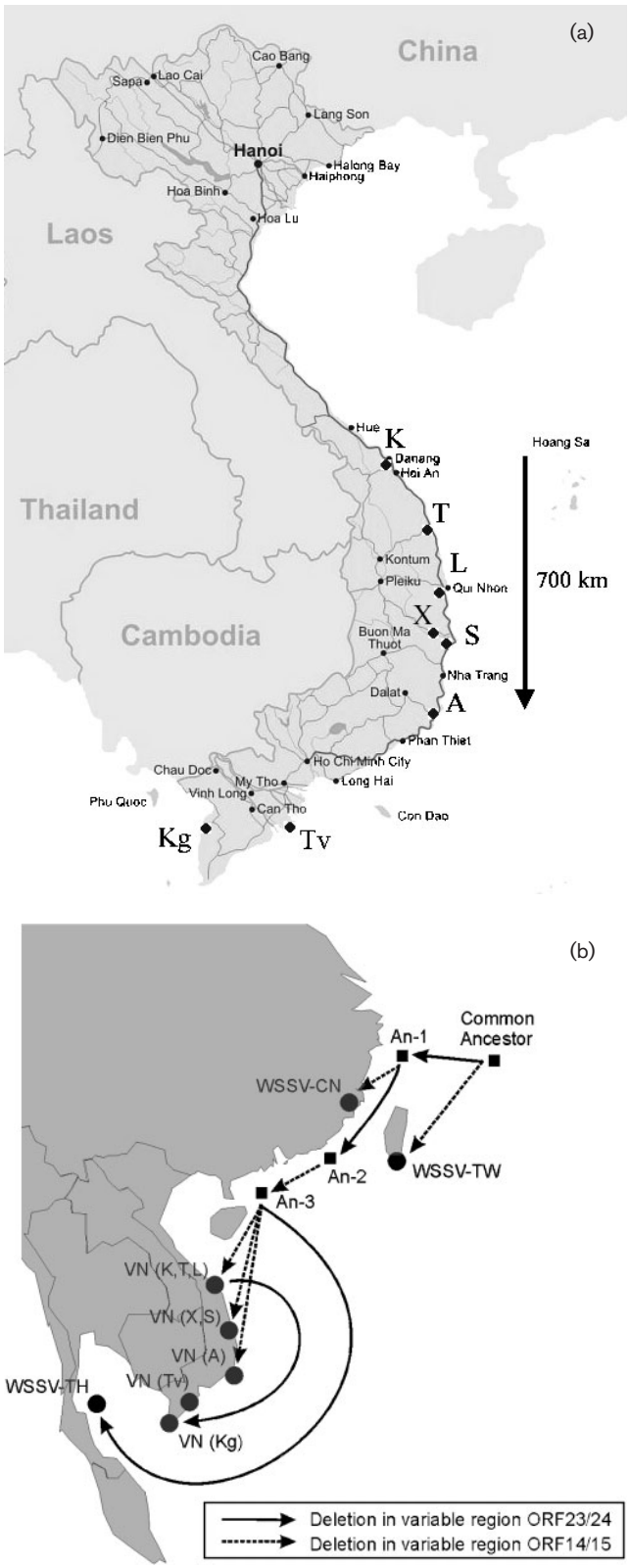
Sequencing and computer analysis. Plasmid clones were sequenced using universal T7 and/or Sp6 primers, and by primer walking for inserts of >1·5 kb (BaseClear). Sequence data were analysed using the software package DNASTAR 4.2 (DNASTAR Inc.) and the output was edited in GeneDoc, version 2.6.000 (Nicholas *et al.*, 1997). Complete WSSV sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) using the accession numbers for WSSV-TW (AF440570), WSSV-CN (AF332093) and WSSV-TH (AF369029). Dot-plot analysis was performed using PIPmaker (http://bio.cse.psu.edu/pipmaker/).

RESULTS

Shrimp (*P. monodon*) infected with WSSV and analysed in this study were collected in 2003/2004 from eight shrimp culture ponds in Vietnam (Table 1). The ponds are distributed over seven different provinces, which are located along the coast from central to south Vietnam (Fig. 1a; K to Kg). Three shrimp from each pond were chosen randomly from juvenile *P. monodon* showing gross signs of WSSV infection. All collected shrimp tested positive for WSSV using a single-step PCR. Therefore, one shrimp was chosen from each pond at random as representative for that pond and used for further analysis. All WSSV-VN isolates, including the abbreviations used in this paper, are listed in Table 1.

The WSSV genomic loci that were shown previously to be variable in their genetic make-up among different WSSV isolates (Marks *et al.*, 2004) were used for our analysis. These loci were studied in detail for each of the VN isolates by PCR amplification, cloning and sequence analysis. The variable loci screened for can be divided into (i) a genomic region prone to large deletions, referred to as 'variable region ORF23/24' (this region was called '13 kb deletion' by Marks *et al.*, 2004), (ii) a genetic variable region, which will be referred to as 'variable region ORF14/15', (iii) a genomic region encoding a putative transposase and (iv) the VNTRs located in ORF75, ORF94 and ORF125. Furthermore, we analysed a conserved genomic fragment encoding part of WSSV DNA polymerase (v). The data for each of these loci will be dealt with separately. To reduce the possibility that the VN isolates have major genetic

differences at loci that were not screened for, a detailed restriction enzyme analysis (RFLP) was performed for one of the VN isolates (WSSV-VN-T) and the result was compared with that for WSSV-TH.



(i) Variable region ORF23/24

Previously, this genomic region was shown to contain deletions of ~1·2 and ~13·2 kb in WSSV-CN and WSSV-TH, respectively, compared with WSSV-TW (Fig. 2a; Marks *et al.*, 2004). Three other unique deletions in this region were reported in Chinese isolates collected in Tong'an and Anhui in south-east China in 2001 (Fig. 2a; WSSV-CN-A to -C; Lan *et al.*, 2002).

We mapped this locus initially in WSSV-VN isolate K by performing PCRs with primer pairs equally distributed over the genomic region 2332–15861 (WSSV-TW coordinates), which harbours the 'variable region ORF23/24'. Based on the primer pairs that failed to give a product, the flanking primer set VR23/24-1 (Table 2; Fig. 2a) was used to pinpoint exactly the coordinates of the deletion. Cloning and sequencing of the ~2·3 kb fragment obtained in the PCR revealed that a deletion of 8539 bp existed in WSSV-VN isolate K compared with WSSV-TW (Fig. 2a). The flanking sequences present in the ~2·3 kb fragment were 100 % identical to the sequences of WSSV-TW and WSSV-CN. Based on this result, a new PCR was performed with primer set VR23/24-screen (Table 2; Fig. 2a) flanking this deletion to detect this deletion specifically in all VN isolates. The amplified fragment had a similar size of 548 bp for the six VN-central WSSV isolates, indicating that they have a deletion of about 8539 bp in this locus compared with WSSV-TW (Fig. 2a). The VN-south isolates (Tv and Kg) failed to give a product in this PCR.

We mapped the variable region ORF23/24 in isolates Tv and Kg using a method similar to that used to map the deletion for WSSV-VN isolate K. Cloning and sequencing of the ~1·6 kb PCR product obtained with primers VR23/24-south-forward and VR23/24-1-reverse (Table 2; Fig. 2a) showed that isolate Tv has a deletion of 11 450 bp relative to the WSSV-TW genome (Fig. 2a). A PCR with the primer pair VR23/24-south (Table 2; Fig. 2a) for isolate Kg resulted in a ~2·6 kb PCR product, which, after cloning and sequencing, showed that this isolate contains a deletion of 12 166 bp relative to the WSSV-TW genome (Fig. 2a). We previously mapped five SNPs and a 1 bp deletion within WSSV-TW coordinates 16447–16773 (flanking the deletion) compared with WSSV-CN and WSSV-TH (Marks *et al.*, 2004). With respect to these genetic differences, isolate Kg is identical to WSSV-CN and WSSV-TH, suggesting that this isolate is more closely related to these isolates than to WSSV-TW.

Fig. 1. (a) Map of Vietnam, showing the geographical origins of the isolates used for this study, indicated by K, T, L, X, S, A, Tv and Kg, according to Table 1. (b) Model of spread of WSSV in South-East Asia from either side of the Taiwan Strait towards the west. Filled circles represent identified isolates, while filled squares represent hypothetical isolates. Each line represents a single-step deletion. Ancestor is abbreviated as An; WSSV-VN isolates are abbreviated as VN.

Dot-plot analysis showed that, except for the *hr*s (van Hulten *et al.*, 2001), the genomic region in WSSV-TW in which these deletions occur contains the most direct and inverted repeats of the entire WSSV genome (Fig. 2b). However, for the deletion in the VN-south isolates Tv and Kg as well as in the six VN-central isolates, no direct repeats were identified within 300 bp flanking the putative recombination sites in WSSV-TW that could be involved in recombination (Fig. 2b; sequence data not shown).

(ii) Variable region ORF14/15

The variable region ORF14/15 is centred in a region of 842 bp in WSSV-CN, of which 257 bp of its 5′ end is only present in WSSV-TH, while the remaining 585 bp of its 3′ end is only present in WSSV-TW (Fig. 3a; Marks *et al.*, 2004). This locus was thought to be a variable region prone to recombination (Marks *et al.*, 2004). However, a partly characterized isolate recently studied by our laboratory contains at least all unique sequences present in this locus, suggesting that WSSV-TW, WSSV-CN and WSSV-TH are derived from a common ancestor by deletions of various sizes (Fig. 3a). Because WSSV-TW, WSSV-CN and WSSV-TH each contain unique sequences, these isolates seem to be distinct and probably evolved separately. Using the same strategy as used for the variable region ORF23/24, this locus was mapped for all VN isolates using primer set VR14/15-screen (Table 2; Fig. 3a). WSSV-TH DNA, taken as a positive control for the PCR, showed the expected fragment of 1254 bp, whereas the VN isolates showed fragments of different sizes ranging from ~500 to ~700 bp (Fig. 3b). Cloning and sequencing of these fragments revealed that all VN isolates had deletions relative to WSSV-TH (Fig. 3a). The flanking sequences of the deletions present in the ~500 to ~700 bp fragments were identical to the sequences of WSSV-TH. The VN isolates K, T, L, Tv and Kg had the same deletion of 714 bp, VN isolates X and S had a deletion of 634 bp, while VN isolate A had the smallest deletion, of 563 bp, compared with WSSV-TH (Fig. 3a).

(iii) A genomic region encoding a putative transposase

The genome of WSSV-TW encodes a putative transposase, which is not present in WSSV-CN or WSSV-TH. Using primer pair Transposase (Table 2) flanking the transposase gene in the WSSV-TW genome, we obtained a PCR fragment of ~150 bp for all VN isolates (data not shown). A fragment of similar size was obtained with WSSV-TH DNA, used as a positive control. Therefore, we conclude that all VN isolates do not contain this particular transposase sequence.

(iv) Genetic variation in VNTR loci

Three non-*hr* unidirectional tandem repeats, in the region encoding ORF75, ORF94 and ORF125, have been shown to be variable in the number of repeat units (RUs) between the WSSV isolates identified so far (Table 3; Wongteerasupaya *et al.*, 2003; Marks *et al.*, 2004). The repeats are positioned in the middle of the ORFs, which have non-repeated 5′ and 3′ ends. For both ORF75 and ORF94, around 50 % of the coding region consists of repeats, while for ORF125 around 20 % of the coding region consists of repeats. Differences in the number of RUs do not cause frameshifts for the respective ORFs, since the length of these RUs is always a multimer of 3 bp. The protein encoded by ORF75 has been shown to be present in WSSV virions (Huang *et al.*, 2002). ORF94 may have a similar function to ORF75, as the repeat units of the two ORFs share a common motif at the protein level consisting of four basic amino acids (arginine or lysine) followed by two alanines, two or three prolines and a stretch of acidic amino acids (aspartate or glutamate).

To study the VN-central isolates for each of these loci, we performed a PCR with a specific primer set (Table 2) flanking the non-*hr* unidirectional tandem repeats. The results for ORF75, ORF94 and ORF125 are shown in Fig. 4. For all three loci, a major band was observed for each isolate, often different in size among isolates. The PCR fragments of all VN isolates were cloned, sequenced and aligned. The sequenced regions flanking the tandem repeats (between the primers used and the actual repeats) on both the 5′ and the 3′ end showed 99·6–100 % nucleotide identity to the corresponding sequences of WSSV-TW, WSSV-CN and WSSV-TH. This indicates that the correct fragment had been amplified for each of the three loci of the VN-central isolates, eliminating the possibility of false annealing of the primers.

ORF75. For all WSSV isolates characterized so far, ORF75 has two types of RUs, with lengths of 102 and 45 bp (Table 3). The first 45 nucleotides of the 102 bp RUs are identical to the RUs of 45 bp. Comparing all RUs within one isolate, they contained SNPs at positions 3, 15, 30, 40, 42 and 44, and the RUs of 102 bp have an extra SNP at position 83. Each of the RUs can be recognized by its specific SNPs.

The number of RUs present in ORF75 of the WSSV-VN-central isolates is summarized in Table 3, together with the exact order of appearance of the 45 and 102 bp RUs. The number of RUs identified for each isolate corresponded to the respective sizes of their PCR fragments shown in Fig. 4. VN isolates K, T, L and X are identical at this point. VN isolate A has an extra RU of 45 bp, which is, based on the SNPs, located after the second repeat unit (sequence data not shown). VN isolate S has a larger number of RUs and, based on the SNPs, more closely resembles the genotype of WSSV-CN (sequence data not shown).

ORF94. In all WSSV isolates characterized so far, ORF94 has tandem RUs of 54 bp with an SNP at position 48 (either guanine or thymine) when comparing the RUs mutually within one isolate (Table 3). The number of RUs was highly variable between the various isolates for

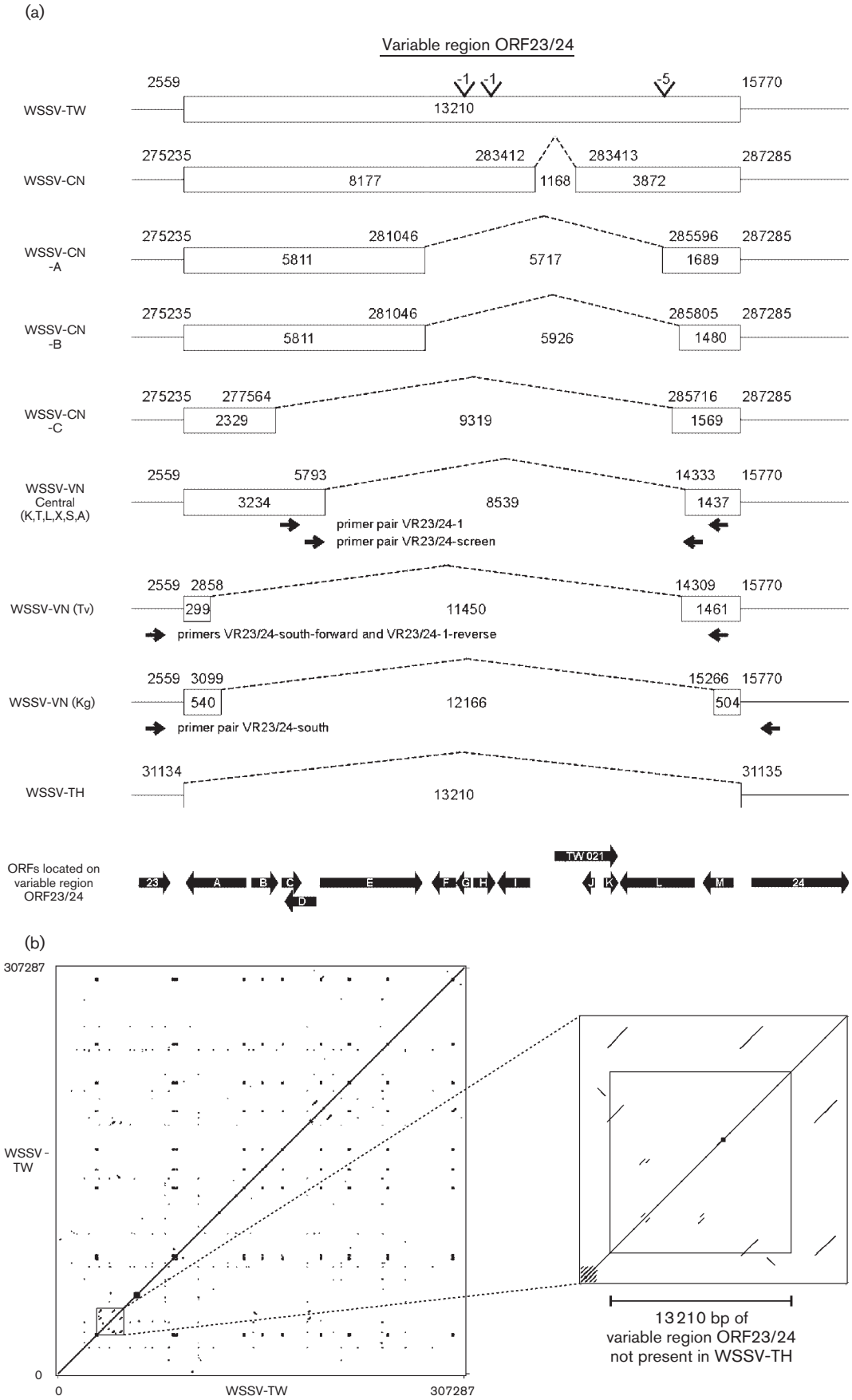

(a)
Variable region ORF23/24
WSSV-TW
2559
-1
-1
-5
15770
13210
WSSV-CN
275235
283412
283413
287285
8177
1168
3872
WSSV-CN -A
275235
281046
285596
287285
5811
5717
1689
WSSV-CN -B
275235
281046
285805
287285
5811
5926
1480
WSSV-CN -C
275235
277564
285716
287285
2329
9319
1569
WSSV-VN Central (K,T,L,X,S,A)
2559
5793
14333
15770
3234
8539
1437
primer pair VR23/24-1
primer pair VR23/24-screen
WSSV-VN (Tv)
2559
2858
14309
15770
299
11450
1461
primers VR23/24-south-forward and VR23/24-1-reverse
WSSV-VN (Kg)
2559
3099
15266
15770
540
12166
504
primer pair VR23/24-south
WSSV-TH
31134
31135
13210
ORFs located on variable region ORF23/24
23
A
B
C
D
E
F
G
H
I
TW 021
J
K
L
M
24
(b)
307287
WSSV-TW
0
0
WSSV-TW
307287
13210 bp of variable region ORF23/24 not present in WSSV-TH

Fig. 2. (a) Schematic representation of the variable region ORF23/24 of WSSV-TW, WSSV-CN, WSSV-TH, three different isolates from China in 2001 (WSSV-CN-A, -B and -C; map numbers are in accordance with WSSV-CN; Lan *et al*., 2002) and the VN isolates. Map numbers, indicated above each isolate, are in accordance with the NCBI database for the genomic sequence of each isolate. Coordinates of the WSSV-VN isolates are according to the WSSV-TW annotation. The length of the fragments is indicated within boxes or sequences. Arrows represent primers. Positions of ORFs located in this region are indicated below by filled arrows, which also represent the direction of transcription. ORFs are numbered in accordance with the numbering used by Marks *et al*. (2004). (b) Dot-plot comparison of the nucleotide sequence of WSSV-TW to itself (the adenine residue at the translation initiation codon of VP28 was designated as the starting point for the numbering of WSSV-TW in this dot-plot), including an enlargement of original WSSV-TW coordinates 425–20425.

which this locus has been characterized: WSSV-TW, WSSV-CN, WSSV-TH and 55 other isolates originating from Thailand. The number of RUs varied from six to 20 repeat units (van Hulten *et al*., 2000a; Wongteerasupaya *et al*., 2003; Marks *et al*., 2004).

The WSSV-VN-central isolates contained between seven and 17 RUs (Table 3), corresponding to the respective sizes of their PCR fragments (Fig. 4). The identity of the nucleotide at position 48 of each of the VN isolates is shown in Table 3. Isolates X and S are identical, while the

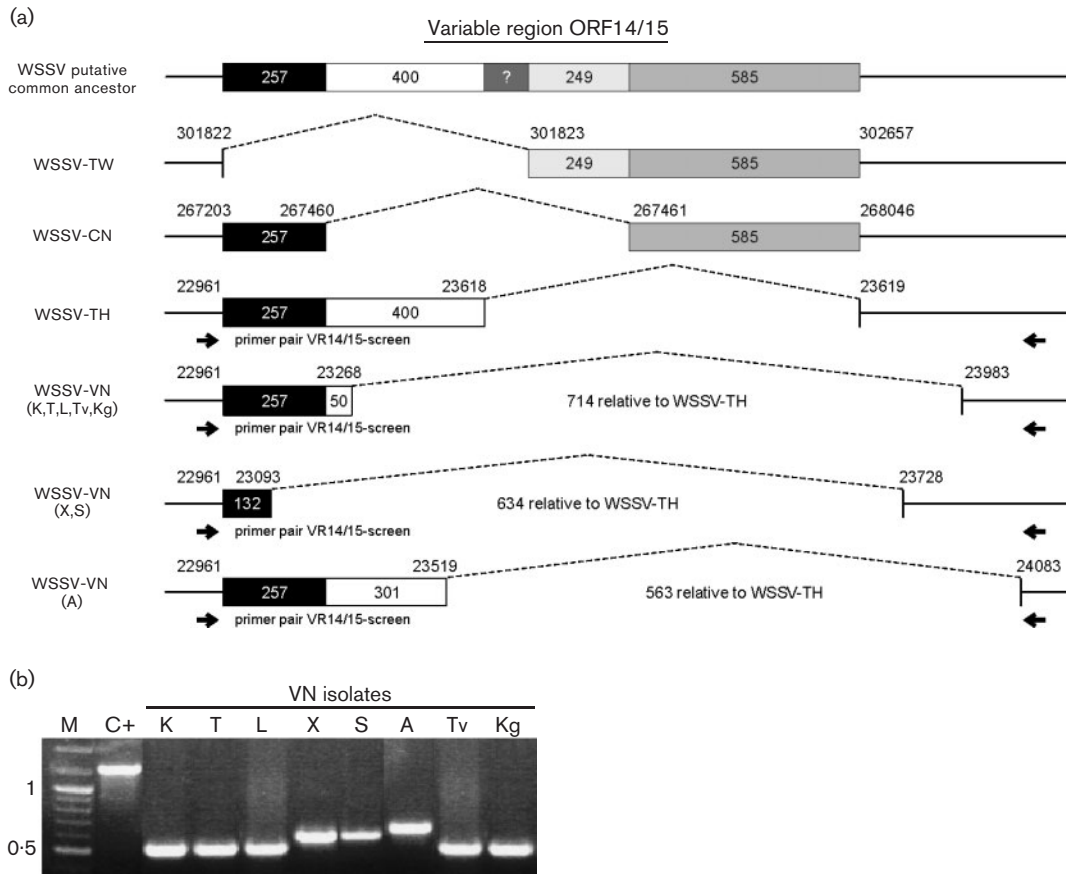


Fig. 3. (a) Schematic representation of the variable region ORF14/15 of the WSSV putative common ancestor, WSSV-TW, WSSV-CN, WSSV-TH and the VN isolates. Different tints represent unique sequences within the WSSV putative common ancestor, WSSV-TW, WSSV-CN and WSSV-TH. Map numbers, indicated above each isolate, are in accordance with the NCBI database for the genomic sequence of each isolate. Coordinates of the WSSV-VN isolates are according to the WSSV-TH annotation. The length of the fragments is indicated within boxes or sequences. Arrows represent primers. (b) PCR on the variable region ORF 14/15 using genomic DNA of the WSSV-VN isolates as template. Lanes are labelled with the VN isolate used. C+ is the same PCR on genomic DNA of WSSV-TH, used as a positive control for the PCR. M, 100 bp DNA marker; some sizes are indicated (in kb).

Table 3. RUs present within the non-*hr* unidirectional repeats of ORF75, ORF94 and ORF125

For each ORF, the length(s) of the RUs and the WSSV-TH coordinates of the total repeat are given. ND, Not determined.

Isolate	ORF75; 45 and 102 bp; 107965–108675		ORF94; 54 bp; 142744–143067		ORF125; 69 bp; 187899–188312	
	Number*	Positioning†	Number	Genotypes‡	Number	Genotypes§
TW	21 (16, 5)	45, 102, 4×45, 102, 3×45, 102, 2×45, 102, 4×45, 102, 2×45	6	T, T, T, G, T, T	8	C, –, E, F, –, H, I
CN	15 (11, 4)	45, 102, 4×45, 102, 2×45, 102, 2×45, 102, 2×45	12	T, T, G, G, G, G, G, G, T, T, T, T	8	C, D, E, –, G, H, –
TH	12 (9, 3)	45, 102, 4×45, 102, 2×45, 102, 2×45	6	T, G, G, G, T, T	6	C, –, –, –, –, H, I
Thai isolates‖						
Sur #1	ND	ND	9	T, T, T, T, G, T, T, G, T	ND	ND
Sur #2	ND	ND	8	T, T, G, T, T, G, G, T	ND	ND
Chu #2	ND	ND	7	T, T, T, G, T, G, T	ND	ND
Chu #3	ND	ND	8	T, T, G, T, T, G, G, T	ND	ND
Chu #4	ND	ND	6	T, T, T, G, G, G	ND	ND
VN-central isolates						
K	5 (3, 2)	102, 45, 102, 2×45	10	G, G, G, T, T, T, G, G, T, T	6	C, –, E, –, –, H, –
T	5 (3, 2)	102, 45, 102, 2×45	17	G, T, T, T, T, G, T, T, T, G, T, G, G, G, G, T, T	5	C, –, –, –, –, H, –
L	5 (3, 2)	102, 45, 102, 2×45	10	G, G, G, G, G, G, G, G, G, G	6	C, –, –, –, –, H, I
X	5 (3, 2)	102, 45, 102, 2×45	7	T, T, T, T, G, T, T	7	C, –, E, –, –, H, I
S	14 (10, 4)	45, 102, 4×45, 102, 45, 102, 2×45, 102, 2×45	7	T, T, T, T, G, T, T	7	C, –, E, –, –, H, I
A	6 (4, 2)	102, 2×45, 102, 2×45	10	G, G, G, T, G, G, T, T, T, T	6	C, –, –, –, –, H, I

*Numbers of 45 and 102 bp RUs, respectively, are given in parentheses.

†Numbers of successive tandem RUs of 45 bp are summarized as $n \times 45$.

‡Genotype of each successive RU at position 48 is shown.

§The order of the RUs is kept, but the RUs are categorized (C–I) by genotype, starting from the third RU (C) to the penultimate RU (I). The genotypes of each RU at positions 8, 18, 25, 66 and 69, respectively, are TGGTC (C, E and F), TTGGT (D, G and H) and CGAGT (I). Where no RU is present, it is indicated by –.

‖Data from Wongteerasupaya *et al.* (2003). These isolates originate from different ponds in Surat Thani or Chumporn (abbreviated as Sur and Chu, respectively), Thailand, 2000.

other isolates, although some have the same number of RUs, all have a unique pattern of nucleotides at position 48. VN isolates K, T and L had a thymine deletion at position 143149 (WSSV-TH coordinates), located in the 3′ end flanking the repeat. As this is outside the coding region, it will not cause a frameshift in ORF94.

ORF125. ORF125 contains tandem RUs of 69 bp, of which the first two as well as the last can be recognized by their specific SNPs when comparing the RUs mutually within one isolate (Table 3). The other RUs (the third to the penultimate) contain SNPs at positions 8, 18, 25, 66 and 69 (Marks *et al.*, 2004). The WSSV-VN-central isolates contained between five and seven RUs (Table 3), corresponding to the respective sizes of their PCR fragments (Fig. 4). VN isolates X and S, as well as VN isolates A and L, are identical in this locus (Table 3). The genotype of the VN isolates A and L is identical to the genotype of WSSV-TH (Table 3).

(v) Fragment encoding part of DNA polymerase

To classify the WSSV-VN isolates further, a PCR was performed on a conserved genomic fragment encoding part of WSSV DNA polymerase using primer set Polymerase (Table 2). Within this genomic fragment, a single nucleotide deletion occurs in WSSV-CN (WSSV-TH coordinate 36030) compared with WSSV-TW and WSSV-TH, causing a frameshift in the polymerase gene (Chen *et al.*, 2002; Marks *et al.*, 2004). The WSSV-VN isolates gave a PCR fragment of a similar size to the positive control WSSV-TH. Cloning and sequencing of the eight PCR fragments from the central and south VN isolates failed to detect an adenine deletion as is present in WSSV-CN. The PCR

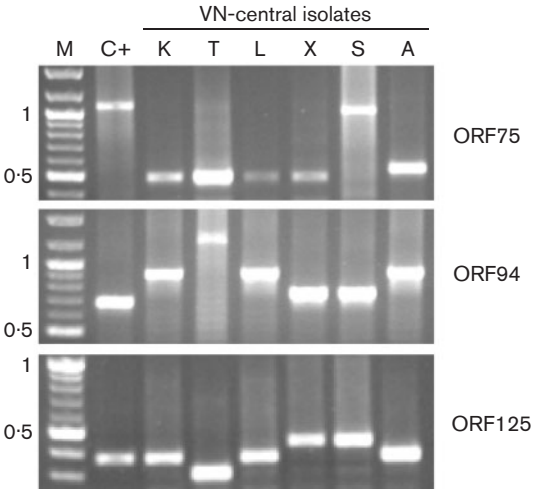


Fig. 4. PCR on the non-*hr* unidirectional repeats of ORF75, ORF94 and ORF125, using genomic DNA of the WSSV VN-central isolates as template. Lanes are labelled with the VN isolate used. C+ is the same PCR on genomic DNA of WSSV-TH, used as a positive control for the PCR. M, 100 bp DNA marker; some sizes are indicated (in kb).

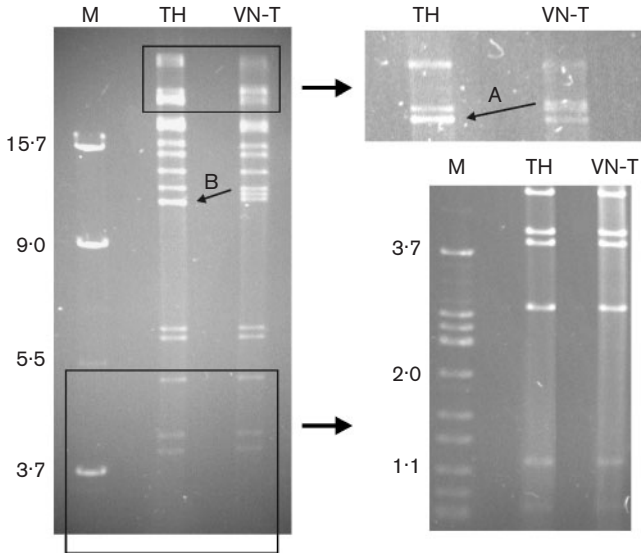


Fig. 5. WSSV genomic DNA of WSSV-TH (TH) and WSSV-VN isolate T (VN-T) digested with *Bam*HI. M, Molecular size standard (lambda DNA digested with *Bam*HI + *Eco*RI + *Hin*dIII); some sizes are indicated (in kb). The enlargements focus on the major (>20 kb) and minor (<4 kb) fragments on similar gels. The clear band shifts between the two isolates are indicated by A and B.

fragments showed 100 % nucleotide identity to the respective fragments of WSSV-TW and WSSV-TH.

Restriction enzyme analysis of VN isolate T

RFLP analysis between WSSV-TH and WSSV-VN-T is shown in Fig. 5. The *Bam*HI restriction pattern of WSSV-TH exactly matches the expected pattern based on the complete nucleotide sequence (van Hulten *et al.*, 2001), except for the three smallest fragments, which are not visible due to their estimated size of <1 kb. Two clear polymorphisms (shifts) are visible between WSSV-TH and VN isolate T, indicated in Fig. 5 by A and B. Shift A, in which a fragment of ~27·5 kb for VN isolate T shifts to ~24·5 kb for WSSV-TH, can be explained by the observed sequence diversity in variable region ORF 14/15 and variable region ORF 23/24, which are both located on this large fragment. The approximately −3 kb discrepancy is the sum of the observed differences in PCR mapping of both variable regions of approximately 0·7 kb and −3·7 kb, respectively (Figs 2a and 3a). Shift B, in which a corresponding fragment has a size of ~11·2 kb for WSSV-TH and of ~11·8 kb for VN isolate T, can be explained by the sequence variation of the repeat in ORF94 (Table 3). The difference of 11 RUs of each 54 bp results in a shift of 594 bp. The differences in the repeats in ORF75 and ORF125 are not clearly visible. ORF75 is located on a large fragment (~20 kb) for which the 350 bp difference in size will only show a minor shift, whereas the difference in the repeats of ORF125 between WSSV-TH and VN isolate T is marginal (138 bp).

DISCUSSION

Genomic analyses of WSSV showed that conserved genes, often used in molecular epidemiological studies to unravel evolutionary relationships by phylogenetic analysis, are too homologous to use for this purpose in the case of WSSV (Marks *et al.*, 2004). For example, the complete DNA polymerase gene of WSSV contains only three SNPs and a 1 bp and 3 bp deletion when comparing this gene for the three completely sequenced WSSV isolates (Chen *et al.*, 2002; Marks *et al.*, 2004). Similar high homologies were found for other conserved WSSV genes (Chang *et al.*, 2001; Marks *et al.*, 2004). Moreover, the major structural protein genes, which for some virus families show a relatively large number of mutations due to antigenic drift or adaptation to different hosts, also show 99·5–100 % nucleotide identity between several geographical WSSV isolates (Moon *et al.*, 2003; Marks *et al.*, 2004). These data indicate that the isolates of WSSV identified so far are very closely related and probably evolved recently from a common ancestor. The DNA polymerase sequences obtained from all VN isolates, showing 100 % identity with WSSV-TW and WSSV-TH, further confirmed this observation. Therefore, we chose the most variable loci of WSSV to classify new WSSV isolates from Vietnam (Marks *et al.*, 2004). The RFLP analysis between WSSV-TH and WSSV-VN-T (Fig. 5) confirmed the high degree of homology among WSSV isolates, but indeed identified the major genomic insertions and deletions in WSSV-VN (isolate T).

Based on both variable region ORF23/24 and variable region ORF14/15, we propose a model to explain the genotypic changes of WSSV during its geographical spread from either side of the Taiwan Strait towards the west to Thailand between 1992 and 1995 (Fig. 1b). In this model, the two loci evolved independently and both deletions in the variable regions showed a progressive increase in length during the spread of WSSV. The WSSV common ancestor (Fig. 1b) contains a genotype similar to WSSV-TW in the variable region ORF23/24 (Fig. 2a) and a genotype similar to the putative common ancestor in variable region ORF14/15 (Fig. 3a). WSSV-TW evolved from this common ancestor by a deletion in variable region ORF14/15, while WSSV-CN evolved by a deletion of ~1·2 kb in variable region ORF23/24 (Fig. 1b; An-1) followed by a deletion in variable region ORF14/15. Based on the observation that the genotypes of the VN isolates seem to have evolved from a genotype similar to WSSV-TH in variable region ORF14/15 by separate unique deletions of different sizes, the VN isolates and WSSV-TH probably have a common lineage, which branched off at an early stage from WSSV-TW and WSSV-CN. However, the extra sequences in the variable region ORF23/24 present in the VN isolates compared with WSSV-TH exclude the possibility that the WSSV-VN isolates are derived from WSSV-TH. Therefore, WSSV-TH and the WSSV-VN isolates probably have a common ancestor, An-3 (Fig. 1b), which could contain the genotype of WSSV-TH in variable region ORF14/15, but the ~8·5 kb deletion similar to the VN-central isolates in variable region ORF23/24. Within the three different WSSV-VN genotypes in variable region ORF14/15, each contains unique sequences and thus probably evolved separately. Therefore, WSSV entered Vietnam by multiple introductions from the common ancestor An-3, from where it spread further within Vietnam (VN isolate Kg; Fig. 1b). WSSV isolates collected further along the coast of South-East Asia [i.e. isolates from North Vietnam, China (Hainan) and Cambodia] should be genotyped to confirm and further detail this model.

The mechanism(s) by which the changes or (gradual) deletions in both variable regions occur is unclear. For WSSV-TH, it was suggested that the deletions in variable region ORF23/24 might have occurred by homologous recombination, as a direct repeat is present at both ends of the deletion in WSSV-TW (Marks *et al.*, 2004). However, no direct repeats that could be involved in recombination were identified for the deletion in the VN-south isolates Tv and Kg or in the six VN-central isolates (Fig. 2b). Maybe the deletions in the variable region ORF23/24 can be explained by genomic pressure on the virus to discard redundant sequences, as Fig. 2(b) shows that WSSV-TW contains a lot of duplicated sequences and ORFs (especially genes of WSSV gene family 4; van Hulten *et al.*, 2001) in this region. It is also possible that the host species or an intermediate host has an effect on the size of the deletion, as WSSV-CN-A (*Metapenaeus ensis*), -B (*P. japonicus*) and -C (*P. vannamei*, *P. monodon*, *P. chinensis*) were isolated from different host species (Fig. 2a; Lan *et al.*, 2002). However, within one host species, WSSV isolates can show different sizes of deletion, as WSSV-TW, WSSV-TH, WSSV-CN-C and the VN isolates were all obtained from *P. monodon* and WSSV-CN and WSSV-CN-B were both isolated from *P. japonicus*. To date, there seems to be no difference in host range between the characterized WSSV isolates (Wang *et al.*, 1998, 1999; Chen *et al.*, 2000; Lan *et al.*, 2002; Hameed *et al.*, 2003).

Based on the genetic make-up in the two variable regions and the thymine deletion shared by isolates K, T and L in the 3′ flanking region of the repeat located in ORF94, three groups of VN-central isolates can be distinguished [(K, T, L), (X, S) and (A); Fig. 1b]. Within these groups, each of the non-*hr* unidirectional tandem repeats located in ORF75, ORF94 and ORF125 seem to have their own, independent genesis in terms of insertion or deletion of repeat units (Table 3). Possibly, insertions or deletions of repeat units are generated during homologous recombination or replication slippage, as is proposed for repeats such as the baculovirus homologous repeats (*hr*s) (Garcia-Maruniak *et al.*, 1996) and the herpesvirus direct repeats (DRs) (Umene, 1991).

Compared to the other two non-*hr* unidirectional tandem repeats (ORF94, ORF125), the repeats in ORF75 seem to be rather conserved within and between the three groups of VN-central isolates. The additional repeat unit in VN isolate A could be explained by a single insertion event. The large number of repeat units present in ORF75 for WSSV-VN-S is surprising, especially because the VN isolates X and S, whose geographical origins are very close (~10 km) and which may even originate from postlarvae from the same supplier, are completely identical in all other loci screened for. Analysis of more WSSV isolates at this locus from different infected shrimp from the same pond may provide clarification of whether this is the common genotype of WSSV isolates derived from pond S or whether it is an irregularity. Also, for the repeats in ORF125, the genotypic differences in VN isolates can be explained by a one-step deletion or insertion of a single repeat unit (Table 3). Analysis of the genotypes present within the WSSV-VN group K, T, L suggests that this locus has a higher mutation frequency than ORF75.

The largest genomic variation among the VN-central isolates was observed for the non-*hr* unidirectional tandem repeats located in ORF94. The number of repeat units within ORF94, as well as the SNP located at position 48, already appeared highly variable for WSSV isolates within Thailand (Wongteerasupaya *et al.*, 2003). Between the isolates characterized within Vietnam, a wide range of genotypic variation was also found for this locus, without any obvious correlation with its geographical location. It is interesting to note that the repeats of ORF94 are highly variable in number, whereas the repeat in ORF75 seems to be more stable, although the two repeat regions share structural properties on the protein level. In conclusion, the repeats of ORF75 and ORF125, each having its own mutation dynamics

different from both more stable variable regions, seem suitable to study WSSV spread on a more local or regional scale.

This paper shows the potential to use genetic markers to study WSSV epidemiology and ecology. However, more information about the mode of spread of WSSV is necessary in order to understand further the relationship between the VN isolates. Often, WSSV infection in a pond can be traced back to the broodstock supplier or the postlarvae producers. Therefore, on a regional scale, most likely the virus spreads in myriad ways during the turnover of shrimp. However, on a global scale, this study provides support for the contention that WSSV originated from either side of the Taiwan Strait and evolved concurrently with its geographical spread over time in South-East Asia.

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