Genotypic Variations in Tandem Repeat DNA Segments between Ribonucleotide Reductase Subunit Genes of White Spot Syndrome Virus (WSSV) Isolates from Vietnam

TRAN TT HOA[1,3], RICHARD AJ HODGSON[1], DANG TH OANH[3], NGUYEN T PHUONG[3], NIGEL J PRESTON[2] AND PETER J WALKER[1]

[1]CSIRO Livestock Industries, St Lucia, Australia

[2]CSIRO Marine Research, Cleveland, Australia

[3]Cantho University, Cantho, Vietnam

ABSTRACT

White spot syndrome is a viral disease that affects most commercially cultivated marine shrimp species. The disease first emerged in East Asia in 1991 and has since spread throughout most shrimp farming regions of Asia and the Americas. Disease outbreaks usually result in high mortalities in affected ponds. However, shrimp may also be infected chronically with no signs of disease and often obtain the infection in hatcheries from infected broodstock. A wide range of other crustaceans can also act as apparently healthy carriers of infection. In this report, variations in the number of a 54 nucleotide tandem repeat sequence (TRS), located between genes encoding the large (RR1) and small (RR2) subunits of ribonucleotide reductase, were used as a WSSV strain-specific genetic marker. The marker was applied to examine the extent of variation among WSSV isolates from *Penaeus monodon* hatcheries and farms in different regions of Vietnam and to obtain a better understanding of the progression of infection in ponds during grow-out. Analysis of approximately 157 WSSV isolates showed common variations in the number of repeats, with some broodstock harbouring more than one genotype. In healthy ponds and in healthy broodstock or postlarval batches collected from hatcheries, WSSV genotypes containing 4-, 5-, 6- 7-, 8- and 9- TRS elements were detected with no evidence of any predominant genotype. However, amongst shrimp sampled from disease outbreak ponds, the 7-TRS genotype dominated. On the other hand, WSSV genotypes containing greater numbers of tandem repeat elements (i.e. 9-, 14- and 23-TRS) were found in unidentified species including a large crab, a small crab and wild shrimp, respectively. High repeat number genotypes (i.e. 23-TRS and 14-TRS) were not detected in cultured shrimp from the same pond. These results suggested that stocked postlarvae rather than invading wild crustaceans were the source of WSSV infection and disease. The results also suggest that genotype analysis in this TRS region will be a useful tool for tracking virulent strains of WSSV.

Hoa, T.T., R A. Hodgson, D.T. Oanh, N. T Phuong, N. J Preston and P.K. Walker. 2005. Genotypic variations in tandem repeat DNA segments between ribonucleotide reductase subunit genes of Whipe Spot Syndrome Virus (WSSV) isolates from Vietnam. *In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 339-351. Fish Health Section, Asian Fisheries Society, Manila.

INTRODUCTION

White spot disease (WSD) is a lethal viral infection of farmed marine shrimp that has caused major economic losses since it first emerged in East Asia in 1992 (Wang *et al*., 1996; Chou *et al*., 1995; Zhan *et al*., 1998). From the original focus of infection in *P. japonicus* in Fujian Province in China, the disease spread rapidly to other farmed shrimp species and, by late 1994, had been observed in most major shrimp farming countries from Japan to India (Nakano *et al*., 1994; Park *et al*., 1998; Mohan *et al*., 1998; Flegel, 1997). Following the first reports from in Texas in 1995 (Lightner *et al*., 1997), WSD has also established in western hemisphere shrimp species and now appears to be endemic over a wide area of the Americas from the Gulf of Mexico to Peru. White spot syndrome virus (WSSV), the causative agent of WSD, can also infect a broad range of wild crustaceans including marine and freshwater shrimp, crabs and crayfish (Flegel *et al*., 1997). In marine shrimp, WSSV can either exist as a chronic infection without visible signs of disease or cause a highly lethal acute infection resulting in up to 100% mortality in a pond within 3-10 days of the first signs of disease (Zhan and Wang, 1998; Lightner, 1996). As no effective vaccines or other preventive or prophylactic treatments are available, infection and disease are presently managed primarily through pathogen exclusion and stress reduction practices.

WSSV is a large, ellipsoid, enveloped DNA virus with an unusual flagellum-like tail (Wongteerasupaya *et al*., 1995). In structure and genome organization, WSSV is distinct from other known viruses and has recently been classified as the type species of the new genus Whispovirus within the new family Nimaviridae (van Hulten *et al*., 2000; Mayo, 2002). The WSSV genome is a circular double-stranded DNA of approximately 300 kb (van Hulten *et al*., 2001; Yang *et al*., 2001). The genome sequence is remarkably conserved amongst isolates from different hosts and different geographic locations (Lo *et al*., 1999) but there is evidence of significant variation at some specific loci. Restriction fragment length polymorphism (RFLP) analysis has been used to show that WSSV isolates identified in a crayfish at the US National Zoological Park was distinct from five isolates identified in shrimp from China, India, Thailand and the USA (Wang *et al*., 2000). Comparison of the complete genome sequence of WSSV isolates from shrimp from Thailand, Taiwan and the Chinese mainland has also revealed a 12.1 kb deletion in one virus. However the overall sequence identity (98-100%) between the isolates was very high (van Hulten *et al*., 2001; Yang *et al*., 2001; GenBank accession numbers: AF369029, AF332093, AF440570). Chang *et al*. (2001) have reported a single point mutation in the large subunit ribonucleotide reductase gene (*rr1*) that distinguished a New Jersey crab (*Callinectes sapidus*) isolate from 17 other WSSV isolates from crabs, shrimp and crayfish from the USA and Asia. Most recently, frequent variations have been reported among WSSV isolates from disease outbreak ponds in Thailand by analysing the copy number and sequence of a 54 base pair (bp) tandem repeat sequence element located between the *rr1* and *rr2* genes (Wongteerasupaya *et al*., 2003).

In this paper, we report variations in the number of *rr1-rr2* 54 bp tandem repeats among WSSV isolates from *P. monodon* broodstock, postlarvae and juveniles collected from hatcheries and grow-out ponds in different provinces of Vietnam, and from other crustaceans collected from ponds. The data indicates a predominance of a 7 tandem repeat sequence (7-TRS) WSSV genotype in diseased shrimp that was not evident in broodstock or postlarval

batches from hatcheries, juvenile *P. monodon* from healthy ponds, or in crabs and wild shrimp collected from diseased ponds

MATERIALS AND METHODS

Origin of crustacean samples

Tissue samples from *Penaeus monodon* broodstock and postlarvae were collected from hatcheries, and *P. monodon* juveniles and other crustaceans were collected grow-out ponds in different provinces of Vietnam between December 2001 and June 2002 (see Tables 2, 3, and 4). Whole shrimp postlarvae, pleopods (broodstock) or whole heads (juvenile shrimp), crabs and wild shrimp were stored in alcoholic preservative (80% ethanol, 20% glycerol) for not more than 2 months before DNA extraction.

DNA extraction and WSSV DNA detection

DNA was extracted from *P. monodon* postlarvae (eyes-removed), broodstock (pleopods) and juveniles (gill and sub-cuticular epidermis), and from small crabs (half cadavers), large crabs (legs) and wild shrimp (half heads) by using the DNA extraction reagents supplied in the IQ-WSSV-2000 (Farming IntelliGene Technology Corp., Taiwan) WSSV detection kit. DNA was extracted conducted according to the manufacturer's protocol and stored in ET buffer (0.1mM EDTA, 0.1mM Tris-HCl pH 7.0) at -70°C until required. WSSV DNA was detected by using the IQ-WSSV-2000 PCR kit (Farming IntelliGene Technology Corp., Taiwan) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) for genotype analysis of WSSV isolates

WSSV genotypes were determined by PCR amplification of the TRS region between the *rr1* and *rr2* genes. One-step and 2-step nested PCR procedures were applied using the primer combinations shown in Table 1. Samples containing higher quantities of WSSV DNA were analysed by the 1-step method. The 1-step reaction (50 µl) contained 1 x *Taq* buffer, 1.5 mM $MgCl_2$, 200 µM dNTP mix, 2.5 U *Taq* DNA polymerase (Promega Corp, Wisconsin, USA), 25 pmol each primer (Wrb6r -F and Wrb6r -R; Wongteerasupaya *et al*., 2003) and 100 ng template DNA. The reaction mixture was placed in a thermocycler (I-Cycler, Bio-Rad Laboratories Inc., California, USA) pre-heated at 85°C ("hot-start" method) and PCR was conducted using 40 cycles of amplification at 94°C/ 20 s, 60°C/ 20 s, 72°C/ 75 s, followed by a final incubation at 72°C/ 10 min. The amplified products were stored at 4°C until analysed by electrophoresis.

Table 1. Sequences of the PCR primers used for WSSV genotype analysis.

Primer	Sequence	Tm (°C)
Wrb6r - F*	5' TCTACTCGAGGAGGTGACGAC 3'	66°C
Wrb6r - R*	5' AGCAGGTGTGTACACATTTCATG 3'	66°C
Geno-WS - F	5' TATTGACCCCGACCACCGCTGC 3'	72°C
Geno-WS - R	5' TCCGCCTCTGCCCACGCATTGA 3'	72°C

* Wongteerasupaya *et al*. (2003).

For samples containing low quantities of WSSV DNA, a 2-step PCR protocol was employed by incorporating an initial PCR using primers (geno-WS-F and geno-WS-R) outside the region amplified in the 1-step PCR protocol. The first amplification was conducted using the same reaction conditions as in the 1-step protocol but employed a "hot-start" at 85°C, and 40 cycles at 94°C/ 20 s, 66°C/ 20 s, 72°C/ 90 s, followed by a final incubation at 72°C/ 10 min. In the second step, 0.5 µl of primary PCR product was used as the DNA template and the reaction was conducted using PCR primers Wrb6r-F and Wrb6r -R under the conditions described above in the 1-step PCR protocol.

PCR products were resolved by electrophoresis in 2% agarose-TAE gels containing 0.5 □ g/ml ethidium bromide and visualized by UV transillumination. Nucleic acid extracts and all PCR reagents were handled in a laminar flow cabinet using aerosol-resistant tips to avoid contamination. Primary PCR products were handled in a separate work area from that in which the nested PCR was performed.

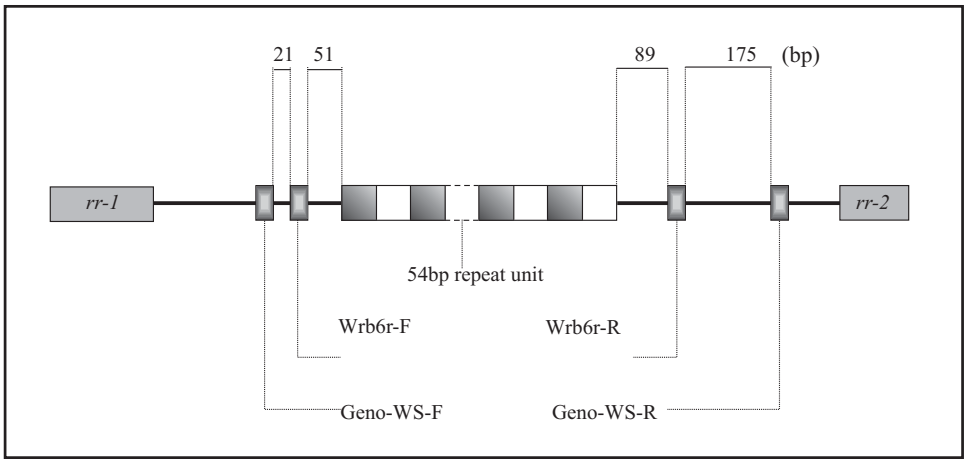

Figure 1. Schematic representation of the variable tandem repeat region of the WSSV genome between the *rr1* and *rr2* genes. Arrows show the location of PCR primer binding sites and the black boxes represent the variable tandem repeat units.

RESULTS

The TRS region analysed in this paper is located between the *rr1* and *rr2* genes encoding the large and small subunits of the WSSV ribonucleotide reductase. The region corresponds to ORF 94 as described by van Hulten *et al*. (2001) and WS178 and WS179 as described by Yang *et al*. (2001) in published complete WSSV genome sequences. Following the first repeat unit of 53 nucleotides (nt), each TRS in this region comprises 54 nt. Sequence variation commonly occurs at a single nucleotide at the first junction of the repeat units (Wongteerasupaya *et al*., 2003). Including the primer and flanking sequences, the length of the amplified PCR products were [XX + 54*n* - 1] nt (outer primers) or [YY + 54*n* - 1] nt (inner primers), where n is the number of repeats (Figure 1).

WSSV isolates from healthy broodstock and postlarvae collected from hatcheries

Tissue samples were obtained from healthy broodstock collected from hatcheries in Kien Giang Province on 26 December 2001 and from pooled postlarval samples collected from hatcheries in Kien Giang, Phan Thiet, Ca Mau, Phan Rang, Cam Ranh and Vung Tau Provinces from 4 January to 20 March 2002. The samples were tested for the presence of WSSV DNA using the IQ 2000 WSSV PCR test. Samples in which WSSV DNA was detected were examined using either the 1-step or 2-step PCR to determine the TRS genotype.

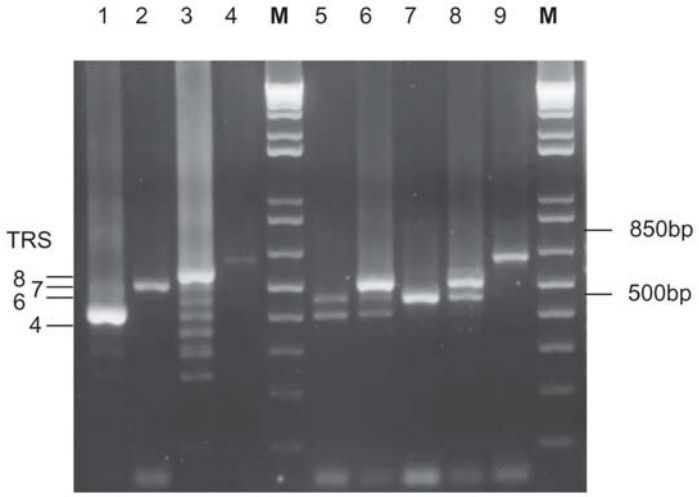

Figure 2. PCR assay of WSSV DNA from different pools of postlarve and different broodstock (*Penaeus monodon*). Lane M: 1kb plus DNA ladder; lanes 1-4: postlarval samples (WS160, WS141, WS63C, WS339, respectively); lanes 5-9: broodstock samples (WS45, WS38, WS40, WS44, WS48, respectively).

The WSSV TRS genotype in each sample is shown in Table 2 and examples of the genotype analysis are shown in Figure 2. Each pooled postlarval sample produced a single PCR product, suggesting infection with a single WSSV genotype. In some samples containing very high levels of target DNA (eg. WS63C, Figure 2, lane 3), a ladder effect was observed below the major PCR product. This appeared to be an amplification artefact as dilution of the sample eliminated the ladder but not the major product. However, the possibility that smaller, minor products may have been obscured by the ladder cannot be excluded. The TRS number in postlarval samples ranged from 4 to 9 copies with no predominance of any one genotype. Genotypes 4-TRS, 6-TRS and 7-TRS were each detected in samples collected from hatcheries in different provinces at different times. For example, a 7-TRS genotype was detected in WS93 collected in Kien Giang on the Gulf of Thailand on 4 January 2002, and in WS63C collected in Phan Thiet on the South China Sea coast on 29 January 2002. Two different genotypes (4-TRS and 6-TRS) were also detected in different postlarval samples (WS152 and WS159) collected from the same province on the same day (Phan Thiet, 18 January 2002).

Table 2. Genotypes of WSSV isolates from healthy broodstock and postlarvae collected from hatcheries in 6 provinces of central and southern Vietnam.

Host	Province of hatchery	Date collected	*Sample ID*	Product size (bp)	TRS1 genotype
P. monodon postlarvae	Phan Thiet	18.2.02	WS 152	399	4
	Ca Mau	18.2.02	WS 160	399	4
	Phan Rang	05.2.02	WS 141	507	6
	Phan Thiet	18.2.02	WS 159	507	6
	Cam Ranh	18.2.02	WS 182	507	6
	Kien Giang	04.1.02	WS 93	561	7
	Phan Thiet	29.1.02	WS 61C	561	7
	Phan Thiet	29.1.02	WS 63C	561	7
	Vung Tau	20.3.02	WS 339	615	8
P. monodon broodstock	Kien Giang	26.12.01	WS 45	399, 453	4, 5
	Kien Giang	26.12.01	WS 46	399, 453	4, 5
	Kien Giang	26.12.01	WS 47	399, 453	4, 5
	Kien Giang	26.12.01	WS 38	399, 453, 507	4, 5, 6
	Kien Giang	26.12.01	WS 39	453	5
	Kien Giang	26.12.01	WS 40	453	5
	Kien Giang	26.12.01	WS 43	453	5
	Kien Giang	26.12.01	WS 44	453, 507, 561	5, 6, 7
	Kien Giang	26.12.01	WS 48	615, 669	8, 9

In contrast to postlarval samples, most DNA extracted from single pleopods of individual broodstock produced 2 or 3 major PCR products. Due to variations in the intensity of the different sized products, the exact number of TRS genotypes in a single sample was often difficult to determine. However, the results clearly indicated that mixed infections with several WSSV genotypes were common. In addition, all genotypes detected in postlarvae from hatcheries in different provinces were represented in the set of genotypes detected in broodstock from Kien Giang Province on 26 December 2001. The data indicates a wide distribution of different WSSV genotypes in broodstock and postlarvae collected from hatcheries in central and southern Vietnam.

WSSV genotypes in from juvenile shrimp from healthy and diseased grow-out ponds

Tissue samples were obtained from 128 juvenile *P. monodon* from 14 disease outbreak ponds in Soc Trang, Ca Mau and Bac Lieu Provinces in the Mekong Delta region of southern Vietnam from 2 January to 6 June 2002. Tissue samples were also obtained from 187 juvenile *P. monodon* from four healthy ponds in Soc Trang Province that were sampled at 30 day intervals following stocking (i.e. 30, 60 and 90 days). WSSV DNA sequences were analysed using the 1-step or 2-step genotyping PCR. The TRS genotype in each sample is shown in Table 3 and examples of the genotype analysis are shown in Figure 3.

Table 3. Genotypes of WSSV isolates from healthy and diseased juveniles from grow-out ponds in 3 provinces of the Mekong Delta of Vietnam.

Province	Date collected	Pond ID	Pond condition	No of samples (positive/tested)	Product size (bp)	*TRS*
Soc Trang	06.06.02	TB	D	11/11	399	4
Soc Trang	14.03.02	N	D	8/8	669	9
Soc Trang	15.01.02	H	D	2/2	561	7
Soc Trang	17.02.02	N7	D	10/10	561	7
Soc Trang	17.02.02	N9	D	10/10	561	7
Soc Trang	06.06.02	TH	D	2/2	561	7
Soc Trang	19.06.02	TH1	D	5/5	561	7
Soc Trang	14.03.02	VC	D	2/2	561	7
Soc Trang	14.03.02	T	D	6/6	561	7
Ca Mau	02.04.02	CN	D	5/5	561	7
Ca Mau	02.04.02	DD	D	3/3	561	7
Bac Lieu	02.01.02	L1 (30)	D	26/31	561	7
Bac Lieu	02.01.02	L1 (30)	D	5/31	561, 669	7, 9
	03.02.02	L1 (60)	D	29/29	561	7
	02.01.02	L1 (60)	D	4/4	561, 669	7, 9
Soc Trang	17.02.02	V5	H	1/21	669	9
Soc Trang	17.02.02	V6	H	1/20	561, 669	7, 9
Soc Trang	17.02.02	V7	H	1/19	561, 615, 669	7, 8, 9
Soc Trang	14.04.02	V8 (90)	H	1/19	615	8
				1/19	669	9
				1/12	561	7

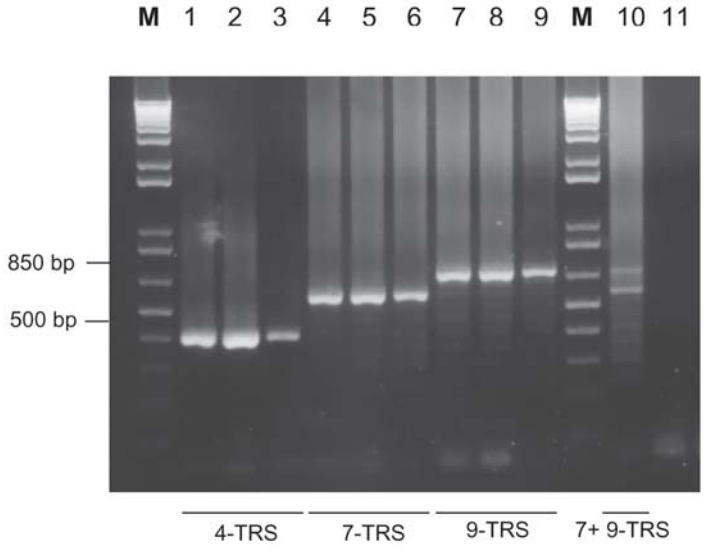

Figure 3. PCR amplification of different tandem repeats DNA fragments from juvenile shrimp in different grow-out ponds in Vietnam. Lanes M, 1kb plus DNA ladder; lanes 1-3, pond TB; lane 4-6, pond N7; lane 7-9, pond N; lane 10, pond L2; and lane 11, negative control.

Most shrimp sampled from diseased ponds appeared to contain a single WSSV TRS genotype. Moreover, except for 2 ponds (L1 and L2) on a single farm in Bac Lieu Province, all shrimp collected from the same diseased pond were infected with the same WSSV genotype. The 7-TRS genotype was detected in 12 of 14 (86%) diseased ponds and in 109 of 128 (85%) diseased juvenile shrimp. Genotype 9-TRS was detected in shrimp from 3 outbreak ponds including 2 ponds (L1 and L2) from one farm that were also infected with genotype 7-TRS. Pond L1 was sampled at day 30 after stocking and again at day 60 during a disease outbreak. At day 30, 26 of 31 (84%) shrimp sampled were infected only with genotype 7-TRS and the remaining shrimp were co-infected with both genotypes 7-TRS and 9-TRS. However, at day 60, only the 7-TRS genotype was detected in all 29 shrimp sampled. In pond H in Soc Trang Province, the 7-TRS genotype was also detected in postlarvae with disease only 4 days after stocking.

In all 4 healthy ponds from the same region (Soc Trang Province), WSSV was detected in only a small proportion of sampled shrimp. Genotypes 7-TRS, 8-TRS and 9-TRS were detected in healthy *P. monodon* from these ponds. Although the number of WSSV-positive shrimp in healthy ponds was small, there was no evident dominance of any single WSSV TRS genotype. However, there was evidence of multiple WSSV genotypes in shrimp from 2 of the 4 ponds and, in pond V7, a single shrimp was co-infected with 3 different TRS genotypes.

Table 4. Genotypes of WSSV isolates from crustacean carriers collected from grow-out ponds in 2 provinces of the Mekong Delta of Vietnam.

Province	Date collected	Sample ID	Pond ID	Pond condition	Species	Product size (bp)	TRS genotype	Shrimp TRS
Bac Lieu	02.01.02	L60SC	L1 (60)	D	Small crab	ND[1]	-	7
Soc Trang	15.01.02	HU	H	D	Wild shrimp	939	14	7
Soc Trang	14.03.02	TU	T	D	Wild shrimp	1423	23	7
		TSC			Small crab	1423	23	7
Soc Trang	17.02.02	V8LC	V8 (30)	H	Large crab	669	9	7

WSSV isolates from crustaceans collected from grow-out ponds

Five samples of individual large crabs, small crabs and wild shrimp (unidentified species) were collected from disease outbreak ponds L2, H and T, and healthy pond V8 from Bac Lieu and Soc Trang Provinces in the Mekong Delta. The samples were tested for the presence of WSSV DNA using the IQ-WSSV-2000 PCR test and with the genotyping PCR as described above for the shrimp samples. The TRS genotype analyses are shown in Figure 4 and the results are summarised in Table 4.

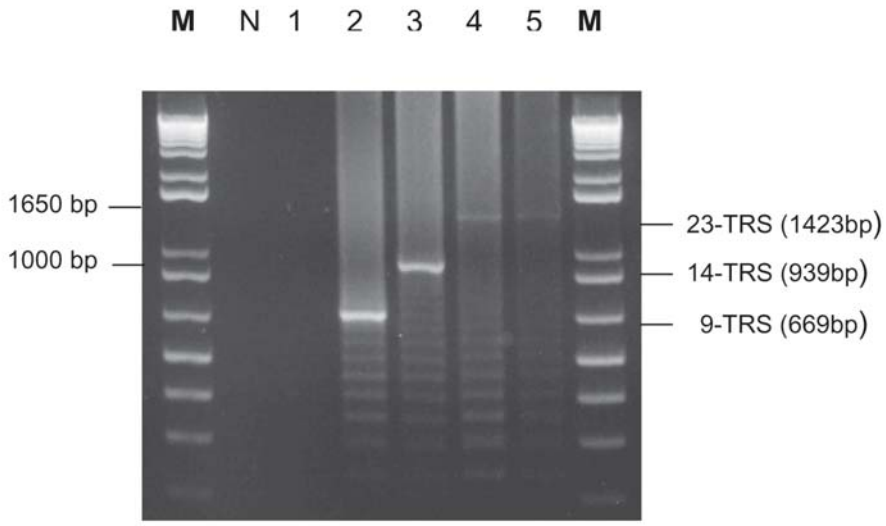

Figure 4. PCR amplification of different tandem repeats of WSSV DNA fragments from crustacean carriers. Lane M, 1kb plus DNA ladder; lane N, negative control; lane1, L60SC; lane2, V8LC; lane3, HU; lane4, TSC; lane5, TU. (SC = small crab; LC = large crab; U = wild shrimp; see Table 4).

A small crab collected from outbreak pond L1 in Bac Lieu Province was weakly WSSV-positive by the IQ-WSSV-2000 test but no product could be amplified using the 2-step genotyping PCR test. The crab sample was taken from the pond at day 60 after stocking during a disease outbreak in which the 7-TRS WSSV genotype was identified in all 29 diseased shrimp sampled from the pond (Table 3). Shrimp pond L1 had also been sampled at day 30 after stocking at which time all 31 shrimp sampled were infected with WSSV. The data indicates that, despite a long-term infection in the shrimp and a contemporaneous disease outbreak in the pond, the level of WSSV infection in the crab was low.

In two other disease outbreak ponds (H and T) from Soc Trang Province, the 7-TRS WSSV genotype was detected in diseased shrimp but different WSSV genotypes were detected in crabs and wild shrimp collected from these ponds during the outbreaks. In pond H, a 14-TRS genotype was detected in wild shrimp. In pond T, a 23-TRS genotype was detected in both wild shrimp and small crabs. In healthy pond V8 sampled at day 30 after stocking, a 9-TRS WSSV genotype was detected in a large crab. Although pond V8 remained healthy throughout grow-out, 7-TRS WSSV genotype was detected in a single shrimp sampled at day 90 after stocking. Overall, this limited study has identified no correlation between the WSSV genotypes present in *P. monodon* and other crustaceans in either healthy or diseased ponds.

DISCUSSION

Genotype analysis to distinguish individual viral isolates has potential to reveal important aspects of the epidemiology of WSSV infection including the identification of hosts and vectors, transmission routes and the sources of disease outbreaks. Variability in the number of a tandem repeat sequence has been applied in this paper to genotype analysis of WSSV isolates in cultured shrimp and wild crustaceans from hatcheries and ponds in several provinces of central and southern Vietnam. Six WSSV genotypes (4-TRS, 5-TRS, 6-TRS, 7-TRS, 8-TRS and 9-TRS) were observed in healthy broodstock and postlarvae. Of these, three genotypes (4-TRS, 7-TRS and 9-TRS) were found in shrimp sampled from disease outbreak ponds. The 9-TRS genotype was also detected in one crab collected from a healthy pond. High repeat number genotypes, 23-TRS and 14-TRS, were detected in unidentified species of small crab and wild shrimp but not in cultured shrimp. Comparison with three other WSSV isolates for which the complete nucleotide sequence is deposited in GenBank indicates that a 1994 isolate from *P. monodon* in southern Taiwan (AF440570) and a 1996 isolate from *P. monodon* in Thailand (AF369029) each have a 6-TRS genotype, and a 1996 isolate from *P. japonicus* in Xiamen Province of eastern China (AF332093) has a 12-TRS genotype. In a study of juvenile *P. monodon* collected from 55 diseased ponds in central and southern Thailand in 2000-2002, a wide range of genotypes (6-TRS to 20-TRS) were identified at the same variable locus (Wongteerasupaya *et al*., 2003). Of these 6-TRS (14.5%), 7-TRS (10.9%), 8-TRS (32.8%) and 9-TRS (14.5%) genotypes were most commonly detected. Very low copy number WSSV genotypes (1-TRS, 2-TRS or 3-TRS) have not yet been observed in these previous studies. As the 6-TRS genotype 1994 Taiwanese isolate is the earliest currently available, the detection of 4-TRS and 5-TRS genotypes in Vietnamese *P. monodon* is of some interest. If there has been a progressive evolutionary expansion of TRS copy numbers since the original emergence of WSD in East Asia in 1992, the common occurrence of low TRS copy numbers amongst Vietnamese isolates suggests that WSSV may have may translocated from East Asia during the initial phase of the panzootic.

In this study, the 7-TRS genotype clearly predominated in juvenile *P. monodon* from disease outbreak ponds in three provinces of the Mekong Delta. However, the predominance of the 7-TRS genotype was not evident either in healthy *P. monodon* broodstock or postlarvae, or in the limited number of WSSV-positive shrimp identified in healthy grow-out ponds. The 7-TRS genotype was also absent from healthy wild crustaceans collected from diseased ponds. The predominance of the 7-TRS genotype in *P. monodon* collected from disease outbreak ponds suggests that a virulence determinant may be associated with the 7-TRS marker. However, as discussed above, the 7-TRS genotype was not predominant in a previous study of diseased *P. monodon* from Thailand (Wongteerasupaya *et al*., 2003) and there is adequate evidence that several other TRS genotypes have caused WSD in Thailand, Taiwan, the Chinese mainland and Vietnam. Further is required to confirm that the Vietnamese 7-TRS genotype is more commonly associated with WSD outbreaks. If so, the virulence determinant associated with this marker may be one that confers increased risk of disease due to an increased sensitivity to environmental stress. As WSSV appears to replicate more efficiently at lower temperatures (Vidal *et al*., 2001), such a determinant could be a higher temperature optimum of the viral polymerase. Comparisons of the virulence of the 7-TRS genotype and other WSSV TRS genotypes in controlled bioassays would assist in resolving

this issue.

Despite the small number of available samples, the analysis of TRS genotypes of WSSV isolates from wild crustaceans was informative. The data indicated that: i) the WSSV genotypes detected in wild crustaceans from healthy and diseased ponds were different from the WSSV genotype in the co-inhabitant *P. monodon*; ii) wild shrimp and crabs from the same diseased pond shared the same uncommon TRS genotype; and iii) crabs from a diseased pond appeared to be infected at a very low level while a disease outbreak and mortalities occurred in the farmed shrimp. The detection of unusually high TRS copy numbers (14-TRS and 23-TRS) in wild crustaceans was also of interest and may have arisen by rapid evolution and adaptation of the virus to a local infection cycle in these host species.

Overall, the data suggests that wild crustaceans are not a common source of WSSV infection or disease in farmed shrimp. This is supported by previous studies (Hsu *et al*., 1999; Withyachumnarnkul, 1999; Peng *et al*., 2001) in which the elimination of infected seed was shown to reduce the risk of disease in ponds significantly. However, a more detailed longitudinal study of ponds from stocking to harvest in various locations will be necessary to more clearly define the origins of WSD. Clearly, genotype analysis will be a very useful tool in studying the dynamics of WSSV infection in the pond environment and assist in developing the most cost-effective strategies for the management of disease.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Robert Lester (The University of Queensland) for his encouragement, and Dr Jeff Cowley and Dr Lisa Leeton (CSIRO Livestock Industries) for their valuable suggestions during this research. Ms Tran TT Hoa was enrolled as a post-graduate research student at the University of Queensland, supported jointly by CSIRO and the Australian Centre for International Agricultural Research (ACIAR).

REFERENCES

Chang, Y.-S., Peng S.-E., Wang, H.-C., Hsu, H.-C., Ho, C.-H., Wang, C.-H., Wang, S.-Y., Lo, C.-F. and Kou, G.-H. 2001. Sequencing and amplified restriction fragment length polymorphism analysis of ribonuleotide reductase large subunit gene of the white spot syndrome virus in blue crab (*Callinectes sapidus*) from American coastal waters. Marine Biotechnology 3, 163-171.

Chou, H.-Y., Huang, C.-Y., Wang, C.-H., Chiang, H.-C. and Lo, C.-F. (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Disease of Aquatic Organisms 23, 165-173.

Flegel, T.W. 1997. Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World Journal of Microbiology and Biotechnology 13, 433-442.

Hsu, H.-C., Lo, C.-F., Lin, S.-C., Liu, K.-F., Peng, S.-E., Chang, Y.-S., Chen, L.-L., Liu, W.-J. and Kou, G.-H. 1999. Studies on effective PCR screening strategies for white spot syndrome virus (WSSV) detection in *Penaeus monodon* brooders. Diseases of Aquatic Organisms 39, 13-19.

Lightner, D.V. 1996. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaied Shrimp. World Aquatic Society, Baton Rouge.

Lightner, D.V., Redman, R.M., Poulos, B.T., Nunan, L.M., Mari, J.L. and Hasson, K.W. 1997. Risk of spread of penaeid shrimp viruses in the Americas by the international movement of live and frozen shrimp. Scientific and Technical Review of the Office International des Epizooties 16, 146-160.

Lo, C.-F., Hsu, H.-C, Tsai, M.-F., Ho, C.-H., Peng, S.-E, Kou, G.-H. and Lightner, D.V. 1999. Specific genomic DNA fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus. Diseases of Aquatic Organisms 35, 175-185.

Mayo, M.A. 2002. A summary of taxonomic changes recently approved by ICTV. Archives of Virology 147, 1655-1656.

Mohan, C.V., Shankar, K.M., Kulkarni S. and Sudha, P.M. (1998). Histopathology of cultured shrimp showing gross signs of yellow head syndrome and white spot syndrome during 1994 Indian epizootics. Diseases of Aquatic Organisms 34, 9-12.

Nakano H., Koube, H., Umezawa, S., Momoyama, K., Hiraoka, M., Inouye, K., and Oseko, N. 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: epizootiological survey and infection trials. Fish Pathology 29, 135-139.

Park, J.H. Lee, Y.S., Lee, S. and Lee, Y. 1998. An infectious viral disease of penaeid shrimp newly found in Korea. Diseases of Aquatic Organisms 34, 71-75.

Peng, S.-E., Lo, C.-F., Lin, S.-C., Chen, L.-L., Chang, Y.-S., Liu, K.-F., Su, M.-S. and Kou, G.-H. 2001. Performance of WSSV-infected and WSSV-negative *Penaeus monodon* postlarvae in culture ponds. Diseases of Aquatic Organisms 46, 165-172.

van Hulten, M.C.W., Tsai, M.-F., Schipper, C.A., Lo, C.-F., Kou, G.-H. and Vlak, J.M. 2000. Analysis of a genomic segment of white spot syndrome virus of shrimp containing ribonucleotide reductase genes and repeat regions. Journal of General Virology 81, 307-316.

van Hulten, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K. and Vlak, J. 2001. The white spot syndrome virus DNA genome sequence. Virology 286, 7-22.

Vidal, O.M., Granja, C.B., Aranguren, F., Brock, J.A. and Salazar, M. 2001. A profound effect of hyperthermia on survival of *Litopenaeus vannamei* juveniles infected with white spot syndrome virus. Journal of the World aquaculture Society 32, 364-372.

Wang C.S., Tang, K.F.J., Kou, G.H. and Chen, S.N. (1996). Yellow head disease-like virus infection in the kuruma shrimp *Penaeus japonicus* cultured in Taiwan. Fish Pathology 31, 177-182.

Wang, Q., Nunan, L.M. and Lightner, D.V. 2000. Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and Southern blot hybridization. Diseases of Aquatic Organisms 43, 175-181.

Withyachumnarnkul, B. 1999. Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). Diseases of Aquatic Organisms 39, 21-27.

Wongteerasupaya, C., Pungchai, P., Withyachumnarnkul, B., Boonsaeng, V., Panyim, S., Flegel, T.W. and Walker, P.J. 2003. High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. Diseases of Aquatic Organisms 54, 253-257.

Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akarajamorn, A., Boonsaeng V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B. and Flegel, T.W. 1995. A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Diseases of Aquatic Organisms 21, 69-77.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X. and Xu, X. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. Journal of Virology 75, 11811-11820.

Zhan, W.B. and Wang, Y.H. 1998. White spot syndrome virus infection of cultured shrimp in China. Journal of Aquatic Animal Health 10, 405-410.