White Spot Syndrome Virus molecular epidemiology: relation with shrimp farming and disease outbreaks

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CONTENTS

ABSTRACT

White spot syndrome virus (WSSV), the causative agent of white spot disease (WSD), has been responsible for most shrimp production losses around the world since the early 1990s. Previous research has focused mainly on the characterization of WSSV genomic variation to gain a better insight in the evolution and spread of the virus at the regional and global levels. Although WSSV genetic variation at small spatial scales has been described, the question is whether there is a correlation between this genomic variation and shrimp farming practices and disease outbreak at the local and farm level. Therefore, the present research has been carried out to describe in detail WSSV genetic variation over space and time and to estimate transmission routes of WSSV genotypes in ponds with different farming regimens. Different molecular markers, natural variation contained within the WSSV genome, were tested to quantify these relationships in semi-intensive, extensive and rice-shrimp farming systems within the Ca Mau and Bac Lieu provinces, both in the Mekong Delta, Vietnam. The outcomes of the research showed that (i) there are differences in genetic structure of WSSV populations in shrimp culture areas, with more variation found in variable number tandem repeat (VNTR) regions, short adjoining repeat sequences, than in genomic regions in which large deletions occur; (ii) a correlation between WSSV population structure, disease outbreak status and pond farming system was observed, providing evidence that the WSSV VNTR structure (in particular the number of repeat units in ORF94) statistically correlate with disease outbreaks and to a lesser extent to farming system; (iii) mixed-genotype WSSV infections of shrimp are correlated with fewer disease outbreaks in ponds; and (iv) it might be possible to use molecular markers (ORF94 and ORF125) to predict the outcome of WSSV infections in shrimp ponds in the future. For field applications, these findings provide important information for the development of specific management strategies to control WSD.

1

- CHAPTER -

GENERAL INTRODUCTION

I. BACKGROUND

1. Aquaculture and shrimp farming in Vietnam

Aquaculture has dramatically grown for over the last 50 years with total annual production currently exceeding 50 million tonnes and with an estimated value of almost US$98.5 billion globally (FAO, 2010). Asia dominates global aquaculture production, accounting for more than 88.9% (Bostock *et al.,* 2010). Within Asia aquaculture production takes place mainly in China and other newly industrialized countries in the Asia-Pacific region, such as Thailand and Vietnam. Aquacultural produce is not only an important source for food consumption but also the main livelihood of 47.5 million people, especially in developing countries (FAO, 2008). Among the different cultured species, carp, oyster and shrimp are heavily cultivated in Asia, while the principal farmed species in Norway, Chile, the United Kingdom and Canada is Atlantic salmon (FAO, 2010).

Among aquaculture industrial sectors, shrimp farming is not an exceptional case with a steady increase of cultivated shrimp production since the early 1980s (FAO, 2000; 2001; Flegel *et al.*, 2008) (Figure 1). Shrimp products are recorded as accounting for 17% of the total internationally traded fishery products, the largest single seafood commodity by value. The black tiger shrimp (*Penaeus monodon*) and the white leg shrimp (*Penaeus vannamei*) are the two species which dominate production and account for approximately 75% of global shrimp aquaculture production (FAO, 2010).

Vietnam is one of the largest aquaculture production countries in the world. In 2008, Vietnam occupied the third place in the list of top 15 aquaculture producers globally (FAO, 2010) (Table 1). This ranking is based on aquaculture production and average annual rate of growth. Over the past two decades, shrimp farming has been transformed into a major industry in Vietnam and has become an important income-generating activity in coastal areas (de Graaf and Xuan, 1998). It generates employment for 4.5 million people, representing more than 5% of Vietnam´s population (Dan and Khoa, 2011).

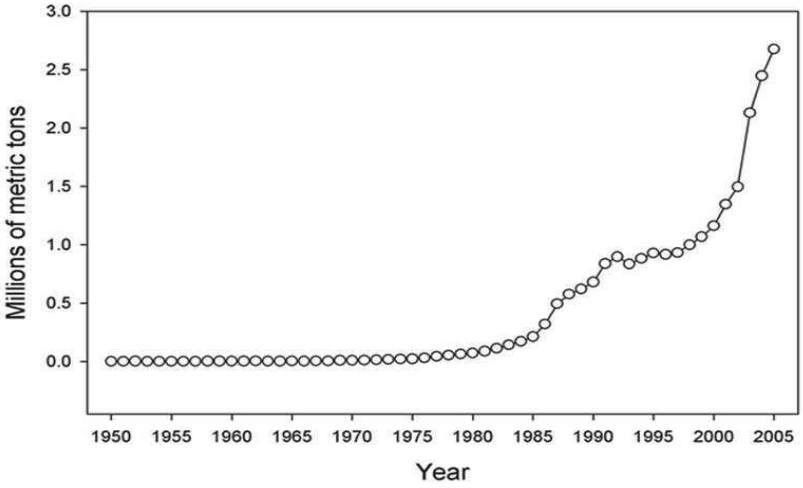

Figure 1. Global production of shrimp from aquaculture 1950 to 2005. Graph is taken from Flegel *et al.* (2008)

Table 1. World aquaculture production: Top 15 of aquaculture producers by production in 2008 and growth

	Production (thousand tonnes)			Average annual rate of growth (%)		
	1990	2000	2008	1990-2000	2000-2008	1990-2008
China	6482	21522	32736	12.7	5.4	9.4
India	1017	1943	3479	6.7	7.6	7.1
Vietnam	160	499	2462	12.0	22.1	16.4
Indonesia	500	789	1690	4.7	10.0	7.0
Thailand	292	738	1374	9.7	8.1	9.0
Bangladesh	193	657	1006	13.1	5.5	9.6
Norway	151	491	844	12.6	7.0	10.0
Chile	32	392	843	28.3	10.1	19.8
Philippines	380	394	741	0.4	8.2	3.8
Japan	804	763	732	–0.5	–0.5	–0.5
Egypt	62	340	694	18.6	9.3	14.4
Myanmar	7	99	675	30.2	27.1	28.8
United states of America	315	456	500	3.8	1.2	2.6
Republic of Korea	377	293	474	–2.5	6.2	1.3
Taiwan	333	244	324	–3.1	3.6	-0.2

Source: FAO, 2010.

Among different aquaculture species, penaeid shrimp are some of the important culture species, and are mostly produced in the Mekong Delta provinces (Figure 2 and Figure 3) (Dan and Khoa, 2011). The provinces involved are located along the shore of the East Sea in the southernmost tip of the Mekong Delta, Vietnam (Figure 4). Prior to 1993, *Penaeus indicus* was the dominant shrimp species for farming. However, in 1997, there was shift in the main cultivated species towards *Metapenaeus ensis* and *Metapenaeus lysianassa* (Clough and Johnston, 1998). By 2010, *P. monodon* had become the main species produced, and exotic *P. vannamei*

was mainly cultivated in intensive systems in Quang Ninh province (North of Vietnam) and Central Vietnam since 2003 (Fisheries Directorate, 2010). This is in contrast to other shrimp-producing countries, where *P. vannamei* is by far the most popular shrimp species prior to 2003.

In Vietnam, the current total brackish shrimp farming area is estimated at 639,115 ha with a production of 469,893 tons. Of this figure, the production of *P. monodon* accounts for over 70 percent (333,174 tons) and that of *P. vannamei* represents about 30% (136,719 tons). An estimated 62,845 ha is under intensive culture and another 550,873 ha is under improved-extensive (extensive plus) culture. In terms of production in 2010, the Mekong Delta provinces ranked as the main shrimp production area of Vietnam, with in ascending order: Tra Vinh (21,120 tons), Ben Tre (27,378 tons), Kien Giang (34,737 tons), Soc Trang (61,160 tons), Bac Lieu (71,236 tons), and Ca Mau (103,900 tons) (Fisheries Directorate, 2010).

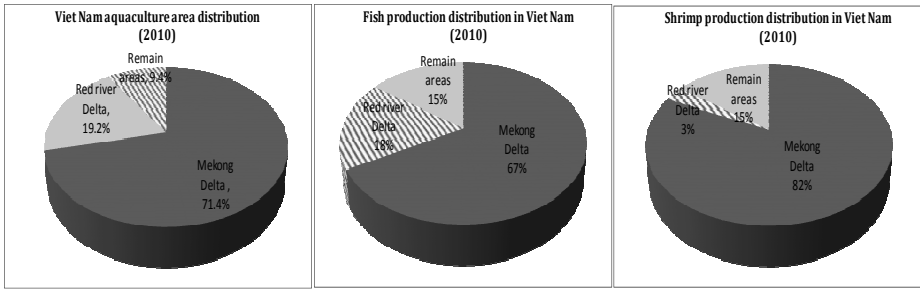

Figure 2. Aquaculture areas and fish/shrimp production achieved from different areas in Vietnam (2010) (Dan and Khoa, 2011).

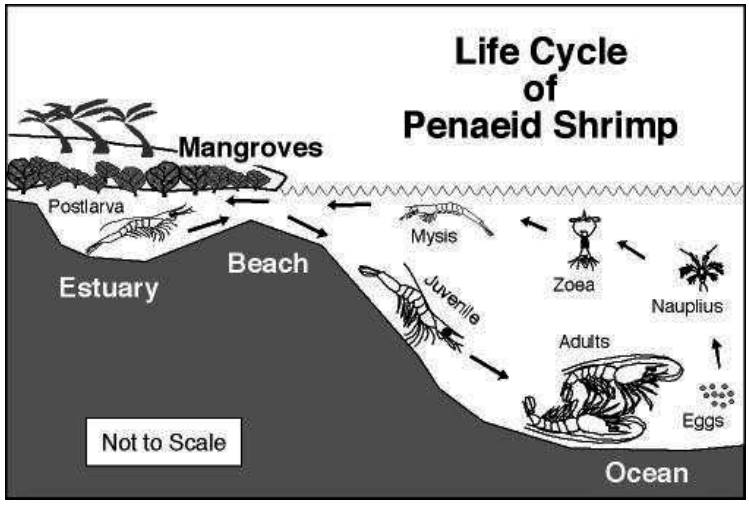

Figure 3. Life cycle of penaeid shrimp (Rosenberry, 2000)

In 2010, different shrimp farming systems were practiced in the cultivated areas of Vietnam, mainly improved-extensive farming systems (320,343 ha), rice-shrimp farming systems (168,633 ha), semi-intensive farming systems and intensive farming systems (41,765 ha), and shrimp-mangrove forestry (48,644 ha) (Figure 4) (Fisheries Directorate, 2010). Improved-extensive farming systems are a modification of extensive system in which stocking seed depends on tidal water inflow and shrimp seed (post larvae –PL) is supplied at low stocking density of about 2 – 4 PL/m^2 (MoFi, 2002). Artificial feeds or other types of food are sometimes supplied in this system. Rice-shrimp farming system, on the other hand, is an alternative system where rice is cultivated in wet seasons and shrimps are cultured in dry seasons. The shrimp stocking density is about 1 – 2 PL/m^2 (Nhuong *et al.,* 2002). Semi-intensive farming systems are well prepared, seeded with high stocking density of 10 – 25 PL/m^2. The systems employ artificial or supplementary feeds and water sources sometimes depend on the spring tide. Another category, intensive farming systems, requires complete diet feeding, and high stocking density (25 – 32 PL/m^2) with pond sizes ranging from 0.3 to 0.9 ha. Ponds are well constructed and well-equipped with aeration systems, inlet and outlet water systems to maintain water quality. Lastly, in mangrove-shrimp farming systems, shrimp is cultured in mangrove forest areas. These farming systems operate year-round without seed supply, or feed supply (IFEP, 2009). In this thesis only improved-extensive, semi-intensive and rice-shrimp systems are considered.

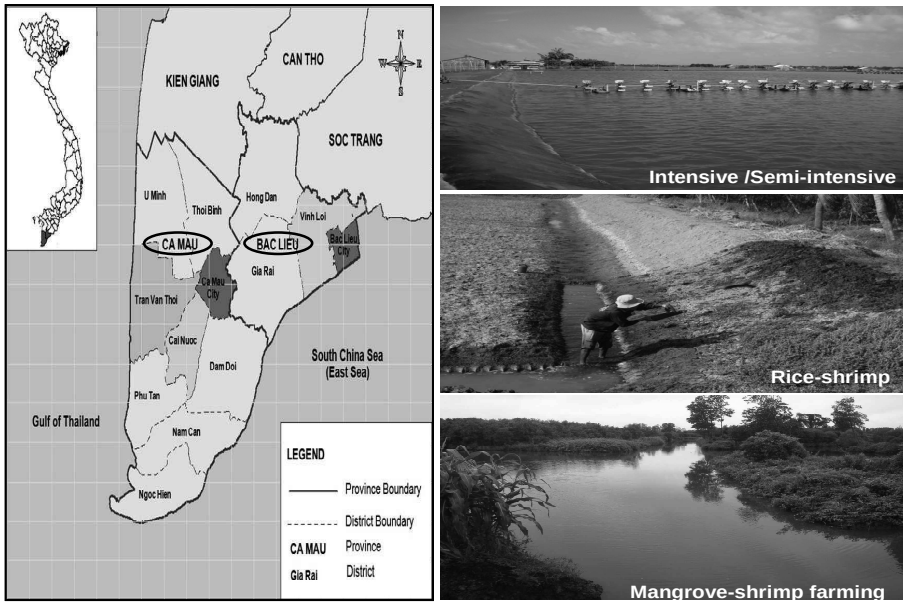

Figure 4. Shrimp farming regions and shrimp farming systems in Mekong Delta of Vietnam (research sites are pointed by circle in the map)

2. Shrimp diseases

The intensification of shrimp farming and the use of higher shrimp stocking densities over the past two decades have been accompanied by an increase in the number of epidemic diseases (Kautsky *et al.,* 2000). Shrimp farming is affected by different types of infectious diseases, namely fungal, parasitic, bacterial and viral diseases (Figure 5) (Flegel *et al.,* 2008). Among these, diseases of viral aetiology are a main cause of substantial economic losses, which are estimated at 3 billion of US dollars per year worldwide (Lundin, 1995; Lightner, 2003). The first shrimp virus was isolated from wild shrimp (*Penaeus duorarum*) collected from the Florida Gulf Coast in the early 1970s (Couch, 1974a,b). Lightner (1992) recorded a list of 11 virus diseases of cultured penaeid shrimp. Since then, more than 20 viruses have been reported to infect shrimp (Lightner, 1996), and the list is expanding with more severe viruses, such as Infectious myonecrosis virus - IMNV, reported in 2002 (Lightner *et al.,* 2004), and Laem-Singh virus - LSNV, reported in 2002 (Sritunyalucksana *et al.,* 2006) (Table 2). White spot syndrome virus (WSSV), yellowhead virus (YHV), and Taura syndrome virus (TSV) are the most devastating shrimp viruses, and are considered as notifiable by the Office Internationale de Epizooties (OIE) and the EC Council Directive 2006/88/EC, adopted during 2008 (OIE, 2001; OIE, 2011; Stentiford *et al.,* 2009). In Asian shrimp farming regions, both WSSV and YHV have caused continuing direct losses of approximately US$ 1 billion per year to the native cultured shrimp industry since 1992 (Flegel *et al.,* 2008).

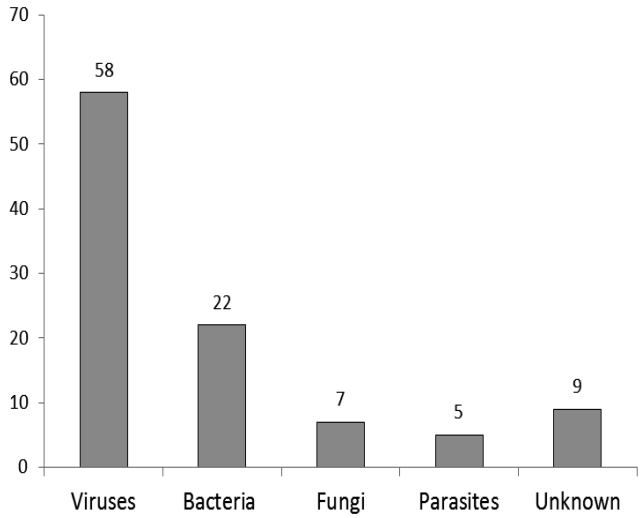

Figure 5. Relative economic loss to disease caused by various pathogen groups in 2001. Adapted from Flegel *et al.* (2008).

Among the six viral pathogens of OIE listed crustacean disease in 2011, five virus species were reported to present in shrimp farming regions in Vietnam, including WSSV (Hao *et al.,* 1997); two types of yellowhead complex viruses (Gill-associated virus – GAV and genotype 3) (Wijegoonawardane *et al.,* 2008); Taura syndrome virus – TSV (Van, 2004); Macrobrachium rosenbergii nodavirus - MrNV (Oanh *et al.,* 2009); Infectious hypodermal and haematopoietic necrosis virus – IHHNV (MARD, 2011).

Table 2. List of emerged viruses in shrimp farming system world wide

Viral nomenclature	Abbreviation	Genome	First year emerged	Taxonomic classification	Known geographic distribution	OIE listed 2007	OIE listed 2011
DNA virus							
Baculovirus penaei	BP	dsDNA	1974	Baculoviridae	Asia	**Yes**	**No**
Monodon baculovirus	MBV	dsDNA	1977	Baculoviridae	Asia-Pacific, Americas, Africa	**Yes**	**No**
White spot syndrome virus	WSSV	dsDNA	1992	Nimaviridae, Whispovirus	Asia, Middle-East, Mediterranean, Americas	Yes	Yes
Infectious hypodermal and haematopoietic necrosis virus	IHHNV	ssDNA	1981	Parvoviridae, Densovirus	Asia-Pacific, Africa, Madagascar, Middle-East, Americas	Yes	Yes
Hepatopancreatic parvovirus	HPV	ssDNA	1983	Parvoviridae, Densovirus	Asia-Pacific, Africa, Madagascar, Middle-East, Americas	**Yes**	**No**
RNA virus							
Yellow head virus	YHV	(+)ssRNA	1990	Nidovirales, Roniviridae, Okavirus	East and Southeast Asia, Mexico	Yes	Yes
Taura syndrome virus	TSV	(+)ssRNA	1992	Picornavirales, Dicistroviridae	Americas, East and Southeast Asia	Yes	Yes
Myonecrosis virus	IMNV	(+)ssRNA	2002	Totivirus (unclassified)	Brazil, Indonesia, Thailand, China	Yes	Yes
Macrobrachium rosenbergii nodavirus	MrNV	(+)ssRNA	1995	Nodavirus (unclassified)	India, China, Taiwan, Thailand, Australia, Caribbean	Yes	Yes
Laem-Singh virus	LSNV	(+) dsRNA	2002	Luteovirus-like (unclassified)	South and Southeast Asia	No	No
Mourilyan virus	MoV	(-)ssRNA	1996	Bunyavirus-like (unclassified)	Australia, Asia	No	No

In Vietnam WSSV was reported to be responsible for serious production losses for shrimp production in 1994 (de Graaf and Xuan, 1998) which has been estimated as the most explosive epidemic in shrimp farming in Vietnam up to now (IFEP, 2009). This mass mortality of cultured shrimp occurred in the Mekong Delta in 1994 and cost about 30 million US dollars (Tam, 1994). WSSV was one of the most prevalent pathogens to be detected in moribund shrimp from endemic areas in 1994 (Hao *et*

al., 1997). Therefore, WSSV was recorded to be present for the first time in Vietnam shrimp farming areas in 1994 (Hao *et al.,* 1997; de Graaf and Xuan, 1998).

WSSV is still the most detrimental virus affecting black tiger shrimp farming areas in Vietnam, especially in the Mekong Delta. In the first shrimp crop of the year 2011, shrimp farming was reported to have been decimated by WSSV, followed by IHHNV, YHV and a new shrimp disease called "hepatopancreatic necrosis syndrome". The disease affected 61.43%, 41.23%, 20-30% of shrimp cultured areas in Soc Trang province, Bac Lieu province, and Ca Mau province, respectively. Among these diseases, WSD has been found to be highly pathogenic and it occurred in all main cultivated shrimps in the period from February to beginning of May, 2011 (MARD, 2011).

Recently, another type of disease has caused mass mortalities in all *P. monodon* shrimp culture areas in Vietnam, namely hepatopancreatic necrosis syndrome. The aetiological agent causing this disease is not known and it is even questionable, whether a pathogen is involved or another yet unknown environmental factor. This new type of disease was first recorded in the 2010 shrimp crop of the Mekong Delta. The disease occurred in both white leg shrimp and black tiger shrimp within one month after stocking. In early stages of the disease, clinical signs are often not very clear. Moribund shrimp have a reduced rate of growth, and show gross clinical signs such as loose shells, atrophied hepatopancreas, discoloration swelling or softening of hepatopancreas, and occasionally black spots in the necrotic hepatopancreas (MARD, 2011; Dan and Khoa, 2011).

3. White spot disease and white spot syndrome virus

WSSV is one of the most widely distributed of all known viruses of shrimp species (Walker and Winton, 2010). Due to its pathogenicity and its epidemic nature, WSSV has been considered notifiable by the OIE. Many other viral agents have been listed and de-listed in the OIE list diseases but WSSV has remained in the list since 2001 (OIE, 2001; OIE, 2003; OIE, 2007; OIE, 2009; OIE, 2011). WSSV ranks in the top three of harmful shrimp viruses and it has caused important losses in the shrimp populations (Dhar *et al.,* 2004). This virus caused continuing direct losses of approximately 10 US$ billion to the native cultured shrimp industry world-wide since its first appearance in 1992 (Stentiford *et al.,* 2009).

Causative agent

White Spot Disease (WSD) is caused by WSSV which is the sole member of the new genus *Whispovirus* within the new family *Nimaviridae* (Vlak *et al.,* 2005). There is genetic variation between WSSV isolates collected at different geographical locations (OIE, 2011). It is worthwhile noting that in the early literature (i.e., in the 1990s), WSSV isolates were given different names based on geographic location of the isolate, clinical signs, and affected species or tissue types (Table 3). It was only

recognized later that all these isolates were representative of a single aetiological agent, WSSV (Mayo, 2002).

WSSV virions have been described as large (120–150 nm in diameter and 270–290 nm in length), ellipsoid to bacilliform in shape, enveloped particles with a flagellum-like tail at one end (Figure 6) (Wang *et al.,* 1995; Wongteerasupaya *et al.,* 1995). However, these tails have never been seen in infected cells or tissues.

Regarding biological characteristics, WSSV virions can remain viable for at least 30 days at 30°C in seawater under laboratory conditions (Momoyama *et al.,* 1998); and is viable in ponds for at least 3–4 days (Nakano *et al.,* 1998). WSSV can be inactivated by heating for <120 minutes at 50°C and <1 minute at 60°C (Nakano *et al.,* 1998).

Table 3. Various reported names of the causative agent of White spot disease.

Abbreviation	Name	Identified geography	Reference
RV-PJ	Rod-shape nuclear virus of *Penaeus japonicus*	Japan	Takahashi *et al.,* 1994
PRDV	Penaeid rod-shaped DNA virus	Japan	Inouye, 1996
HHNBV	Hypodermal and haematopoietic necrosis baculovirus	China	Huang *et al.,* 1995
CBV	Chinese baculovirus	China	Lu *et al.,* 1997
SEED	Shrimp explosive epidermic disease	China	Cai *et al.,* 1995
SEMBV	Systemic ectodermal and mesodermal baculovirus	Thailand	Woongteerasupaya *et al.,* 1995
PmNOBII	*Penaeus monodon* non-occluded baculovirus II	Thailand	Woongteerasupaya *et al.,* 1995
PmNOBIII	*Penaeus monodon* non-occluded baculovirus III	Taiwan	Wang *et al.,* 1995
WSBV	*White spot baculovirus*	Taiwan	Lo *et al.,* 1996

Genomic WSSV

The WSSV genome is a circular double-stranded DNA molecule. The three WSSV isolates, ranging in size from 292 to 307 kbp, have been fully sequenced. WSSV is in the list of top ten largest viruses which has been identified so far. All large viruses with large double-stranded DNA genomes, e.g. WSSV, are often called giruses (Van Etten *et al.,* 2010). More specifically, a WSSV isolate from *P. monodon* collected in Thailand (WSSV-TH; GenBank accession number AF369029.2) was found to have a 292,967 nucleotide genome sequence (van Hulten *et al.,* 2001a). Yang *et al,* (2001) reported that WSSV isolated from moribund *Penaeus japonicus* in China had a genome of 305,107 bp (WSSV-CN; GenBank accession number AF332093.1 or NC_003225.1). The complete 307,287 bp nucleotide sequence was determined by Lo and Kou (2001) for an isolate from *Penaeus monodon* in Taiwan

(WSSV-TW; GenBank accession number AF440570.1). The size difference among these WSSV isolates is due primarily to two major indels (Marks *et al.,* 2004).

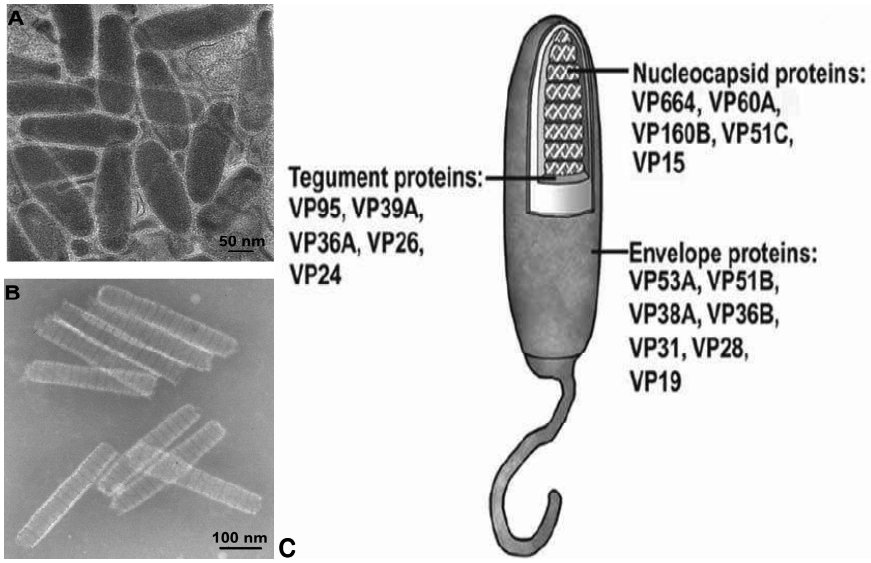

Figure 6. WSSV by transmission electron microscopy. A. intact enveloped virions; B. nucleocapsids appeared as stacked, ringed structures (Reproduced from Li *et al.,* 2007); C. a schematic diagram on WSSV virion structures containing envelope, tegument, and nucleocapsid with major identified proteins (Reproduced from Leu *et al.,* 2009).

The WSSV sequence contains a total of 531 putative open reading frames (ORFs) (Yang *et al.,* 2001) or 684 ORFs (van Hulten *et al.,* 2001a), of which at least 181-184 ORFs are thought to encode functional proteins. These ORFs are larger than 50 aa and non-overlapping. Only 45 of the protein-encoding genes resemble known proteins (>20% amino acid identity) including immediate early genes, latency-related genes, ubiquitination-related genes, and anti-apoptosis genes. A number of ORFs contains recognizable motifs and is mainly involved in nucleotide metabolism and DNA replication. The known genes encode: DNA replication (ribonucleotide reductase large and small subunits, thymidylate synthase, a novel chimeric protein of thymidine kinase and thymidylate kinase and DNA polymerase), dUTPase, transcription factors, virion proteins and protein modification enzymes (protein kinases) (Tsai *et al.,* 2000a; b; van Hulten *et al.,* 2001a; b; Zhang *et al.,* 2002). Nine regions of homologous repeats (hr's) containing 47 repeated mini-segments were identified and found to be dispersed throughout the WSSV genome (Leu *et al.,* 2009). WSSV is also unique amongst known viruses in that it contains a collagen gene and "a gene encoding an extracellular matrix protein of animal cells that has never been found in any virus" (Yang *et al.,* 2001;

van Hulten *et al.,* 2001a). It has been suggested that the synthesis of this collagen-like protein (ORF30, van Hulten *et al.,* 2001a) might help protect WSSV from environmental factors and allow it to exist in shrimp ponds for long periods of time (Huang *et al.,* 2001; Yang *et al.,* 2001). Finally, a protein of about 664 kDa has been detected in WSSV nucleocapsids, which is the largest eukaryotic protein to date, An internal nucleotide sequence of this gene serves as target for a widely used WSSV detection kit (IQ2000) (Leu *et al.,* 2005).

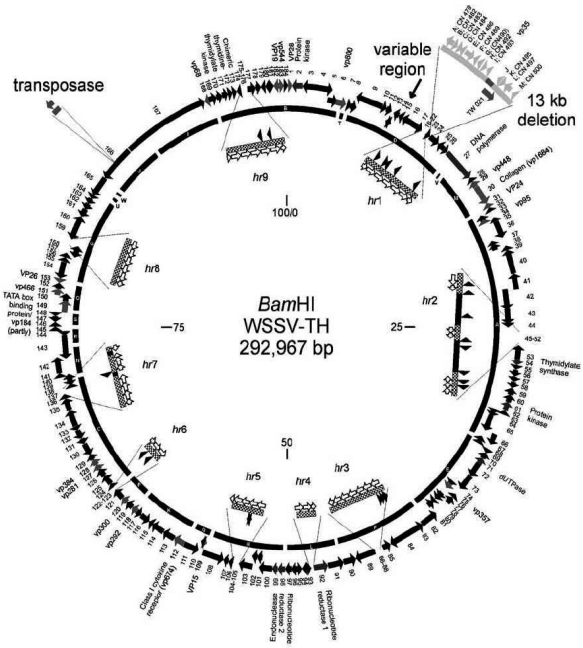

Figure 7. Schematic map of the WSSV-TH DNA genome showing the three major differences (13 kb deletion, variable region and transposase) among WSSV-TH, WSSV-CN and WSSV-TW. Reproduced from Marks *et al.* (2004).

All the three WSSV complete genomes have been studied and their genetic comparisons have revealed a high degree of genetic similarity (99.5%; Marks *et al.,* 2004). Differences among these three WSSV genomes were identified: (i) indel-I region: a difference of about 750 bp involves a genetically variable region; (ii) indel-II region: a deletion of approximately 13 kb (WSSV-TH) and 1 kb (WSSV-CN) compared to WSSV-TW, located between ORF23 and ORF24 (WSSV-TH) (Figure 7); (iii) a unique transposase sequence of 1,337 bp in WSSV-TW; (iv) Variation in the number of repeat units within homologous regions and direct repeats (Table 4); (v) single nucleotide mutations, and single nucleotide polymorphism (Marks *et al.,* 2004). A putative ancestor to these three sequenced viruses was found in Thailand, named WSSV-TH-96-II and contains the largest WSSV genome reported to date (approx. 312 kbp; Marks *et al.,* 2005).

Table 4. Comparison of repeat units among three sequenced WSSV isolates. Table from Marks *et al.* (2004)

Genomic position (WSSV-TH coordinates)	Length of repeat units (bp)	Number of repeat units present (length of units)		
		WSSV-TH	WSSV-CN	WSSV-TW
11167-11454[a] (Only in WSSV-TW; TW 021)	9	-	-	32
44830-48294 (ORF30)	63	55.5	55.5	55.5
93118-93219 (ORF65)	3	34	33	33
97066-97400 (ORF67)	56/84	2.5 (84) and 2 (56)	2.5 (84) and 2 (56)	2.5 (84) and 2 (56)
107965-108675 (ORF75)	45/102	3 (102) and 9 (45)	4 (102) and 11 (45)	5 (102) and 16 (45)
119018-119311 (ORF84)	84	3.5[b]	3.5	2.5
142744-143067 (ORF94)	54	6	12	6
176987-177100 (ORF116)	42	2.75	2.75	2.75
180619-180834 (ORF119)	66/6	3 (66) and 4 (6)	3 (66) and 7 (6)	3 (66) and 7 (6)
187899-188312 (ORF125)	69	6	8	8
286717-286878 (ORF177)	54	3	3	3

[a] WSSV-TW coordinates

[b] One repeat unit has 3bp extra in WSSV-TH

Bold: non-hr unidirectional repeats which contain differences between the three isolates

Underlined: non-hr unidirectional repeats with a different number of repeat units between the three isolates

WSSV endemic and geographic distribution

WSSV was first emerged in East Asia in 1992. In Japan an outbreak occurred in *Penaeus japonicus* in 1993 and this was believed to have originated from China via imported shrimp (Inouye *et al.,* 1994; 1996; Nakano *et al.,* 1994). WSSV infection is posing a major threat in almost all shrimp producing countries in Asia and the Americas (Subasinghe *et al.,* 2001). The importation of live animals and uncooked, harvested shrimps are probably responsible for the spread of WSSV among WSSV reported countries (Nunan *et al.,* 1998; Durand *et al.,* 2000; Rodgers *et al.,* 2011).

WSSV has now been officially reported from 14 countries in the Asia–Pacific region, namely Taiwan, China, Japan, Korea, Thailand, Vietnam, Bangladesh, India, Malaysia, Indonesia, Pakistan, Singapore, Sri Lanka, Philippines. From Asia, the virus subsequently spread and reached 9 countries in the Americas (Colombia, Ecuador, Guatemala, Honduras, Mexico, Nicaragua, Panama, Peru and the United States) (NACA, 2002; OIE, 2003), Brazil (Muller *et al.,* 2010) and Middle East via live shrimp movements (Lightner, 2011). WSSV has been confirmed to be present in the European continent, namely in Greece, Italy, Spain and Turkey (Stentiford and Lightner, 2011). Very recently (2011) WSSV was also detected from Mozambique (Dr. Rohana Subasinge, personal communication).

Host range and transmission pathway of WSSV

WSSV can infect a broad range of farmed and wild crustaceans including marine and freshwater shrimp, crabs and crayfish (Escobedo-Bonilla *et al.,* 2008). This reported host ranges include natural hosts, those hosts found to be infected in the field, and hosts

found to be susceptible to infection in experimental studies. WSSV can spread and infect shrimps of any stage of grow-out, asymptomatically affecting all life cycle stages, from eggs to broodstock (OIE, 2011).

WSSV can be transmitted to healthy, susceptible shrimp, within or between shrimp farms by vertical and horizontal transmission pathways. Vertical transmission from spawner to offspring has been observed in experimental infections. PCR and *in situ* hybridization analyses have shown that WSSV can be transferred from carrier female brooders to larval, post-larval and juvenile shrimp (*P. monodon*) (Lo *et al.,* 1997; Satoh *et al.,* 1999).

The major transmission of WSSV is horizontal: (i) ingestion of WSSV infected shrimp, (ii) via the water route (Chou *et al.,* 1998; Zhan *et al.,* 1999) due to the fact that WSSV can persist in the pond at least 3 – 4 days (Nakano *et al.,* 1998). In experimental studies, WSSV transmission by ingestion of infected material is much more efficient than transmission by the water route (Soto and Lotz, 2001; Wu *et al.,* 2001)

The use of live food as *Artemia*, microalgae, rotifers and their resting eggs was implied in the transmission of the disease in the hatcheries (Sahul Hameed *et al.,* 2002; Liu *et al.,* 2007; Li *et al.,* 2004; Yan *et al.,* 2004; Zhang *et al.,* 2010). Furthermore, mud crabs (Kanchanaphum *et al.,* 1998) and seabirds (Vanpatten *et al.,* 2004), krill *Acetes* sp. (Supamattaya *et al.,* 1998), Ephydridae insect larvae (Lo *et al.,* 1996), polychaete worms (Vijayan *et al.,* 2005), and marine molluscs (OIE, 2009) have also been implied as a vector in the transmission of WSSV to shrimp ponds.

However, none of the above studies reached out beyond the anecdote. They are often individual cases and a qualitative description. No statistical methodology was used to quantify the transmission rates in these cases and to determine the relative impact of the various transmission routes on the development of disease. Even the transmission rate as such has not been determined.

Diagnostic methods for WSSV

Gross signs of WSD

WSD usually occurs after 1-2 months of stocking (Corsin *et al.,* 2002). Before the onset of the disease, infected shrimp are observed to be lethargic and display reduced feeding. Infected shrimp sometimes swim slowly near the pond surface and have some white spots of 2-3 mm in size, which are primarily observed on the carapace and/or the six abdominal segments of the shrimp cephalothorax (Figure 8A). In some cases, infected or moribund shrimp have a reddish coloration due to expansion of chromatophores (Figure 8B). WSSV can cause up to 100% mortality within a pond within 3-10 days after the onset of the above symptoms (Chou *et al.,* 1995).

To date, the diagnosis of WSSV infection involves, depended on the supposed health status of shrimp, different approaches used to screen apparently healthy populations or, on the other hand, to confirm a suspected outbreak. A number of techniques have been developed and applied in particular WSSV infection stages (Table 5). Samples of whole larvae, post-larvae or samples from the pleopods, gills, haemolymph, stomach or abdominal muscle of juveniles and adults are recommended for WSSV diagnosis. In the case of broodstock, pleopod, pieces of gill or ablated eyestock are the best organs for WSSV detection (OIE, 2011).

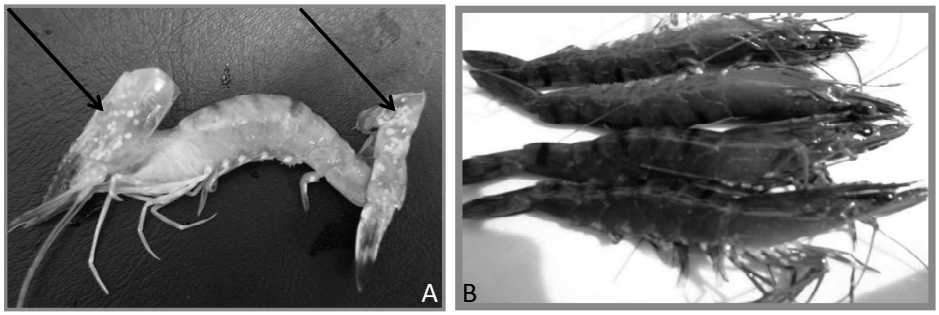

Figure 8. Gross signs of WSSV infected in black tiger shrimp A. Arrow show the position where white spots appeared in the epidermis of the carapace and the 6th abdominal segment; B. Moribund shrimps with reddish coloration.

Table 5. Recommended methods for WSSV diagnosis at different stages

Method	Target surveillance				Exploratory WSD diagnosis	Comfirmatory WSD diagnosis
	Larvae	Post-larvae	Juveniles	Adults		
Gross signs	-	-	+	+	+	-
Bioassay	-	-	-	-	+	++
Direct microscope	-	-	+	+	+	+
Histopathology	-	+	+	+	+++	+++
Transmission EM	-	-	-	-	-	+++
Antibody-based assays	-	-	+	+	+++	++
DNA probes - in situ	-	-	+	+	+++	+++
PCR	-	++	+++	+++	+++	+++
Sequencing	-	-	-	-	-	+++

PCR: polymerase chain reaction; EM: electron microscopy
+++ = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;
++= the method is a standard method with good diagnostic sensitivity and specificity;
+ = the method has application in some situations, but cost, accuracy, or other factors severely limits its application;
- = the method is presently not recommended for this purpose.
Sources: modified from OIE (2011).

Preventive methods for white spot disease

As no effective treatment methods for WSD treatment are available, only preventive methods have so far been developed to control the disease throughout different stages of the shrimp production cycle. In the hatchery stage, for example, washing eggs and nauplii with a combination of Iodiphor and running seawater would be an appropriate method for clearing WSSV in the offspring (OIE, 2011). In grow-out farms, screening of post-larvae for the presence of WSSV prior to stocking, avoidance of stocking in the cold season, and the application of strict biosecurity measures in water and culture systems were applied and recommended for WSD prevention (Withyachumnarnkul, 1999; MPEDA/NACA, 2003; Mohan *et al.,* 2008).

Disease outbreaks may be triggered by different stressors, e.g. rapid changes in salinity and water temperature (Fegan and Clifford, 2001; Tendencia *et al.,* 2010). However, water temperatures exceeding 32^{o}C could reduce WSD outbreaks, as shown in some laboratory experiments (Vidal *et al.,* 2001; Granja *et al.,* 2003; Rhahman *et al.,* 2006). A further experiment clearly showed that maintaining white leg shrimp constantly at 32 ±1^{o}C for 7 days resulted in the elimination of WSSV infection (Wongmaneeprateep *et al.,* 2010). This may be due to the inability of WSSV DNA polymerase to function properly at this temperature. Research activities on the role of the Janus kinase and signal transducers and activators of transcription (Jak/STAT) in the shrimp response to viral pathogens may also open new venues for WSD prevention (Liu *et al.,* 2007; Chen *et al.,* 2008). Additionally, probiotics and immunostimulants (beta-glucan, vitamin C, seaweed extracts (fucoidan)) promise the enhancement of WSD preventation by boosting shrimp immunity (Chang *et al.,* 2003; Chotigeat *et al.,* 2004).

Currently, there are no commercially available vaccines that protect shrimp against WSSV infection. However, different vaccine prototypes and vaccine inoculation methods have been tested in the laboratory, in an attempt to find the approach that confers the best protection (Johnson *et al.,* 2008). Experiments have shown the survival rate of infected shrimp increases if the shrimp are injected with inactivated WSSV virions or fed with bacterially expressed WSSV envelope protein VP28 (Witteveldt *et al.,* 2004) with food pellets, treated with orally administered, bacterially expressed VP28 dsRNA (Sarathi *et al.,* 2008) or transduced upon feeding with baculovirus expressing VP28 under the control of the WSSV ie1 promoter (Musthaq and Kwang, 2011). None of these approaches led to robust protection, but efforts are continuing to improve on the prototype vaccines.

Molecular epidemiology of white spot syndrome virus

Among the viruses detected in crustaceans, WSSV has been best studied in terms of its epidemiology (Flegel *et al.,* 2008). Of these aspects, characteristics of causative agent (Wongteerasupaya *et al.,* 1995; van Hulten *et al.,* 2001a; Yang *et al.,* 2001),

prevalence of WSSV infection (Lo *et al.,* 1996; Otta *et al.,* 1999; Nunan *et al.,* 2001; Thakur *et al.,* 2002), mode of transmissions, and host range (Lo *et al.,* 1997; Chou *et al.,* 1998; OIE, 2011) were extensively researched.

The term ´molecular epidemiology´ was first proposed in 1973 for influenza virus (Kibourne, 1973). Molecular epidemiology is the integrative research discipline that combines molecular biology techniques with traditional epidemiology approaches to better understand the aetiology and prevalence of a disease, and to inform prevention efforts (Snow, 2011).

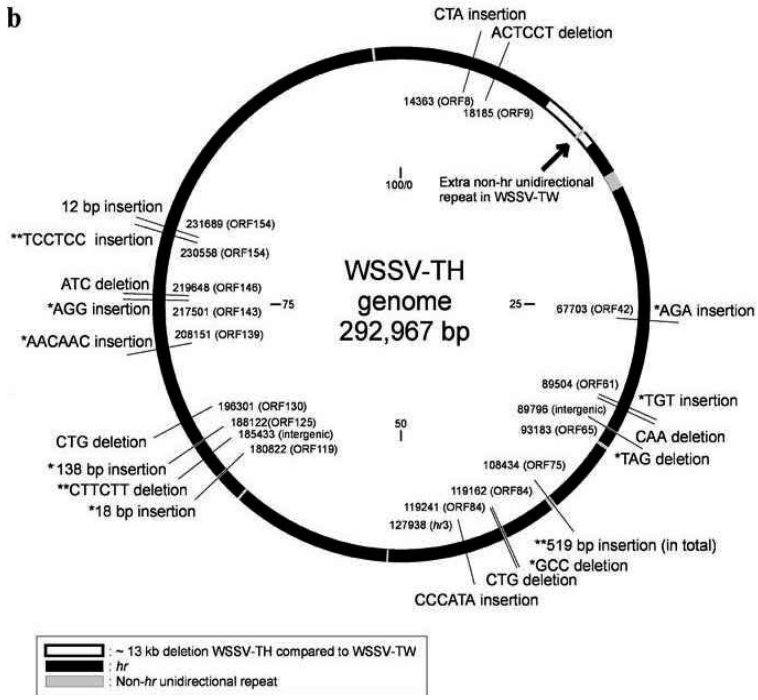

Figure 9. Schematic map shows 13kb deletion in WSSV-TH compared to WSSV-TW. Reproduced from Marks *et al.* (2004).

Although the WSSV genome appears relatively stable, some genomic variation which can be applied in molecular epidemiology studies has been found (Marks *et al.,* 2004). Among them, the variable genomic regions include variable number tandem repeat regions (VNTRs) located at the open reading frames- ORF75, ORF94, ORF125 (Table 4); the indel-I region of 5,3kb located between ORFs 14 and 15 of WSSV-TH96-II which was absent in the genome of WSSV-TH; and the indel-II region of 13kb located in ORF23 and ORF24 of WSSV-TW which absent in the genome of WSSV-TH (Figure 9) (Marks *et al.,* 2004). These specific regions have been used for different approaches of WSSV molecular epidemiological studies. Although regions with multiple repeats were identified early on, Wongteerasupaya *et al.* (2003) were the first to exploit this information for epidemiology studies.

Shekar *et al.* (2005) also suggested that thirteen microsatellite and three mini-satellite loci could be used as molecular marker tools for an understanding of the genetic and epidemiological relationship among WSSV isolates.

First, molecular genotyping methods were developed for WSSV strain discrimination. Restriction fragment length polymorphism (RFLP) analysis (Nadala *et al.,* 1998) distinguished a WSSV isolate from *P. japonicus* (China) and isolates from *P. monodon* (Indonesia) and *P. setiferus* (USA). Restriction analysis and southern blot hybridization analysis have also been used to show that a WSSV isolate identified in a crayfish at the US National Zoological Park (Washington) was distinct from isolates identified in shrimps from China, India, Thailand, South Carolina and Texas (Wang *et al.,* 2000). Single point mutations in the *ribonucleotide reductase* large subunit (*rr1*) gene distinguished a New Jersey crab (*Callinectes sapidus*) isolate from 17 other WSSV isolates from crabs, shrimp and crayfish from the USA and Asia (Chang *et al.,* 2001). Recently, PCR and sequence-based genotyping using variable number tandem repeats (VNTRs) has emerged as a faster genotyping method with a higher discriminatory power than the above-mentioned methods (Woongterasupaya *et al.,* 2003; Marks *et al.,* 2004; Dieu *et al.,* 2010; Muller *et al.,* 2010; Tan and Shi, 2011).

VNTRs are polymorphisms associated with tandem repeat elements DNA (Nakamura *et al.,* 1987). PCR-based methods are now used for VNTR typing, and employ primer sets specific for the regions flanking the tandem repeats. Amplicon sizes are then measured by gel electrophoresis, which is used to estimate the number of tandem repeats present at the respective locus (van Soolingen *et al.,* 2007). VNTRs have employed in WSSV epidemiology in the different main shrimp farming countries in the world, including Thailand (Wongteerasupaya *et al.,* 2003), Vietnam (Dieu *et al.,* 2004), India (Pradeep *et al.,* 2008b), Brazil (Muller *et al.,* 2010) and China (Tan and Shi, 2011).

Second, the variable genomic regions are not only useful for WSSV strain identification but also provide insights into WSSV pathogenesis. For example, the indel-II of 13 kb may be linked to differences in virulence of WSSV strains (Lan *et al.,* 2002; Zwart *et al.,* 2010a). A direct comparison in virulence between two WSSV isolates, different from each other of more than 13kb deletion at the indel-II, has been conducted by using competition assay during 5 serial passages in shrimp (Marks *et al.,* 2005). At the beginning of the experiment, shrimp were inoculated with a 1:1 mixture of virions of the two viruses. After the fifth passage, the virus with the larger genome could not be detected any longer. As suggested in the paper, it could be that the smaller virus has a replication advantage, or that the larger virus contains genes which make the virus less virulent.

The VNTR regions may also have a relationship to WSSV pathogenesis and virulence. For example, the ORF94 VNTR shows a high level of variation at small temporal or spatial scales in *P. monodon* isolates from different outbreaks of WSD

in Thailand (Wongteerasupaya *et al.,* 2003) and in India (Musthaq *et al.,* 2006; Pradeep *et al.,* 2008b), where the high frequency of 8 repeats genotype was found in outbreak farms. In the laboratory, preliminary studies suggest a difference in virulence between different strains of WSSV with different repeat unit numbers in the ORF94 VNTR. An example is WSSV isolates with high repeat numbers in ORF94 (ORF94-12 and 18) cause less mortality in shrimp than isolates with low repeat unit numbers (ORF94-8 and ORF94-9) (Waikhom *et al.,* 2006). However, in these cases no rigorous statistics has been employed to confirm these correlations.

The occurrence of host adaptation has also been suggested using VNTRs. Waikhom *et al.* (2006) examined the stability of the VNTR in ORF94 of the WSSV genome after passaging through different hosts. The authors concluded that the change in this VNTR resulted from host selection rather than geographical isolation. After a single passage in a laboratory experiment, the WSSV isolate with ORF94-12 genotype yielded ORF94-8 genotype in crabs (*Portunus pelagicus*) and ORF94-18 genotype in shrimps (*Penaeus monodon*). In addition, two other isolates with ORF94-9 genotype still retained ORF94-9 in shrimp (*Penaeus monodon*) and crabs (*Portunus sanguinolentus*) but yielded ORF94-8 when passaging through other crab species (*Portunus pelagicus*) and ORF94-12 in prawn (*Macrobrachium rosenbergii*). In addition, the pathogenicity of WSSV was reported to be changed after passaging through different animals (Waikhom *et al.,* 2006).

WSSV indels have been employed to confirm and reconstruct patterns of WSSV spread from its site of origin (most likely China) (Dieu *et al.,* 2004; Pradeep *et al.,* 2008a; Dieu *et al.,* 2010) and virus spread across continents (Zwart *et al.,* 2010a). Based on the 13kb deletion in indel-II, Dieu *et al.* (2004) mapped the migration of WSSV from Taiwan or China through Vietnam to Thailand. Pradeep *et al.* (2008a) mapped the movement of WSSV to India based on the same region. Dieu *et al.* (2010) subsequently showed that indel-II can also be used as a marker at smaller spatiotemporal scales, in this case the movement of this virus to other shrimp farming regions in Vietnam. Moreover, these indel markers were not only useful in studying the spread of WSSV at regional and national scales (Dieu *et al.,* 2004; Pradeep *et al.,* 2008a), but also in explaining the rapid worldwide spread of WSSV and its evolution over time and space (Zwart *et al.,* 2010a). The indels were evaluated as good molecular markers for epidemiological studies at intermediate spatiotemporal scales (Dieu *et al.,* 2010), although polymorphisms at these indel sites do not appear to be neutral (Marks *et al.,* 2005; Zwart *et al.,* 2010a).

II. THESIS RATIONALE AND OUTLINE

The overall objective of the present study was to find out whether any of these molecular markers (indels, VNTRs) could be used to correlate WSSV genotype and population structure with WSSV epidemiology, outbreak status, regional occurrence and farming system. Such correlations, however, would require the

application of statistical methods to corroborate conclusions. It would also require a large set of samples, much larger than had been used in earlier studies.

The research was conducted to achieve a number of specific objectives (i) determining the nature, origin and variation of WSSV within Ca Mau and Bac Lieu provinces from ponds of high and low-density shrimp culture using different molecular markers; (ii) analyzing the correlation of WSSV population structure to virulent disease outbreaks and shrimp farming systems; (iii) applying informative molecular markers to the study of WSSV transmission routes and transmission rates in and between ponds.

To address the specific objectives, the following research questions were put forward and covered in subsequent chapters.

(i) To what extent can molecular markers be used to estimate the diversity of WSSV genotypes within and between shrimp ponds?

(ii) Are molecular markers suited for studies of WSSV epidemiology in relation to WSD outbreaks and shrimp farming systems?

(iii) Which molecular markers can be used best for WSSV transmission studies within and between shrimp ponds?

(iv) Which transmission routes are the most important in each type of shrimp farming system?

In **chapter 2**, the VNTR of ORF94 was first studied as a marker 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.

In **chapter 3**, the VNTRs of ORF75, ORF94 and ORF125 were all employed to investigate the genetic variability among natural populations of WSSV in the three main shrimp farming systems in Bac Lieu and Ca Mau provinces, semi-intensive, improved-extensive and rice-shrimp farming, and to see if there is a correlation between VNTR structure on the one hand and farming system and disease status on the other.

In **chapter 4**, the utility of the two variable regions located between ORF14/15 and ORF23/24 in the WSSV genome in epidemiological studies at farm scale has been explored. In this chapter, we analyzed the genomic deletions in WSSV isolated from shrimp produced in different farming systems and with different disease status from two shrimp-producing areas in the Mekong delta, Vietnam. The research provides more insight into the potential application of WSSV indels for epidemiological studies at a small spatiotemporal scale, and test whether these indels have potential utility as molecular indicators of disease status of ponds and farming systems.

In **chapter 5**, we tested whether VNTR loci are suitable for detecting mixed-genotype WSSV infections by a PCR-based method. We tested whether mixed-genotype WSSV infections occur in shrimp ponds using WSSV variable loci as molecular makers, and estimated the prevalence of mixed-genotype infections in individual shrimp.

In **chapter 6**, an analysis on the combined results of all three VNTR marker loci made it possible to follow transmission pathways of WSSV in the respective shrimp farming systems. The chapter also describes the application of molecular markers and mathematical models to estimate and explain WSSV transmission routes in two different farm settings: an improved-extensive shrimp farming system in Ca Mau Province and a semi-intensive shrimp farming system in Bac Lieu Province.

Finally, in **chapter 7** a synopsis and a reflection on the results obtained are given. Moreover, a perspective is given on the future use of molecular markers to study WSSV epidemiology, transmission and disease in shrimp farming systems.

2
- CHAPTER -

Genotypic variations in tandem repeat DNA segments between *ribonucleotide reductase* subunit genes of white spot syndrome virus (WSSV) isolates from Vietnam

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ABSTRACT

White spot syndrome is a viral disease that affects most commercially cultivated marine shrimp species. The disease first emerged in East Asia in 1992 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.

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.,* 2001a; 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.,* 2001a; 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 two 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 (GeneReach Biotechnology Corp., Taipei, 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^{o}C until required. WSSV DNA was detected by using the IQ-WSSV-2000 PCR kit (GeneReach Biotechnology Corp., Taipei, 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^{o}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 0.5µg/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.

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.* (2001a) 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).

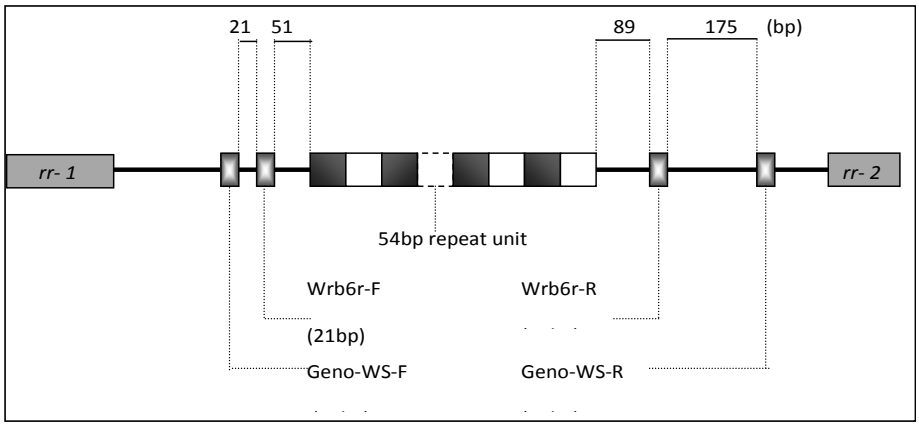

Figure 1. Schematic representation of the variable tandem repeat region of the WSSV genome between the *rr1* and *rr2* genes. Arrows show the location of PCR primer binding sites and the black boxes represent the variable tandem repeat units.

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.

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

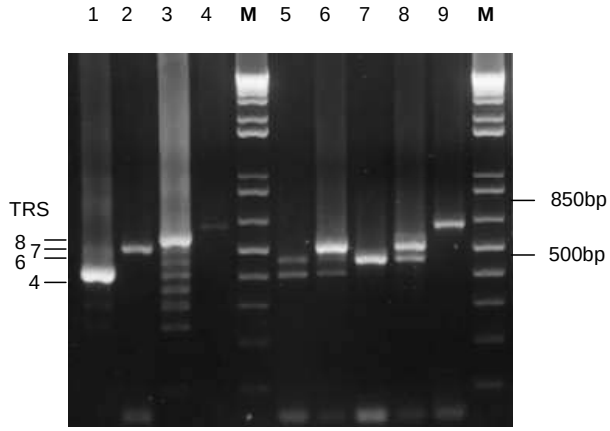

Figure 2. PCR assay of WSSV DNA from different pools of postlarve and different broodstock (*Penaeus monodon*). Lane M: 1kb plus DNA ladder; lanes 1-4: postlarval samples (WS160, WS141, WS63C, WS339, respectively); lanes 5-9: broodstock samples (WS45, WS38, WS40, WS44, WS48, respectively).

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 PCR genotyping. The TRS genotype in each sample is shown in Table 3 and examples of the genotype analysis are shown in Figure 3.

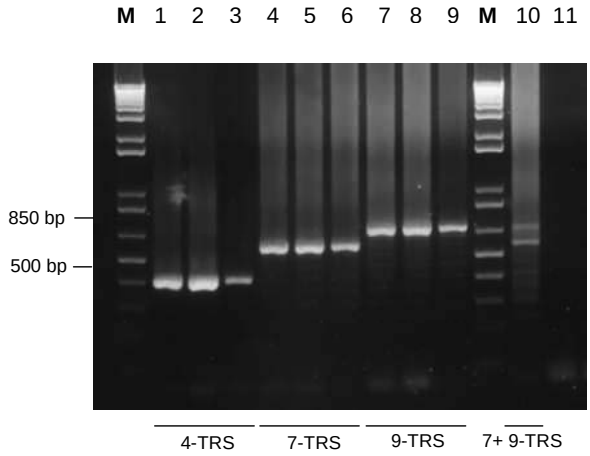

Figure 3. PCR amplification of different tandem repeats DNA fragments from juvenile shrimp in different grow-out ponds in Vietnam. Lanes M, 1kb plus DNA ladder; lanes 1-3, pond TB; lane 4-6, pond N7; lane 7-9, pond N; lane 10, pond L2; and lane 11, negative control.

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 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
	02.01.02	L1 (30)	D	5/31	561, 669	7, 9
	03.02.02	L1 (60)	D	29/29	561	7
Bac Lieu	02.01.02	L2	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
				1/19	615	8
				1/19	669	9
Soc Trang	14.04.02	V8 (90)	H	1/12	561	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 PCR genotyping as described above for the shrimp samples. The TRS genotype analyses are shown in Figure 4 and the results are summarised in Table 4.

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

[1]ND = WSSV detected using the IQ-WSSV-2000 detection kit but no product detected in the PCR genotyping.

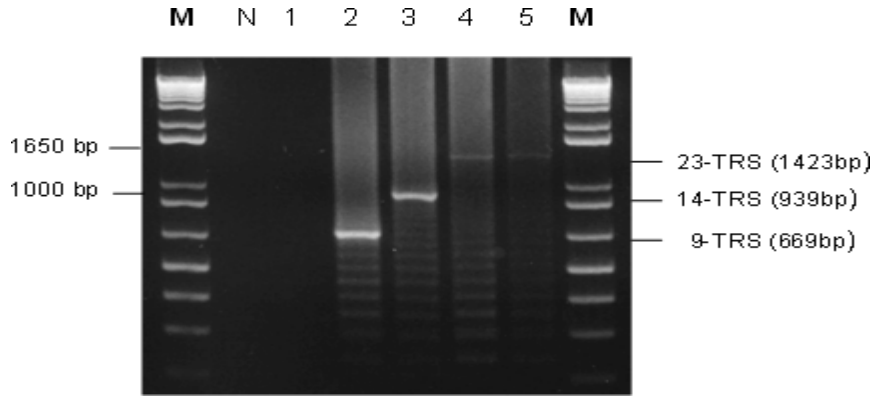

Figure 4. PCR amplification of different tandem repeats of WSSV DNA fragments from crustacean carriers. Lane M, 1kb plus DNA ladder; lane N, negative control; lane1, L60SC; lane2, V8LC; lane3, HU; lane4, TSC; lane5, TU. (SC = small crab; LC = large crab; U = wild shrimp; see Table 4).

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 PCR genotyping 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.

3

- CHAPTER -

Low numbers of repeat units in VNTR regions of White spot syndrome virus are correlated with disease outbreaks

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ABSTRACT

White spot syndrome virus (WSSV) is the most important pathogen in shrimp farming systems worldwide including the Mekong Delta, Vietnam. The genome of WSSV is characterized by the presence of two major 'indel regions' found at ORF14/15 and ORF23/24 (WSSV-Thailand) and three regions with variable numbers tandem repeats (VNTR) located in ORF75, ORF94 and ORF125. In the current study we investigated whether or not the number of repeat units in the VNTRs correlates with virus outbreak status and/or shrimp farming practice. We analyzed 662 WSSV samples from individual WSSV-infected *Penaeus monodon* shrimp from 104 ponds collected from two important shrimp farming regions of the Mekong Delta: Ca Mau and Bac Lieu. Using this large data set and statistical analysis we found that for ORF94 and ORF125 the mean number of repeat units (RUs) in VNTRs was significantly lower in disease outbreak ponds than in non-outbreak ponds. Although a higher mean RU number was observed in the improved-extensive system than in the rice-shrimp or semi-intensive systems, these differences were not significant. VNTR sequences are thus not only useful markers for studying WSSV genotypes and populations, but specific VNTR variants also correlate with disease outbreaks in shrimp farming systems.

INTRODUCTION

White spot syndrome virus (WSSV) is an important pathogen in farmed crustaceans, especially in penaeid shrimp (Flegel, 1997). Since the first recognized outbreaks of WSSV in Southern China and Taiwan in 1992, several studies have described the worldwide WSSV spread and the underlying reasons for the epizootic nature of WSSV (Dieu *et al.,* 2004; Flegel and Alday-Sanz, 1998; Lightner *et al.,* 1997; Nakano *et al.,* 1994; Zwart *et al.,* 2010a). WSSV has a large double-stranded DNA genome of approximately 300 kbp and has been accommodated in the Nimaviridae family (Vlak *et al.,* 2005). The full-length genome sequence has been determined for three WSSV isolates: the 292.9 kbp isolate (AF369029) from Thailand (WSSV-TH), the 307.2 kbp isolate (AF440570) from Taiwan (WSSV-TW) and the 305.1 kbp isolate (AF332093) from China (WSSV-CN) (van Hulten *et al.,* 2001a; Lo and Kou, 2001; Yang *et al.,* 2001).

The differences among the three genomes encompass only approximately 0.7% of the shared nucleotides, including five well-known molecular genetic markers regions. There are two large insertion / deletions sequence regions, indel-I and indel-II and three regions each with a variable number of tandem repeats (VNTR) (Marks *et al.,* 2004). The indel-I and indel-II variable regions (ORF14/15 and ORF23/24 in WSSV-TH) have been shown to be useful molecular markers for epidemiological studies on regional and global scales (Dieu *et al.,* 2004; Marks *et al.,* 2004; Pradeep *et al.,* 2008a; Dieu *et al.,* 2010). The indel sizes at these loci are thought to be positively correlated to WSSV virulence (Marks *et al.,* 2005; Zwart *et al.,* 2010a).

The three WSSV VNTR regions are the 54 bp repeat unit (RU) region in ORF94, the 69 bp RU region in ORF125, and the compound 45 bp and 102 bp RUs region in ORF75 (van Hulten *et al.,* 2001a; Wongteerasupaya *et al.,* 2003; Marks *et al.,* 2004). These regions appear to be highly variable, and a range of RU numbers have been found in WSSV isolates originating from a wide range of hosts, including post-larvae and juveniles of cultured shrimp and wild crustaceans (Wongteerasupaya *et al.,* 2003; Hoa *et al.,* 2005; Pradeep *et al.,* 2008b; John *et al.,* 2010). ORF94 appears to harbor the greatest diversity in RU numbers, whereas the compound repeats (45bp and 102bp) in ORF75 show the least diversity (Pradeep *et al.,* 2008b; Muller *et al.,* 2010; Hoa *et al.,* 2011a). These VNTRs appeared to be useful markers for identification of WSSV isolates in the field, but only at small spatial scales due to their high variability (Wongteerasupaya *et al.,* 2003; Hoa *et al.,* 2005; Pradeep *et al.,* 2008b; Dieu *et al.,* 2010).

Multiple experimental studies show differences in virulence between WSSV isolates, where infectivity of virions, median lethal time, or both have been used as measures of virulence (Marks *et al.,* 2005; Zwart *et al.,* 2010a). WSSV populations within ponds and within shrimp are often composed of multiple genotypes (Hoa *et al.,* 2011a) and the genetic structure of WSSV populations entering farms may

determine the outcome of an infection and development of disease outbreaks. For example, field studies have shown a positive correlation between mixed-genotype infections in single shrimp and the absence of disease outbreaks (Hoa *et al.,* 2011a), whereas shrimp from outbreaks usually have a single WSSV genotype. This suggests that there may be a relationship between virus genotypes and virulence. It is therefore of prime importance to understand the nature of differences in WSSV virulence, and to learn whether and how virus genotypes interact with the host environment to control disease outbreaks. Moreover, genetic markers of WSSV virulence would be valuable from an epidemiological and applied perspective, to enable tracking, tracing and perhaps ultimately farm-level screening for highly virulent genotypes. Ideally the population structure of variable genomic loci would correlate with virulence, fitness traits, outbreak status or shrimp culture practices. Genomic information could then be used for disease management and perhaps even shrimp health certification (Marks *et al.,* 2004).

Variability in VNTR RU numbers have been found to have wide application in (i) studying genotypic variation in populations of bacteria (van Belkum *et al.,* 1997; Kim *et al.,* 2001), (ii) in studying the epidemiology (Falk *et al.,* 1995) and virulence (Perdue *et al.,* 1997) of viruses, and (iii) in identification of pathogenic bacteria (Klevytska *et al.,* 2001; Peak *et al.,* 1996) and their virulence factors (Saunders *et al.,* 2000). Various studies have suggested a link between WSSV VNTRs and virulence. ORF94 has been suggested to be a useful marker for virulent WSSV variants, with low RU numbers correlating to virulence (Hoa *et al.,* 2005; Waikhom *et al.,* 2006; Pradeep *et al.,* 2008b). The current study is more focused on field studies in different shrimp farming systems and disease outcomes of shrimp ponds. We characterized WSSV isolates using VNTRs as genetic markers and used statistical analyses to determine whether a correlation between WSSV genotype(s) and disease outbreak or shrimp farming systems exists. We found that also in the field the presence of low RU numbers in VNTRs is significantly correlated with disease outbreaks, and thus possibly with virulence of the virus, but that there is no statistically supported correlation between RU numbers and shrimp farming system.

MATERIALS AND METHODS

Study areas and sample collection

In Vietnam, the majority of shrimp farms and production areas are located in a few provinces in the Mekong Delta, including the provinces Bac Lieu and Ca Mau. WSSV infected shrimp samples were collected from 104 grow-out ponds in these two provinces in 2006, 2008 and 2009. *Penaeus monodon* (Fabricius) juveniles and/or wild crabs (not identified species) were collected from 52 improved-extensive shrimp ponds, 44 semi-intensive shrimp ponds and 8 rice-shrimp ponds. Shrimp samples with clinical signs of WSD were collected either in ponds at outbreak time or in ponds during stocking periods. Individual shrimp or crab samples were

collected and transported immediately to the College of Aquaculture and Fisheries, Can Tho University, Vietnam. The samples were either preserved in absolute ethanol at room temperature or in a liquid nitrogen container before being transferred to -80 °C for storage. Additionally, information on farm management and diseases status was also recorded at the time of sampling.

WSSV isolates and PCR detection

A total of 662 WSSV-infected shrimp (*Penaeus monodon*) were obtained from different shrimp farming systems and different locations (Ca Mau and Bac Lieu): 390 samples from improved-extensive systems, 233 from semi-extensive systems and 39 samples from rice-shrimp systems. DNA was extracted from gill tissue and WSSV DNA was detected with a nested PCR using the IQ2000™ WSSV kit, according to the manufacturer's instructions (GeneReach Biotechnology Corp., Taipei, Taiwan). The IQ2000™ primer sets were designed in such a way as to yield WSSV-specific amplicons of 296 bp and/or 550 bp, respectively. Amplification of an 848-bp template within a crustacean housekeeping gene (supplied in the kit) allowed for confirmation of correct DNA isolation when no WSSV-specific sequences were detected.

WSSV genotyping

Genotyping of WSSV-positive samples was done using a PCR-based method. In detail, PCR with specific primers was used to amplify the VNTR loci as described elsewhere for ORF75 (Dieu *et al.,* 2004), ORF94 (Wongteerasupaya *et al.,* 2003) and ORF125 (Hoa *et al.,* 2011a). All PCR reactions were performed in an Applied Biosystems (Foster City, USA) thermal cycler using GoTaq Flexi DNA Polymerase (Promega; Madison, USA), and PCR products were analyzed by electrophoresis in 1% agarose gels. The size of the PCR product was estimated based on 100bp plus DNA markers (Fermentas; Burlington, Canada). We report our genotyping results as the length of the PCR amplicon(s) for the ORF75 repeat region (because here we have two compound RU), and ORF94-X or ORF125-Y, where X and Y are the respective number of RUs detected in ORF94 and ORF125.

Cloning and sequencing

PCR products were ligated into the pGEM-T easy vector (Promega) and transformed into competent *Escherichia coli* DH5α by electroporation. Insert size of white colonies was confirmed by colony PCR and plasmid length analysis. Plasmid DNA was purified from clones with the GeneJET™ plasmid miniprep kit (Fermentas, Burlington city, Ontario, Canada), and sequenced using universal T7 and/or Sp6 primers. Sequence chromatograms were analyzed using Chromas software (version 1.45). The sequences were then aligned using BLAST and multiple sequence alignment (Corpet, 1988). Referenced WSSV sequences were obtained from the NCBI GenBank database (AF369029.2, AF440570.1 and AF 332093.1) and other available VNTRs data in the literature resource

(Wongteerasupaya *et al.,* 2003; Dieu *et al.,* 2004; Hoa *et al.,* 2005; Musthaq *et al.,* 2006; Waikhom *et al.,* 2006; Pradeep *et al.,* 2008b; Muller *et al.,* 2010; John *et al.,* 2010).

Statistical analysis

VNTR genotyping data (number of RUs) were coded and the median genotype (i.e., the genotype with the median number of RUs) was calculated taking ponds as units. To test whether the structure of VNTRs was correlated with farming system, sampling provinces, sampling year and disease status, the median genotype was analyzed using generalized linear models (GLM) with a Gaussian distribution for the dependent variable and an identity link function. For all analyses a p value <0.05 was considered statistically significant. Data analysis was performed with STATA statistical software version 10 (Stata Corp., College Station, Texas, USA).

RESULTS

Distribution of WSSV VNTR variation in Bac Lieu and Ca Mau shrimp farming

We carried out analysis of a large set of WSSV isolates, representing a sampling regimen with a high spatial resolution from two geographic regions. We used the variable VNTR regions WSSV as molecular markers (Fig. 1) in order to test whether population structure correlated with disease status and farming system.

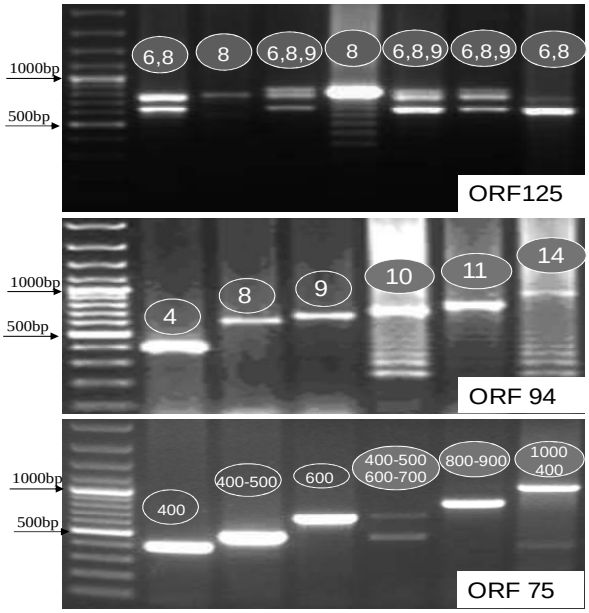

Figure 1. Example agarose gel showing PCR amplification products of ORF125 with multiples WSSV genotypes originating from virus of a single shrimp, ORF94 from 4 to 14 RUs and ORF75 repeats from 400bp to over 1000bp amplicons.

Similar to previous results (Pradeep *et al.,* 2008b; Muller *et al.,* 2010; Hoa *et al.,* 2011a), the range of RU numbers or amplicon size was greatest in ORF94 and least in ORF75 (Table 1). The results from the analyses of WSSV samples showed that in ORF94, 18 different RU configurations were found, ranging from ORF94-3 (i.e., ORF 94 with 3 repeats) to ORF94-20, with ORF94-7 being most commonly observed (Fig. 2). In ORF125, 14 genotypes were observed ranging from ORF125-2 to ORF125-17, except ORF125-11. For ORF125 the 6 and 7 RU variants of were observed most frequently. The compound repeat regions of ORF75 displayed 8 types of amplicons (ranging from 400bp to approximately 1000bp). In some cases two or more PCR products were observed for a DNA sample derived from a single infected shrimp (25/662 cases for ORF75; 71/662 cases for ORF94; 116/662 cases for ORF125) (Fig. 1). Multiple products indicate mixed-virus infection of a single host and mixed genotypes, as previously shown (Hoa *et al.,* 2011a).

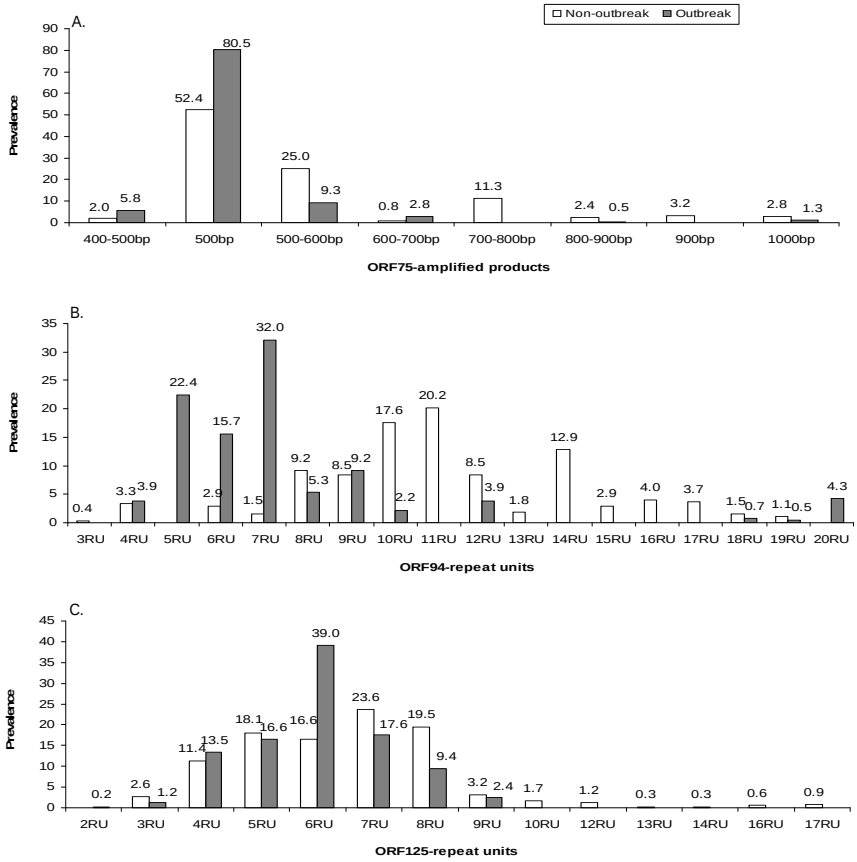

Figure 2. Distribution of WSSV genotypes among WSSV populations isolated from outbreak and non-outbreak shrimp ponds based on markers of ORF75, ORF94 and ORF125

Table 1. Range of WSSV genotypes based on RUs present in the region encoding ORF75, ORF94 and ORF125.

	Rice-shrimp	% n = 39	Semi-intensive	% n = 233	Improved - extensive	% n = 390
ORF75	_		400-500	9.8	400-500	1.3
(bp)	500	12.8	500	82.6	500	59.9
	500-600	87.2	500-600	7.2	500-600	20.6
	_		600-700	0.4	600-700	4.5
	_		_		700-800	6.7
	_		_		800-900	1.6
	_		_		900	2.1
	_		_		1000	3.2
ORF94	_		_		3	0.2
(RUs)	4	12.8	4	4.4	4	2.1
	_		5	1.6	5	26.5
	6	12.8	6	18.1	6	5.3
	7	38.5	7	46.2	7	1.7
	8	23.1	8	4	8	6.7
	_	_	9	9.2	9	8.8
	_	_	10	2.8	10	11.9
	_	_	_		11	13.1
	12	12.8	12	4.4	12	5.5
	_	_	_		13	1.2
	_	_	_		14	8.4
	_	_	_		15	1.9
			_		16	2.6
			_		17	2.4
			18	1.2	18	1.0
			19	0.8	19	0.7
			20	7.2		
ORF125	_		2	0.4	_	
(RUs)	_		3	1.3	3	2.3
	_		4	11.2	4	14.2
	_		5	18.9	5	17.9
	6	100	6	24	6	25.7
	_		7	23.6	7	20.3
	_		8	16.3	8	14
	_		9	4.3	9	2.3
	_		_		10	1.2
	_		_		12	0.8
	_		_		13	0.2
	_		_		14	0.2
	_		_		16	0.4
	_		_		17	0.6

_: Genotype not detected in the farming system but identified from other farming system in the study

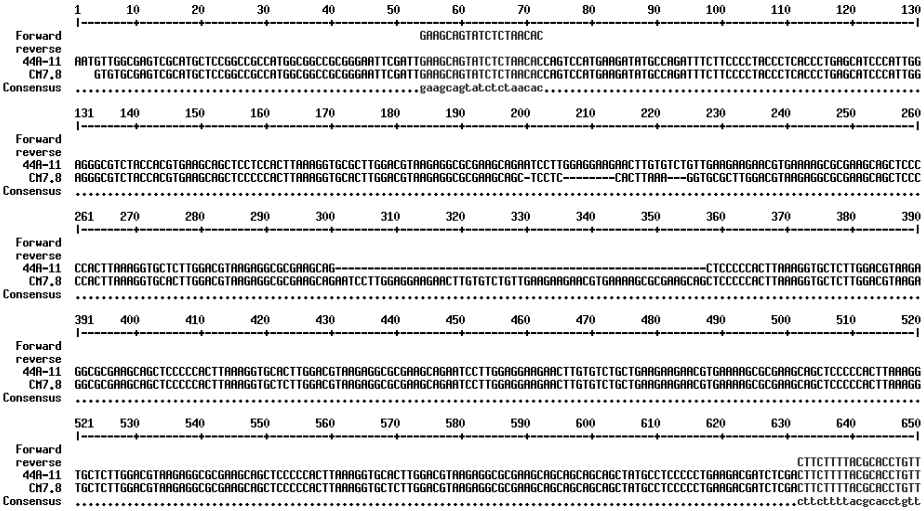

Figure 3. Multiple sequence alignment of compound repeat sequence of two WSSV isolates (CM7.8 and 44A-11) from Ca Mau province with ORF75-forward and reverse primers.

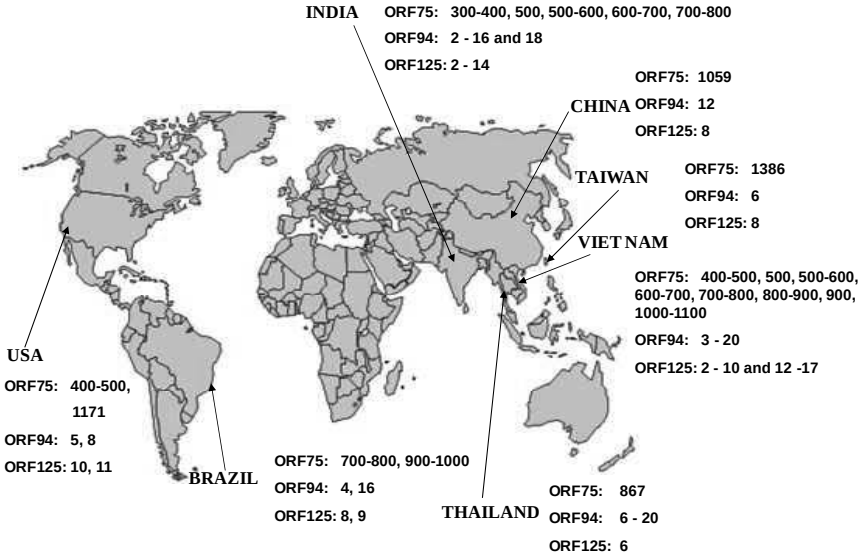

Figure 4. World map of different WSSV identified from Vietnam and other countries based on VNTRs markers (Wongteerasupaya *et al.,* 2003; Hoa *et al.,* 2005; Musthaq *et al.,* 2006; Waikhom *et al.,* 2006; Pradeep *et al.,* 2008b; Muller *et al.,* 2010 and referenced WSSV in GenBank). The number of WSSV isolates characterized per country and per locus varies, and variation in sample size probably affects the number of genotypes found. All values for ORF75 are the amplicon length in bp. All values for ORF94 and ORF125 are number of RUs.

For ORF75, the 500 bp amplicon occurred with a high frequency (69.8%, Table 1), and we therefore sequenced two isolates. We found 4 indels and 20 single nucleotide polymorphisms (SNPs) in the two sequences (Fig. 3). ORF75 therefore appears to harbor much variation at the sequence level, although this variation is invisible with PCR genotyping. DNA sequence analysis confirmed the number of repeat units derived from PCR genotyping ORF94 and gel analysis (data not shown). For our data and other available Vietnamese sequences on ORF94 (i.e., Dieu *et al.,* 2004), the SNP (T/G) in the first repeat was always a G, whereas for all other samples from other countries, with the exception of India, this SNP is a T (Table 2). For the three VNTRs, all RU numbers reported for isolates from other countries were present in the two main shrimp farming areas under investigation in *Vietnam*(Supplementary online material, Fig. 4).

VNTR markers in relation to disease status

We observed differences between outbreak and non-outbreak ponds: there was a higher prevalence of variants with a low number of RUs in outbreak ponds than in non-outbreak ponds (Fig. 2). This difference was most pronounced for the median RU number of ORF94, for which outbreak ponds had, on average, 4.00 RUs less than non-outbreak ponds. The 95% confidence interval for the difference was 1.94 - 6.04, and the difference was significant ($p < 0.001$). The frequency of ORF94-5, ORF94-6 and ORF94-7 from outbreak ponds was remarkably different from that from non-outbreak ponds (Fig. 2B). For example, the ORF94-7 genotype was observed in 32% of the cases in outbreak ponds as compared to 1.5% in non-outbreak ponds. On the other hand, in non-outbreak ponds ORF94-10RU and ORF-11RU were present at the highest frequencies, but these variants were not found in outbreak ponds. For ORF125, outbreak ponds had, on average, 0.82 RUs less than non-outbreak ponds. The 95% confidence interval for the difference was 0.10-1.55 and the difference was also significant ($p = 0.026$). There is no one particular RU number, for which there are very big differences between outbreak and non-outbreak farms (Fig. 2C). For ORF75, the median number of RUs between outbreak and non-outbreaks ponds was not statistically significant ($p = 0.86$), which is not surprising given the apparent similarity of the RU distributions (Fig. 2A). Moreover, the genotypes in WSSV samples from non-outbreak ponds had a greater range of RU numbers than those from outbreak ponds: 16 genotypes for non-outbreak ponds as opposed to 11 genotypes in outbreak ponds for ORF94, 13 genotypes as opposed to 8 genotypes for ORF125, and 8 genotypes as opposed to 6 genotypes for ORF75.

We also tested whether sample collection site (i.e., province) and time of collection (i.e., year) had an effect on RU numbers. For all three VNTRs there were significant differences between the two provinces ($p < 0.001$ for ORF75, $p = 0.03$ for ORF94 and $p = 0.04$ for ORF125) and among sampling years ($p < 0.001$ for all three VNTRs

regions). These differences between years and provinces were included in the model when estimating the difference between outbreak and non-outbreak ponds.

Table 2. Comparison of single nucleotide polymorphism at the nucleotide position 48 of the 54bp RUs of ORF94 among geographical WSSV isolates.

Region	ORF94 RUs number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
China	GenBank																
AF332093	T	T	G	G	G	G	G	G	T	T	T	T					
Taiwan	Genbank																
AF440570	T	T	T	G	T	T											
Thailand	GenBank																
AF369029	T	G	G	G	T	T											
	Wongteerasupaya *et al.,* 2003																
Surat #1	T	T	T	T	G	T	T	G	T								
Chum #2	T	T	T	G	T	G	T										
Chum #4	T	T	T	G	G	G											
Surat #2	T	T	G	T	T	G	G	T									
Chum #3	T	T	G	T	T	G	G	T									
Brazil	Muller *et al.,* 2010																
S.Cat. 2005	T	G	T	T	G	T	G	G	G	G	T	G	G	G	G	G	
S.Cat. 2007	T	G	T	T	T	T	T	T	T	T	T	T	G	G	T	G	
S.Cat. 2008	T	G	T	G	T	T	T	T	T	T	T	T	T	T	T	T	
Bahia, 2008	T	G	G	G													
India	Musthaq *et al.,* 2006																
ORF94-13	G	T	T	T	G	G	G	T	T	T	T	T	T				
ORF94-11	G	G	G	G	G	G	T	T	T	T	T						
ORF94- 9	G	T	G	G	G	G	G	T	T								
ORF94-7	G	T	T	T	G	G	T										
Vietnam	This study																
Ca Mau	T	T	T	T													
Ca Mau	G	T	T	T	G	T	G										
Ca Mau	G	T	T	T	G	T	G										
Bac Lieu	G	T	G	G	G	T	T										
Bac Lieu	G	T	G	G	G	T	T										
	Dieu *et al.,* 2004																
K	G	G	G	T	T	T	G	G	T	T							
T	G	T	T	T	T	G	T	T	T	G	T	G	G	G	G	T	T
L	G	G	G	G	G	G	G	G	G	G							
X	T	T	T	T	G	T	T										
S	T	T	T	T	G	T	T										
A	G	G	G	T	G	G	T	T	T	T							

VNTR markers in relation to shrimp farming systems

The distribution of WSSV genotypes was also analyzed based on shrimp farming system: semi-intensive, rice-shrimp and improved-extensive systems. We found no correlation between farming system and VNTR RU distribution ($p = 0.34$ for ORF75; $p = 0.54$ for ORF94 and $p = 0.98$ for ORF125). Although there is no significant influence of farming practice on VNTR variants found in individual ponds, we also considered the genotypic variation found in the different farming systems (Table 1). The highest genotype variation was found in improved-extensive systems, where almost the full spectrum of variation was found for all three VNTRs. For ORF94, there appears to be a bimodal distribution of RU numbers for the semi-intensive system (Table 1), somewhat similar to the distribution in non-outbreak ponds (Fig. 2B). The semi-intensive system had less variation in terms of number of RUs in ORF94. The variation in number of RUs in ORF75 was much less than for ORF125. In the rice-shrimp system WSSV had the smallest range for all three loci (ORF75, ORF94 and ORF125). The smallest number of samples (n=39) was, however, collected from the latter system.

DISCUSSION

Molecular markers associated with WSSV virulence may be of value for monitoring shrimp health, as WSSV is often ubiquitous irrespective of the disease status of a pond (e.g. Hoa *et al.,* 2011a), and for understanding the genetic basis for virulence. A number of studies have suggested that low RU numbers for WSSV ORF94 are associated with virulent disease outbreaks (Hoa *et al.,* 2005; Waikhom *et al.,* 2006; Pradeep *et al.,* 2008b), but these studies did not employ statistical analyses and should therefore be considered anecdotal (case study). Here, we investigated whether there was a statistically supported correlation between one or more WSSV VNTRs and disease status, farming system, year of sample collection and geographic location, using a large number (N=662) of WSSV isolates collected on Vietnamese shrimp farms.

After fitting a statistical model to the data, we found that ORF94 was significantly correlated to disease outbreaks, with high RU numbers correlating with non-outbreak pond status and low RU numbers with outbreaks. Our results therefore confirm previous observations, but now using a much larger data set and rigorous statistical testing. Moreover, we also found that ORF125 is also correlated with disease status, although the correlation is weaker than for ORF94. Combining the analysis for ORF94 and ORF125 made the correlations even stronger. A striking phenomenon is that the ORF94 RU distribution appears to be bimodal, with short variants (< 8 RUs) predominating in outbreak ponds, and long variants (> 9 RUs) predominating in non-outbreak ponds (Fig. 2B).

These results are in good agreement with previous results, in the laboratory and field. In an experimental study, Waikhom *et al.* (2006) observed that WSSV isolates

with high repeat numbers in ORF94 (ORF94-12 and 18) cause less mortality in shrimp than isolates with low repeat numbers (ORF94-8 and ORF94-9). Pradeep *et al.* (2008b) typically found that genotypes with less than 8 RUs in outbreak ponds, whereas genotypes with more 9 RUs were found in non-outbreak ponds. Hoa *et al.* (2005) found that a 7 RU genotype of ORF94 dominated in outbreak ponds, but without supporting statistical analyses. In general, it therefore appears that WSSV genotypes with less than 9 RUs in ORF94 are dominant in outbreak ponds. However, there is evidence of the predominance of genotypes 8RUs and 18 RUs in a progressive series of disease outbreaks from a study in India (Walker *et al.,* 2011a). Genotype analysis for tracking white spot disease in small-holder farming could be influenced by local conditions and could vary from crop to crop (Walker *et al.,* 2011a). Therefore, the combination of the two significant VNTR regions, as used in our studies, might be a more useful approach to correlate with virulence/disease outbreaks.

Although we have established a statistical correlation between ORF94 RUs and disease outbreaks, the causal relationship between these two variables must still be elucidated. Data of Waikhom *et al.* (2006) suggest that low repeat numbers may be causing higher virulence. However, crude host tissue was used to initiate infection and differences in virion dose were not controlled in this study. Musthaq *et al.* (2006) found no differences in virulence between isolates with different number of RUs in the ORF94 VNTR. However, the design of the bioassays, data and statistical analysis were not reported making it difficult to verify the strength of their claims. More evidence from controlled laboratory experiments is therefore needed. Recombinant WSSV genotypes with specific RU numbers cannot yet be engineered, and as such the best approach at present would be to compare the virulence of a panel of isolates with low and high RU numbers for ORF94, while having a similar genotype for the other variable loci. John *et al.* (2010) performed such as experiment, and report that RU number in the ORF94 VNTR had no effect on virulence. However, here the authors did not challenge shrimp with equal virion doses. Dependence of shrimp mortality on WSSV dose has been established in many studies (e.g., Prior *et al.,* 2003; Escobedo-Bonilla *et al.,* 2005). The existence of differences in virulence between WSSV isolates is therefore a more cogent explanation for these results, because isolates containing low copy numbers of virus genome caused high mortality and vice-versa (John *et al.,* 2010).

If low RU number variants of ORF94 are causing higher virulence – which has yet to be demonstrated – then what mechanisms might be responsible? Although a direct link between RU number and virulence is not unthinkable (e.g., Peak *et al.,* 1996), we consider it more likely that the region surrounding ORF94 might be involved in the determining WSSV virulence. The repeat region of ORF94 is located between ribonucleotide reductase 1 and 2 (van Hulten *et al.,* 2000). Ribonucleotide reductase is an enzyme that catalyzes the formation of deoxyribonucleotide

precursors, which are involved in DNA replication process (Elledge *et al.,* 1992). Ribonucleotide reductase has been implied in the virulence of herpes viruses and pox viruses (Child *et al.,* 1990; Lembo and Brune 2009; Gammon *et al.,* 2010). Given that these loci are adjacent we expect strong linkage, and this may explain why the repeat structure of ORF94 is correlated to WSSV virulence. However, this possible explanation needs experimental support.

We also explored whether RU number correlated with province, year of sample collection, and farming system. Statistically significant differences were found for province ($p = 0.00$ for ORF75; $p = 0.03$ for ORF94 and $p = 0.04$ for ORF125) and year ($p = 0.00$ for ORF75; ORF94 and ORF125), which suggest WSSV populations are in a constant state of flux, even when considered on small spatiotemporal scales. We did not find a correlation between RU number and farming system for any of the VNTRs. Nevertheless, farming system does appear to have an effect on the range and number of RU variants present, with the greatest variety of RU variants present on improved-extensive farms. WSSV populations can persist in improved-extensive ponds, because these ponds are typically not ploughed. Therefore, we expect to see a more stable WSSV populations in these ponds than in those of other farming systems, and this has indeed been observed (Dieu *et al.,* 2011; Hoa *et al.,* 2011b). If the population is more stable than we expect to see then, in the absence of purifying selection (Graur and Li, 2000), standing genetic variation is conserved and *de novo* variation can become established in the virus population, leading to a higher number of genotypes present and therefore more mixed-genotype infections in improved extensive ponds. We previously reported more mixed-genotype infections in improved-extensive ponds than in semi-intensive ponds (Hoa *et al.,* 2011a), and here we confirm that there is a higher number of genotypes present (Table 1). WSSV populations in the improved-extensive system therefore behave as we would expect them to, in a qualitative sense, from a population genetic perspective.

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4

- CHAPTER -

Indel-II region deletion sizes in the white spot syndrome virus genome correlate with shrimp disease outbreaks in southern Vietnam

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ABSTRACT

Sequence comparisons of the genomes of white spot syndrome virus (WSSV) strains have identified regions containing variable-length insertions/deletions (i.e. indels). Indel-I and Indel-II, positioned between open reading frames (ORFs) 14/15 and 23/24, respectively, are the largest and the most variable. Here we examined the nature of these two indel regions in 313 WSSV-infected *Penaeus monodon* shrimp collected between 2006 and 2009 from 76 aquaculture ponds in the Mekong Delta region of Vietnam. In the Indel-I region, two WSSV genotypes with deletions either 5,950 bp or 6,031 bp in length compared to a reference strain from Thailand (WSSV-TH-96-II) were detected. In the Indel-II region, four WSSV genotypes with deletions 8,539 bp, 10,970 bp, 11,049 bp or 11,866 bp in length compared to a reference strain from Taiwan (WSSV-TW) were detected, with the 8,539 bp and 10,970 bp genotypes predominating. Indel-II variants with longer deletions were found to correlate statistically with WSSV-diseased shrimp originating from more intensive farming systems. Like Indel-I lengths, Indel-II lengths also varied based on the Mekong Delta province from which farmed shrimp were collected.

INTRODUCTION

White spot syndrome virus (WSSV) is one of the most widely distributed of all known viruses of aquatic crustaceans. The first outbreaks of white spot disease (WSD) were reported in 1992 in Taiwan and China (Chou *et al.,* 1995; Huang *et al.,* 1995). The disease quickly spread to Japan and Korea (Inouye *et al.,* 1994; Kim *et al.,* 1998), and to countries throughout Southeast Asia, the Indian continent, and to Central and South America (Escobedo-Bonilla *et al.,* 2008). Recently, WSSV has been detected in shrimp farmed in Southeast Europe and the Middle East (Lightner, 2011; OIE, 2009). Comparisons of WSSV strains examined at different times from different geographical locations have revealed similarities in disease symptoms and histopathology (Flegel, 1997; Wang *et al.,* 1999; Leu *et al.,* 2009), but differences in virus morphology (Leu *et al.,* 2009), genome structure (Marks *et al.,* 2004; Shekar *et al.,* 2005) and virulence (Marks *et al.,* 2005; Zwart *et al.,* 2010a). Except for two genome regions in which major insertions/deletions (indels) occur, nine regions containing homologous repeats and three regions containing variable-number tandem repeat (VNTR) sequences, the genome nucleotide sequences of different WSSV strains are highly (>99.5%) conserved (Nadala and Loh, 1998; Marks *et al.,* 2004).

The two major genomic indels are located between open reading frames (ORFs) 14/15 (Indel-I) and 23/24 (Indel-II) of the sequenced Thai strain WSSV-TH-96-I (van Hulten *et al.,* 2001a; Marks *et al.,* 2004; Marks *et al.,* 2005). Compared to another sequenced Thai strain (WSSV-TH-96-II), WSSV-TH-96-I has a 5.3 kb deletion in Indel-I (Marks *et al.,* 2005), and differs from a strain from Taiwan (WSSV-TW) by possessing a 13.2 kb deletion in Indel-II (Marks *et al.,* 2004). Sequence analyses of these two indel regions in other Asian WSSV strains have identified intermediate-length deletions (Dieu *et al.,* 2004; 2010; Zwart *et al.,* 2010a). For example, WSSV strains from China contain shorter Indel-II deletions (1,168 bp to 5,928 bp) compared to WSSV-TW, while strains from Vietnam contain a medium-length Indel-II deletion (~8,539 bp) not yet found in strains examined from other geographic regions (Dieu *et al.,* 2004; Yang *et al.,* 2001; Lan *et al.,* 2002; Tan and Shi 2011).

Indel-I and Indel-II regions are now being used as molecular markers to study WSSV epidemiology (Dieu *et al.,* 2004; 2010; Zwart *et al.,* 2010a), with Indel-II proving particularly useful for examining spatiotemporal spread of WSSV at regional (Dieu *et al.,* 2010) and intercontinental levels (Zwart *et al.,* 2010a). Data on Indel-II support a hypothesis that all WSSV strains affecting farmed shrimp have a recent common ancestor. This virus presumably circulated in China or Taiwan before spreading to shrimp farms in Vietnam, from there to Thailand (Dieu *et al.,* 2004) and from there to India (Pradeep *et al.,* 2008a) and other Asian countries (Zwart *et al.,* 2010a). However, as a WSSV strain originating from Thailand in 1996 (WSSV-TH-96-II) possesses the longest genome of all strains

characterized to date (Marks *et al.,* 2005), it is possible that WSSV might have been introduced initially into China and/or Taiwan through imports of live shrimp from Thailand.

VNTRs have been used to study WSSV epidemiology in different crustacean hosts at small (farm or pond) spatiotemporal scales (Wongterasupaya *et al.,* 2003; Hoa *et al.,* 2005; Pradeep *et al.,* 2008b), to detect mixed WSSV infections in shrimp (Hoa *et al.,* 2011a) and to examine WSSV transmission within and between aquaculture ponds (Pradeep *et al.,* 2008b; Walker *et al.,* 2011). Data on VNTRs in conjunction with epidemiological models have also been used to quantify different transmission routes of WSSV within and between farm ponds (Hoa *et al.,* 2011b).

In a preliminary longitudinal study of WSSV genotypes affecting farmed shrimp, the Indel-II region was useful for tracking genotypes over time (Dieu *et al.,* 2011). WSSV genome lengths estimated from analyses of Indel-I and Indel-II lengths suggest that genome shrinkage rates since 1992 are decreasing over time (Zwart *et al.,* 2010a). While increased Indel-I and Indel-II deletion lengths have been associated with increased WSSV virulence (Marks *et al.,* 2005; Zwart *et al.,* 2010a), an early study reported reduced virulence with increased Indel-II deletion lengths (Lan *et al.,* 2002).

Here we report a spatiotemporal study examining Indel-I and Indel-II deletion lengths in WSSV strains detected in *Penaeus monodon* shrimp collected between 2006 and 2009 from different farm types in two regions of the Mekong Delta in Vietnam. The data associated increased Indel-II deletion lengths with WSSV disease and thus with higher intensity farming systems from which most diseased shrimp were collected over the study period.

MATERIALS AND METHODS

Shrimp samples and classification of farming system

In total, 313 WSSV-infected shrimp (*Penaeus monodon*) collected from 35 aquaculture ponds in Bac Lieu Province and 41 ponds in Ca Mau Province, Mekong Delta, Vietnam, in 2006, 2008 and 2009 were examined. Shrimp were sampled either at stocking (n = 140) or at the time of disease outbreaks when ponds were emergency harvested (n = 173). Whole shrimp were stored either in 100% ethanol at room temperature or in liquid nitrogen before being transferred to -80°C.

Farm management systems from which shrimp were collected were classified as either improved-extensive, rice-shrimp or semi-intensive. Improved-extensive farms primarily recruit wild *P. monodon* postlarvae (PL) and juveniles, but sometimes supplement these with PL from hatcheries. Rice-shrimp farms tend to be stocked at low densities (0.5-2 PL/m^2), have shrimp at different developmental stages, use little or no feed and only operate during the dry season when paddies are not being used to grow rice. Semi-intensive farms tend to use aerated ponds that are drained and cleaned before stocking, hatchery PL seeded at higher

stocking densities (12-20 PL/m^2) and industrial shrimp feed. Shrimp were sampled from 41 improved-extensive ponds (37 in Ca Mau province and 4 in Bac Lieu province), 27 semi-intensive ponds (4 in Ca Mau and 23 in Bac Lieu) and 8 rice-shrimp plots (all in Bac Lieu). The farming systems from which shrimp were sampled in Ca Mau and Bac Lieu provinces were quite distinct (Chi-square = 227.40; degrees of freedom = 2; p value < 0.001) (Table S1). In reality there are also differences in the farm systems used in these two provinces; there are relatively more semi-intensive ponds and rice-shrimp plots in Bac Lieu than in Ca Mau, and relatively less improved-extensive ponds in Ca Mau than in Bac Lieu (Anonymous, 2009).

Table S1. Association of shrimp farming types with provinces.

Shrimp source	Ca Mau (%)	Bac Lieu (%)
Improved-extensive ponds	162 (96%)	7 (4%)
Semi-intensive ponds	17 (16%)	88 (84%)
Rice-shrimp plots	0 (0%)	39 (100%)

Pearson´s Chi-square indicates a significant correlation between province and farming type (p < 0.001)

WSSV PCR

WSSV DNA was extracted from gill tissues and amplified by two-step PCR using the IQ2000™ WSSV kit (GeneReach Biotechnology Corp., Taipei, Taiwan) according to the manufacturer's instructions. The primer sets were designed to amplify WSSV-specific products 296 bp and/or 550 bp in length, as well as a 848 bp crustacean-specific product to confirm the integrity of the DNA.

PCR amplification across WSSV indels

WSSV Indel-I and Indel-II regions were amplified by PCR using the primer sets VR14/15-screen, VR23/24-screen, VR23/24-1 and VR23/24-south (adapted from Dieu *et al.,* 2004; 2010), and 14/15-complete (adapted from Marks *et al.,* 2005) (Table S2). Approximate 200 ng DNA was amplified in a 25 µl reaction containing PCR master mix (Promega; Madison, USA), using the thermal cycling conditions 94°C for 5 min, 30 cycles of 94°C for 30 s, 49-55°C (primer dependent) for 30s and 72°C for up to 7 min depending on the expected amplicon length. PCR products were resolved in 1% agarose gels beside a 100 bp Plus DNA marker (Fermentas; Burlington, Canada).

PCR product sequence analysis

Representative PCR products were sequenced to confirm that indel lengths had been estimated accurately. PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and sequenced by Macrogen Inc. (Seoul, South Korea). Sequence chromatograms were examined using Chromas

Version 1.45, and edited using BioEdit 7.0.9 (Hall, 1999). BLASTn was used to align Indel-I and Indel-II sequences to reference WSSV strains WSSV-TH-96-II (AY753327) and WSSV-TW (AF440570), as well as to other available WSSV sequences (AF369029, AF332093).

Table S2. Sequences of PCR primers used to amplify WSSV Indel regions

Primer set name	Sequence (5'- 3')	Anneal temp (ºC) / Elongation time (s)	WSSV-TW *
Indel-I			
VR14/15-screen (F)	GAGATGCGAACCACTAAAAG	49 / 75	301765-301784
VR14/15-screen (R)	ATGGAGGCGAGACTTGC		303179-303195
14/15-complete (F)	AATATGGAACGACGGGTG	55 / 420	301090-301107
14/15-complete (R)	GACCAGCGCCTCTTCAG		303735-303751
Indel-II			
VR23/24-screen (F)	CACACTTGAAAAATACACCAG	49 / 65	5503-5523
VR23/24-screen (R)	GTAAGTTTATTGCTGAGAAG		14571-14590
VR23/24-1(F)	ATGGGCTCTGCTAACTTG	50 / 360	4359-4376
VR23/24-1 (R)	ATGATTGTATTCGTCGAAGG		15172-15191
VR23/24-south (F)	GTAGTGCATGTTTCTCTAAC	49 / 100	2356-2375
VR23/24-south (R)	GTAAGTTTATTGCTGAGAAG		14571-14590

F = forward ; R = reverse ; nucleotide sequence
*Coordinates of the region amplified

Statistical analysis

Indel lengths were coded as small (5,950 bp for Indel-I and 8,539 bp for Indel-II) or large (6,031 bp for Indel-I and 10,970 bp to 11,866 bp for Indel-II). A 2 × 2 contingency table was constructed by counting the numbers of each WSSV Indel type with disease status or sampling location of each shrimp, and a 2 × 3 contingency table was constructed by counting the numbers of each WSSV Indel types and the farming system from which each shrimp was collected. The relationship between farming system or disease status and Indel-I or Indel-II type detected was quantified using Pearson's Chi-squared test. All probability tests were right sided with a significance level of 0.05.

RESULTS

WSSV Indel-I variants in shrimp from Ca Mau and Bac Lieu provinces

WSSV ORF14/15 Indel-I lengths were determined for 313 shrimp that tested positive in the WSSV IQ2000™ PCR (Fig. 1). Among the two Indel-I PCR primer sets used, set VR14/15-screen amplified products for 159 of 214 samples tested and set 14/15-complete amplified products for 15 of 38 samples tested. Sequence analyses confirmed that products amplified by either primer set represented 5,950 bp or 6,031 bp deletion types (Fig. 2A) in comparison to the reference strain containing the maximum-length indel sequence, Thai strain WSSV-TH-96-II (Marks *et al.,* 2004; Dieu *et al.,* 2004). Statistical analyses determined that neither Indel-I

deletion type was correlated with the improved-extensive, rice-shrimp or semi-intensive farming system from which the shrimp originated (Chi-square = 2.38; degrees of freedom = 2; p value = 0.305), or whether shrimp were healthy or diseased (Chi-square = 0.85; degrees of freedom = 1; p value = 0.358), but that Indel-I types segregated based on the Ca Mau or Bac Lieu provinces from which shrimp were collected (Chi-square = 13.15; degrees of freedom = 1; p value < 0.001).

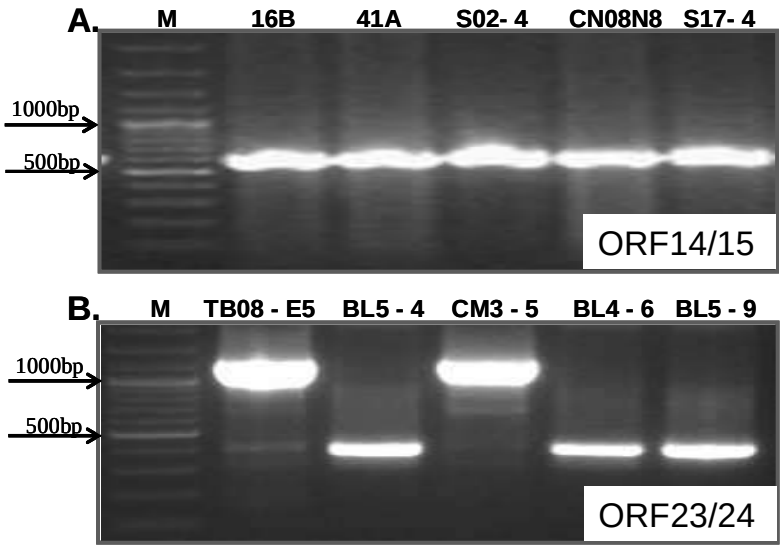

Figure 1. An agarose gel showing WSSV-specific PCR products amplified from individual shrimp (various identification codes) using either (A) primer set VR14/15-screen (600 bp) for Indel-I or (B) primer set VR23/24-south (400 bp or 1200 bp) for Indel-II. M = DNA marker.

WSSV Indel-II variants in shrimp from Ca Mau and Bac Lieu provinces

The various PCR primer sets used were only able to determine ORF23/24 Indel-II lengths for 206 of the 313 WSSV-positive samples tested. For the others, either no PCR product was amplified or multiple PCR products were amplified that could not be distinguished clearly. PCR primer set VR23/24-screen failed to amplify a product of the expected size (548 bp; Dieu *et al.,* 2004) from any WSSV-positive shrimp. Set VR23/24-1 amplified a ~2.3 kb product from 109 of 216 shrimp that was confirmed by sequence analysis to contain a 8,539 bp deletion (Indel-II nucleotide region 5793-14332) compared to the reference strain containing the maximum-length indel sequence, Taiwanese strain WSSV-TW (Marks *et al.,* 2004). Of 119 samples amplified using primer set VR23/24-south, 97 generated products either ~400 bp and/or ~1200 bp in length (Fig. 1). These were confirmed by sequence analysis to possess Indel-II deletions 11,866 bp (n = 4), 10,970 bp (n = 70) or both (n = 22) (Indel-II nucleotide regions 2704-14570 or 3372-14342), or

11,049 bp (n = 1) (Indel-II nucleotide region 3387-14436) in length compared to WSSV-TW (Table 1).

Of the putative proteins encoded within the 13 kb Indel-II region of WSSV-TW, two (wssv006 and wssv025) are retained even in those genotypes identified to contain the longest (11,866 bp) deletion (Table 2, Fig. 2B). These ORFs reside adjacent to an essential early viral gene, wssv004 (Han *et al.,* 2007), and do not exist in the virulent Thai strain WSSV-TH-96-I or other Asian strains detected more recently in WSSV-diseased shrimp (Zwart *et al.,* 2010a). Characteristics of WSSV proteins in this region predicted from *in silico* analyses, including non-essential transmembrane protein wsv479 that can be found in virions (Yang *et al.,* 2001), are summarized in Table 2.

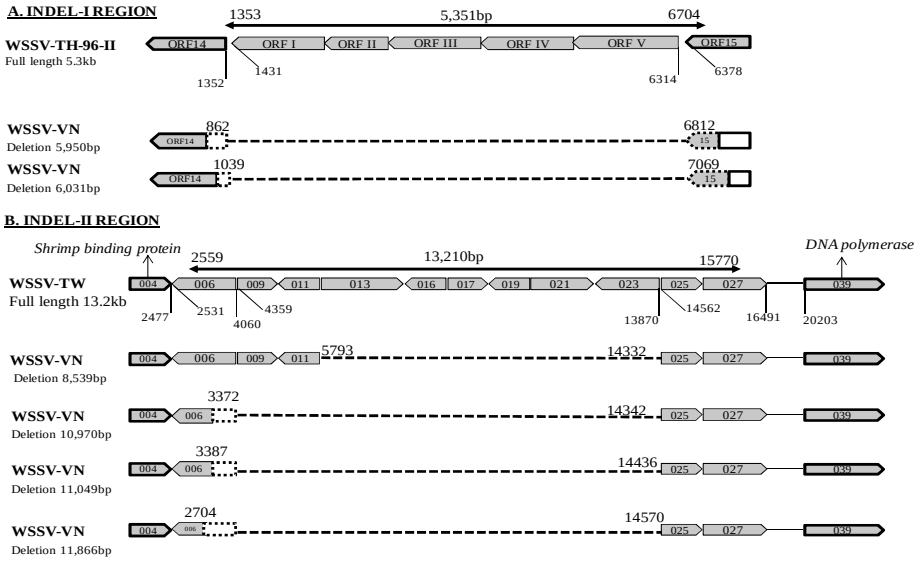

Figure 2. A. Schematic representation of the Indel-I region of the putative ancestral WSSV strain WSSV-TH-96-II, which contains the longest known Indel-I region (Marks *et al.,* 2005), and two representative WSSV-VN strains detected in shrimp sampled from the Mekong Delta, Vietnam. The numbers indicate genome nucleotide positions in WSSV-TH-96-II (AY753327), and dashed lines present deleted sequence. B. Schematic representation of the Indel-II region of the putative ancestral WSSV strain WSSV-TW, which contains the longest know Indel-II region, and four representative WSSV-VN strains. The numbers indicate genome nucleotide positions in the WSSV-TW (AF440570) and dashed lines represent deleted sequence.

Table 1. Indel-I and Indel-II deletion lengths identified among WSSV strains analysed here and in other studies

Country/WSSV strain	Deletion length (bp)		Position Indel-II	References
	Indel-I*	Indel-II**		
Thailand				
WSSV-TH	5,316	13,210	2559-15770	van Hulten *et al.,* 2001a
China				
WSSV-CN	5,131	1,168	283412-283413	Yang *et al.,* 2001
WSSV-CN-A	ND	5,717	281048-285596	Lan *et al.,* 2002
WSSV-CN-B	ND	5,926	281048-285805	Lan *et al.,* 2002
WSSV-CN-C		9,319	277564-285716	Lan *et al.,* 2002
WSSV98NB2	4,749	11,093	275879-285805	Tan and Shi, 2011
Brazil				
WSSV-Brazil	ND	11,453	2719-14172	Muller *et al.,* 2010
India				
WSSV-India	5,892	**10,970**	3372-14342	Pradeep *et al.,* 2008a
Vietnam				
WSSV-VN-Central	**5,950**	**8,539**	5793-14333	Dieu *et al.,* 2004
WSSV-ND-North	6,031	9,631	4860-14493	Dieu *et al.,* 2010
WSSV-HP-North	6,031	**11,048**	3342-14342	Dieu *et al.,* 2010
WSSV-LA-South	5,950	**10,970**	3372-14342	Zwart *et al.,* 2010
WSSV-TV-South	6,031	11,450	2858-14309	Dieu *et al.,* 2004
WSSV-HT-South	5,138	11,886	2704-14570	Dieu *et al.,* 2010
WSSV-KG-South	6,031	12,166	3099-15266	Dieu *et al.,* 2004
WSSV-BR-South	5,316	13,210	2559-15770	Dieu *et al.,* 2010
South Vietnam				
VN-Extensive-CN08N5	5,950	ND		This study
VN-Extensive-TB08B4	ND	10,970	3372-14342	This study
VN-Extensive-CM7.8.1	ND	**11,049**	3387-14436	This study
VN-Extensive-TB08-M20	5,950	10,970	3372-14342	This study
VN-Intensive-CM3.5.13	ND	10,970	3372-14342	This study
VN-Intensive-CM3.5.14	ND	10,970	3372-14342	This study
VN-Intensive-17A	**6,031**	**8,539**	5793-14332	This study
VN-Intensive-31A	**5,950**	**10,970**	3372-14342	This study
VN-Intensive-S9.1	ND	10,970	3371-14342	This study
VN-Intensive-S20.2	5,950	10,970	3372-14342	This study
VN-Intensive-S15	5,950	ND		This study
VN-Intensive-S26	5,950	10,970	3372-14342	This study

Numbers in bold indicate deletion lengths found both here and in other studies.

* Compared to WSSV-TH-96-II sequence (AY753327)

** Compared to WSSV-TW sequence (AF440570)

ND = Not determined or no PCR product amplified

Table 2. Characteristics of Indel-II deletions in WSSV reference strains and those detected in Mekong Delta shrimp

WSSV-Taiwan	WSSV-China	Characteristics	TH-I-96[a] & VN-BR[b] (13,210 bp)	TH-II-96[c] (0 bp)	IN & VN-LA[d] VN[f] (10,970bp)	VN-ST[b], VN[f] (11,866bp)	VN-K[e], VN[f] (8,539bp)	VN[f] (11,049bp)
wssv004	**wsv477**	Protein interaction with host actin[g]	ORF23					
wssv005	*wsv478*	Early gene & GTP-binding motif[h], ZF[i], TM[i]						
wssv006	**wsv479**	Expression at 12 hpi[j]	X					
wssv007	*wsv480*	TM[i], Glu-rich cluster				X		
wssv008	**wsv481**		X		X	X		X
wssv009	**wsv482**	TM[i]	X		X	X		X
wssv010	**wsv483**		X		X	X		X
wssv011	**wsv484**	TM[i]	X		X	X		X
wssv012	*wsv485*							
wssv013	**wsv486**	TM[i]	X		X	X	X	X
wssv014	**wsv487**		X		X	X	X	X
wssv015	*wsv488*							
wssv016	**wsv489**		X		X	X	X	X
wssv017	**wsv492**		X		X	X	X	X
wssv018	*wsv491*							
wssv019	**wsv493**	Viral envelop protein (vp35)[j]	X		X	X	X	X
wssv020	*wsv494*		X		X	X	X	X
wssv021	**wsv495**		X		X	X	X	X
wssv022	**wsv496**		X		X	X	X	X
wssv023	**wsv497**	TM[i]	X		X	X	X	X
wssv024	*wsv499*							
wssv025	**wsv500**	ATP/GTP	X			X		X
wssv026	*wsv501*	binding motif[i], ZF[i]						
wssv027	**wsv502**	ATP/GTP binding motif A[i]	ORF24					
wssv028	*wsv503*	ZF[i]						
wssv029	*wsv504*	Expression at 18 hpi[k]						
wssv030		Cell attachment sequence[l], TM[i] and SP[i]						
	wsv508	TM[i]	ORF25					
wssv036	**wsv513**	TM[l]	ORF26					
wssv039	**wsv514**	DNA polymerase[i,l], TM[h]	ORF27					

X indicates an ORF has been deleted, ZF indicates a Cys2/Cys2-type zinc finger is present, and TM indicates a putative transmembrane protein. References are indicated by letters in superscript and given below the table.

[a] Marks *et al.* (2004), [b] Dieu *et al.* (2010), [c] Marks *et al.* (2005), [d] Pradeep *et al.* (2008a), see also Zwart *et al.* (2010a), [e] Dieu *et al.* (2004), [f] this study, [g] Chen (2008), [h] Han *et al.* (2007), [i] Yang *et al.* (2001), [j] Chen *et al.* (2002), [k] Tsai *et al.* (2004), [l] van Hulten *et al.* (2001).

Table 3. Frequency at which WSSV Indel-II deletion types were detected in newly stocked healthy shrimp and in diseased shrimp from emergency harvests from different farming systems across two provinces in the Mekong Delta, Vietnam

Shrimp source	Deletion Length - Indel-II				
	8,539 bp	10,970 bp	11,049 bp	11,866 bp	10,970 bp and 11,866 bp
Emergency harvest	77 (48%)	65 (41%)	0 (0%)	4 (3%)	13 (8%)
Pond stocking	32 (68%)	5 (11%)	1 (2%)	0 (0%)	9 (19%)
Improved-extensive ponds	50 (68%)	6 (8%)	1 (1%)	4 (5%)	13 (18%)
Semi-intensive ponds	22 (23%)	64 (67%)	0 (0%)	0 (0%)	9 (10%)
Rice-shrimp plots	37 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Ca Mau province	61 (76%)	7 (9%)	1 (1%)	0 (0%)	11 (14%)
Bac Lieu province	48 (38%)	63 (50%)	0 (0%)	4 (3%)	11 (9%)

Relationship between WSSV Indel-II type, disease, farm systems and location

Contingency tables were constructed to determine whether Indel-II deletion type might correlate with WSSV disease status, farming system and farm location. However, as farming systems used in the two Mekong Delta provinces studied vary in number (Anonymous, 2009), as emergency harvests due to WSSV disease outbreaks will mostly occur in more stringently managed semi-intensive farm systems, and as samples were not collected specifically to examine these variables, correlations among them or to Indel-II types need to be interpreted with caution at best. Of the 313 WSSV PCR-positive shrimp examined, 173 were collected from 33 ponds at the time of stocking when there was no evidence of disease and 140 shrimp were collected from 43 ponds at times of emergency harvested due to outbreaks of WSSV disease. Of the Indel-II types detected in 206 of these 313 shrimp (Table 3), the 10,970 bp deletion was detected in 65 of the 159 (41%) diseased shrimp and in 5 of the 47 (11%) healthy shrimp. When grouped into either shorter (8,539 bp) or longer Indel-II deletion (10,970 bp to 11,866 bp) types, a 2 x 2 contingency table showed longer deletion types to be correlated significantly with WSSV disease (Chi-square = 5.63; degrees of freedom = 1; p value = 0.018) (Table 4).

Table 4. Association of WSSV Indel-I and Indel-II deletion types with shrimp disease status, farming system and farm location

A. Indel-I

Shrimp source	Number (%) with Deletion Lengths	
	5,950 bp	6,031 bp
Emergency harvest[a]	136 (96%)	5 (4%)
Pond stocking[a]	25 (93%)	2 (7%)
Improved-extensive ponds[a]	43 (96%)	2 (4%)
Semi-intensive ponds[a]	79 (94%)	5 (6%)
Rice-shrimp plots[a]	39 (100%)	0 (0%)
Ca Mau province[c]	53 (88%)	7 (12%)
Bac Lieu province[c]	108 (100%)	0 (0%)

B. Indel-II

Shrimp source	Number (%) with Deletion Lengths	
	8,539 bp	10,970 bp + 11,866 bp
Emergence harvest[b]	77 (48%)	82 (52%)
Pond stocking[b]	32 (68%)	15 (32%)
Improved-extensive ponds[c]	50 (68%)	24 (32%)
Semi-intensive ponds[c]	22 (23%)	73 (77%)
Rice-shrimp plots[c]	37 (100%)	0 (0%)
Ca Mau province[c]	61 (76%)	19 (24%)
Bac Lieu province[c]	48 (38%)	78 (62%)

Pearson´s Chi-square was used to test for significant effects of shrimp sources: [a] indicates $p > 0.05$, [b] indicates $p < 0.05$, and [c] indicates $p < 0.001$.

Indel-II deletion types were assessed similarly against whether shrimp had originated from rice-shrimp plots (n = 37), improved-extensive ponds (n = 74) or semi-intensive ponds (n = 95) (Table 3). The 8,539 bp Indel-II deletion type occurred in all 37 shrimp from rice-shrimp plots and 50 of the 74 (68%) shrimp from improved-extensive ponds. The 10,970 bp Indel-II deletion type occurred in 64 of the 95 (67%) shrimp from semi-intensive ponds. Mixed Indel-II deletion types were detected in 13 of the 74 (18%) of shrimp from improved-extensive ponds, 9 of the 95 (10%) of shrimp from semi-intensive ponds. Construction of a 2 x 3 contingency table showed that the shortest (8,539 bp) Indel-II deletion type was correlated significantly with shrimp sampled from improved-extensive ponds and rice-shrimp plots (Chi-square = 73.06; degrees of freedom = 2; p <0.001) (Table 4).

The 8,539 bp Indel-II deletion was detected in 61 of the 80 (76%) shrimp collected from Ca Mau province and 48 of the 126 (38%) collected from Bac Lieu province. In contrast, the 10,970 bp Indel-II deletion was detected in 63 of the 126 (50%) of shrimp from Bac Lieu province but only 7 of the 80 (9%) shrimp from Ca Mau province. A 2 x 2 contingency table constructed with these data showed Indel-II types to differ significantly based on the province from which shrimp were collected (Chi-square = 28.59; degrees of freedom = 1; p <0.001) (Table 4).

DISCUSSION

Genomic indels and VNTR sequences are being used increasingly as epidemiological tools to predict disease and trace WSSV spread to different shrimp farming regions within and beyond Asia, including the Western hemisphere (Dieu *et al.,* 2004; Pradeep *et al.,* 2008a; Muller *et al.,* 2010). For example, certain repeat structures in ORF125 and particularly in ORF94 have been found to correlate with disease outbreaks (Hoa *et al.,* 2011b; Dieu *et al.,* 2011). Although no specific repeat unit (RU) numbers have been associated with particular farming systems (Hoa *et al.,* 2012), WSSV types in shrimp being reared in extensive systems appear to remain more stable than in shrimp reared in semi-intensive systems (Hoa *et al.,* 2011b; Dieu *et al.,* 2011). These findings may contribute to our understanding of how WSSV population structures influence disease.

To examine whether different WSSV indel variants might correlate with disease status, location and system used to farm *Penaeus monodon* in the Mekong Delta region of Vietnam, shrimp were sampled over a 3 year period at times of pond stocking and when emergency harvested due to WSSV disease occurring. For Indel-I within the WSSV ORF14/15 region, deletions of 5,950 bp or 6,031 bp were detected as found previously among WSSV strains infecting shrimp farmed in central and southern regions of Vietnam (Dieu *et al.,* 2004; 2010). These indel types appear to be quite stable and unique to WSSV strains in Vietnam, but have not been found to be associated with disease or farm system or to be useful for distinguishing strain characteristics at regional (Dieu *et al.,* 2010) and

intercontinental (Zwart *et al.,* 2010a) scales. While the two Indel-I types did segregate to either the Ca Mau or Bac Lieu provinces from which shrimp were collected in the Mekong Delta, as nothing is known about the origin of PL seeded into semi-intensive or the other pond types, and as farming systems from which shrimp were sampled from the two provinces differed substantially, the significance of this putative geographic segregation of WSSV strains based on Indel-I type will need to be investigated more thoroughly.

For Indel-II in the WSSV ORF23/24 region, 4 deletion types were detected as found previously among *P. monodon* shrimp examined from northern, central and southern regions of Vietnam (Dieu *et al.,* 2004; 2010; Zwart *et al.,* 2010a). In these studies, WSSV strains with the 11,049 bp deletion type were found in shrimp being farmed in northern Vietnam and the 10,970 bp and 11,866 bp deletion types were found in shrimp being farmed in southern Vietnam. The 10,970 bp deletion type, which was found here at high frequency, has also been detected in WSSV strains infecting *P. monodon* shrimp in India (Pradeep *et al.,* 2008a). The 8,539 bp deletion type found previously in WSSV strains infecting shrimp farmed in central Vietnam (Dieu *et al.,* 2010) was the most common type detected here among shrimp being farmed across the Mekong Delta (Table 3). It is possible that WSSV strains with this Indel-II deletion type have been transferred from central to southern Vietnam through infected postlarvae. However, based on the limited and fragmented spatiotemporal data on Indel-II, it is equally plausible that WSSV strains with the 8,539 bp deletion type have existed in shrimp or other crustaceans in southern Vietnam for some time and been simply missed until this more comprehensive study of *P. monodon* farmed across the Mekong Delta.

On various spatiotemporal scales, Indel-I and Indel-II types have been used to investigate how WSSV might have spread and evolved (Marks *et al.,* 2004; 2005; Dieu *et al.,* 2004; 2010; Pradeep *et al.,* 2008a; Tan *et al.,* 2009; Tan and Shi, 2011; Zwart *et al.,* 2010a). While these studies have relied generally on WSSV genotyping data on relatively small numbers of shrimp, often sourced from distant locations, here WSSV strains were examined from a large number of shrimp collected over a three year period from many farms spread widely across two neighboring provinces in the Mekong Delta. Variations in Indel-I and Indel-II types were limited and stable temporally, suggesting WSSV genotypes predominating in this region could have been determined quite accurately from far fewer samples. However, due to the large number of samples examined, existing PCR primer sets were used, and based on the finding that these primers sets failed to amplify a product for samples that tested positive in the robust commercial IQ2000 PCR for diagnosing WSSV infection, it is possible that these primer sets limited detections to those Indel-I and Indel-II reported previously.

Univariate tests were undertaken with Indel-I and Indel-II types detected across the limited number of WSSV-infected shrimp that genotyped successfully to

identify whether these might correlate with disease status, farming system and farm location. However, as different farming systems predominate in either province studied and as emergency harvests due to WSSV disease occur predominantly at semi-intensive farms (Hoa *et al.,* 2011a; b), where most diseased samples examined here originated from, the pertinence of these correlations need to be viewed with caution. Indel-I and Indel-II types segregated based on province, somewhat like as found with Indel-II types of WSSV strains detected in shrimp from northern, central and southern regions of Vietnam (Dieu *et al.,* 2010), although to be completely consistent with this, only Indel-II variants with larger deletions would have been expected to be detected. Another major limitation of the sample set was that healthy shrimp were collected only as PL/juveniles at pond stocking, and diseased shrimp were collected from emergency harvested ponds. This sampling regime is likely to have biased correlations of disease with Indel-I and Indel-II deletion types, as few diseased shrimp originated from improved-extensive ponds, with shrimp from these primarily sampled at the time of stocking when healthy.

The 10,970 bp Indel-II deletion was correlated most strongly with disease, followed by the 8,539 bp Indel-II deletion, although this deletion type was also detected commonly in healthy PL/juveniles sampled when ponds were stocked. Such correlations concur with previous evidence of specific WSSV genotypes associating with disease (Hoa *et al.,* 2005; Musthaq *et al.,* 2006) and with WSSV strains with shorter genomes being more virulent (Marks *et al.,* 2005; Pradeep *et al.,* 2009; Zwart *et al.,* 2010a). However, why WSSV strains with longer Indel-II deletions seem to cause or result from disease remains to be elucidated.

Short Indel-II deletions were found most frequently among WSSV strains detected in healthy shrimp sampled when improved-extensive ponds and rice-shrimp plots were stocked. In contrast, longer WSSV Indel-II deletions were found most frequently in healthy shrimp sampled when semi-intensive ponds were stocked. Shrimp continually inhabit improved-extensive ponds that are never drained, and in rice-shrimp plots, shrimp are present throughout the dry season. It is possible, therefore, that low virulence WSSV variants can persist in these shrimp populations (Dieu *et al.,* 2011), which might lead to higher frequencies of mixed-genotype infections (Hoa *et al.,* 2011a). In contrast, in semi-intensive farming systems, the high densities of shrimp likely promote transmission, stress and more virulent WSSV variants causing disease outbreaks. Moreover, the relatively short crop durations interspersed by pond draining and cleaning provide no opportunities for shrimp sub-populations to persist, and thus no advantage to WSSV for persisting in such stocks (Dieu *et al.,* 2011). While these factors could explain why more virulent WSSV strains with longer Indel-II deletions and shorter overall genomes (Zwart *et al.,* 2010a) were found more frequently in semi-intensive ponds systems, no data was collected on the hatchery sources of PL,

which might also influence what genotypes were detected. In any case, our data support those of other studies in which farming practices have been found to influence the risk of disease (Joffre and Bosma, 2009) and what WSSV genotypes exist in the shrimp (Dieu *et al.,* 2011).

Examining WSSV Indel-I and Indel-II types in a large number of healthy and diseased shrimp collected over a 3 year period from various farm types in two provinces in the Mekong Delta, Vietnam, found that Indel-I to have little epidemiological value but that longer Indel-II deletions correlated significantly with disease shrimp emergency harvested at semi-intensive farms.

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5
- CHAPTER -

Mixed-genotype white spot syndrome virus infections of shrimp are inversely correlated with disease outbreaks in ponds

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ABSTRACT

Outbreaks of white spot syndrome virus (WSSV) in shrimp culture and the relation between the virus and virulence are not well understood. Here, we provide evidence showing that WSSV mixed-genotype infections correlate with lower outbreak incidence and that disease outbreaks correlate with single-genotype infections. We tested 573 shrimp samples from 81 shrimp ponds in the Mekong Delta with outbreak or non-outbreak status. The variable number tandem repeat (VNTR) loci of WSSV were used as molecular markers for the characterization of single- and mixed-genotype infections. The overall prevalence of mixed-genotype WSSV infections was 25.7%. Non-outbreak ponds had a significantly higher frequency of mixed-genotype infections than outbreak ponds for all VNTR loci, both at the individual shrimp as well as at the pond level. The genetic composition of WSSV populations appears to correlate with the health status of shrimp ponds. The causal relationship between genotypic diversity and disease outbreaks can now be experimentally approached.

INTRODUCTION

White spot disease (WSD) is a highly contagious viral disease that can rapidly spread throughout an area and cause high levels of mortality in many species of cultured penaeid shrimp, especially *Penaeus monodon* (Flegel and Alday-Sanz, 1998; Walker and Mohan, 2009). White spot syndrome virus (WSSV) is the causative agent of this disease (Inouye *et al.*, 1994). WSSV has a large dsDNA genome of about 300 kbp which shows relatively little genetic variation, except for significant nucleotide sequence variation at five loci (Marks *et al.*, 2004; Dieu *et al.*, 2004). Comparison of the complete genome sequence of shrimp-derived WSSV isolates from Thailand, Taiwan and China (GenBank Accession numbers: AF369029.2, AF440570.1 and AF332093.1) revealed three variable number tandem repeat (VNTR) loci on the WSSV genome, overlapping with ORF75, ORF94 and ORF125, and two variable regions in which major deletions occur, the ORF14/15 and ORF23/24 regions (Marks *et al.*, 2004). These variable regions have now been used as genetic markers in a considerable body of epidemiological research. Studies employing one or more VNTR loci have found high levels of variation, even at very small spatial scales, suggesting that these loci are useful markers at such small scales to study genomic variation (Wongteerasupaya *et al.*, 2003; Hoa *et al*., 2005; Pradeep *et al.*, 2008b). On the other hand, the ORF14/15 and ORF23/24 variable regions have proven useful for inferring patterns of spread on larger spatial scales, such as the spread of WSSV to and in Vietnam (Dieu *et al.*, 2004, 2010a) and in India (Pradeep *et al*., 2008a). These results show that PCR genotyping is a useful tool for tracing the movement of WSSV and to better understand the epidemiology of this virus.

Hoa *et al*. (2005) and Pradeep *et al.* (2008b) reported different PCR amplicons in a single reaction when amplifying VNTR loci from viral DNA from individual shrimp. These different amplicons could indicate mixed-genotype WSSV infections of individual shrimp, although this was never confirmed by further molecular analysis. Mixed-genotype infections are common for many viruses (e.g., Smith and Crook, 1988; Davis *et al.*, 1999; Cory *et al.*, 2005). Moreover, complementation between genotypes within the host can increase virulence (Símon *et al.*, 2006; Vignuzzi *et al*., 2006). However, for WSSV the following information is needed for further studies: (i) confirmation of whether mixed-genotype infections occur, and if so, whether they are rare or abundant, and (ii) what the role of mixed-genotype infections is in WSSV epidemiology and evolution. In this report, we first investigated whether VNTR loci are suitable for detecting mixed-genotype WSSV infections by a PCR-based method. We then tested whether mixed-genotype WSSV infections occur in shrimp ponds using WSSV variable loci as molecular makers, and estimated the prevalence of mixed-genotype infections in individual shrimp. The disease status of these shrimp cultures in ponds was also recorded, allowing

us to test to what extent single- or mixed-genotype infections are correlated with the occurrence of disease outbreaks.

MATERIALS AND METHODS

P. monodon juveniles and wild crabs were collected from grow-out ponds in Bac Lieu and Ca Mau provinces, Vietnam, during 2006–2009 and were transported immediately to Can Tho University, Vietnam. During that period, a total of 573 shrimp samples were taken from 81 sites (ponds). The samples were stored either in 100% ethanol at room temperature, or in a liquid nitrogen container before transfer to –80 °C. DNA was extracted from gill tissues and WSSV DNA was detected with a two-step PCR using the IQ2000TM WSSV kit, according to the manufacturer's instructions (Farming IntelliGene Technology Corporation, Taipei, Taiwan). The primer sets were designed to yield WSSV-specific amplicons of 296 bp and/or 550 bp, respectively. Amplification of a 848-bp amplicon from a crustacean housekeeping gene allowed for confirmation of correct DNA isolation, when no WSSV-specific sequences were detected.

Genotyping of WSSV-positive samples was done using a PCR-based method, based on different molecular markers, tandem repeat sequences (ORF94 and ORF125) and compound repeat units (RUs) (ORF75) located along the WSSV genome. In detail, PCR with specific primers and conditions (Table 1) was used to amplify the VNTR loci ORF94 (54bp RU) (Wongteerasupaya *et al.*, 2003), ORF125 (69bp RU) (This study), and ORF75 (compound RUs of 45bp and 102bp) (Dieu *et al.*, 2004). All PCR reactions were performed in an Applied Biosystems (Foster City, USA) thermal cycler using GoTaq Flexi DNA Polymerase (Promega; Madison, USA), and the PCR products were analyzed by electrophoresis in 1% agarose gels.

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

Site	Primer name	Sequence (5'- 3')	Annealing temperature / Elongation time
ORF75	ORF75-flank-F	GAAGCAGTATCTCTAACAC	49^0C/80s
	ORF75-flank-R	CAACAGGTGCGTAAAAGAAG	
ORF94	ORF94 - F	TCTACTCGAGGAGGTGACGAC	52^0C/60s
	ORF94 - R	AGCAGGTGTGTACACATTTCATG	
ORF125	Geno125 - F	ACAGTGACCACACGATAATACCA	60^0C/60s
	Geno125 - R	TCGTTCACCATATCCATTGCCCT	

To determine whether different PCR products are really indicative of mixed-genotype infection, the ORF75 PCR products of different sizes derived from one individual shrimp were cloned. This analysis was performed for two WSSV-positive samples, selected because they rendered multiple PCR amplicons. PCR products were ligated into the pGEM-T easy vector (Promega) and transformed into competent *Escherichia coli* DH5α by electroporation. White colonies were selected for plasmid isolation and colony PCR with the ORF75-flank primer pair (Table 1). Plasmid DNA was purified from clones representative of all insert sizes

observed by PCR with the GeneJET™ plasmid miniprep kit (Fermentas). Plasmid clones were sequenced using universal T7 and/or Sp6 primers, and sequence chromatograms were analyzed using Chromas software (version 1.45). The sequences were then aligned using NCBI BLAST and Genedoc software. Referenced WSSV sequences were obtained from the NCBI Genbank database (AF369029.2, AF440570.1 and NC_003225.1). Molecular cloning can induce variation in WSSV RU number (Dieu *et al.*, 2010), but the frequency of variants is low (< 10% of clones) and these variants only have a decreased number of RUs.

RESULTS AND DISCUSSIONS

Both WSSV samples analyzed by cloning and sequencing of ORF75 PCR products gave evidence of patent mixed-genotype infections. Individual *E. coli* clones contained WSSV variants with a different number of repeat units (Fig. 1), which corresponded to the amplicon lengths observed in the initial PCR of the shrimp samples. Two distinct genotypes were present in sample A (Fig. 1A), and three genotypes were present in sample B (Fig. 1B). Sequencing of selected clones confirmed (Fig. 2) that the variants originating from a single shrimp sample were so different from each other that they are not likely to be artefacts from the experimental procedure. i.e., these variants could not be generated by the addition or deletion of a single repeat unit, or a single recombination event. Identical cloning and sequencing analyses were performed for two WSSV isolates which had rendered multiple PCR bands for ORF94 (four and nine RU variants) and ORF125 (four and seven RU variants), resulting in sequences of both variants being found for each isolate (data not shown). These data demonstrate that this approach works for all three WSSV VNTRs and, moreover, confirms that mixed-genotype WSSV infections occur.

To determine the sensitivity of the VNTR PCR assay, PCR products from different RU variants were purified (Gel Band Purification kit, GE Healthcare kit, Buckinghamshire, UK) and mixed in the following ratios of variants: 1:1000, 1:100; 1:10, 1:1, 10:1, 100:1, 1000:1. A total of 50 ng of DNA was then used as the template for standard individual PCR-genotyping (ORF75, ORF94, ORF125). The detection threshold ranged from 1:10 (ORF75, ORF94) to < 1:1000 (ORF125). The smaller RU variant was more readily detected for all three VNTRs, indicating that the sensitivity of the assay depends on the exact combination of RU variants. When a large number of field samples were analyzed, more mixed-genotype infections were detected for ORF125 than for ORF75 or ORF94 (see Table 1). The rate of failure for PCR amplification of the VNTR is also lower for ORF125 (15/573) than for ORF75 (17/573) and ORF94 (33/573). Although PCR for all three VNTRs can be used to detect mixed-genotype infections, the ORF125 PCR has the highest sensitivity, also for mixed-genotype infections. ORF125 is therefore probably best suited for investigating mixed-genotype WSSV infections and for epidemiological studies tracking virus populations over space and time at small scales.

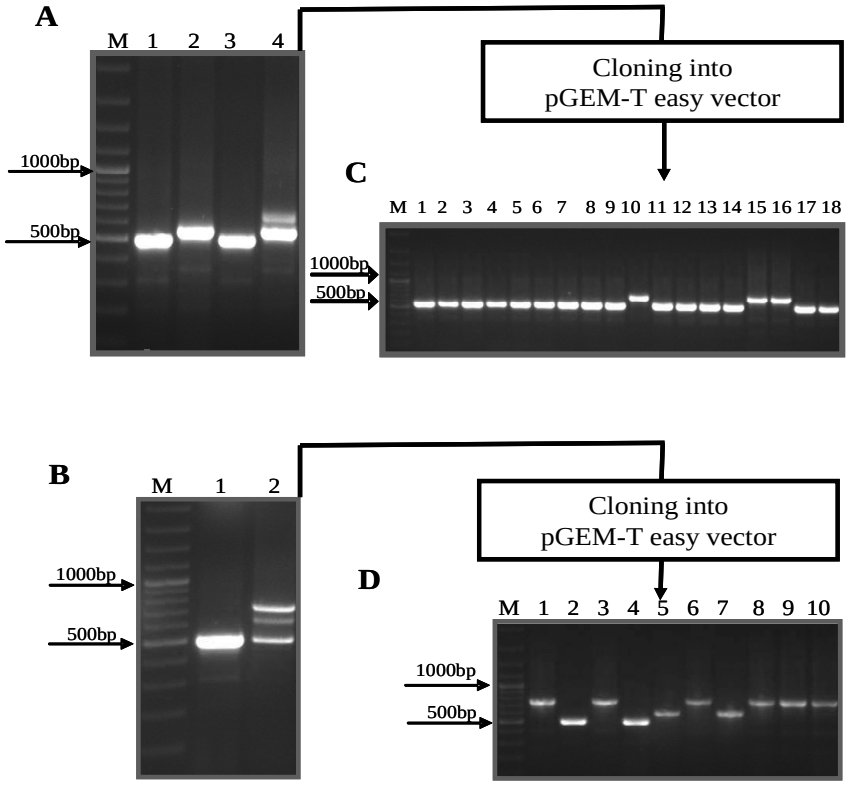

Figure 1. Electrophoresis of PCR-genotyping (ORF75). Two ORF75 PCR products were ligated into the pGEM-T easy vector (Promega) and transformed into competent *Escherichia coli* DH5α by electroporation. White colonies were selected for plasmid isolation and colony PCR with ORF75 primer. (A) PCR products (PCR genotyping of repeat sequences at ORF75) of DNA shrimp samples (lane 4) containing two WSSV genotypes at approximately 500bp and 650bp. (B) PCR products (PCR genotyping of repeat sequences at ORF75) of DNA shrimp samples (lane 2) containing three WSSV genotypes at approximately 500bp, 600bp and 700bp. (C) PCR products (PCR genotyping of repeat sequences at ORF75) of 18 colonies from cloning of PCR product contained 500bp and 650bp inserts in PGEM-T easy vector that showed an individual band at approximately 500bp (lanes 1-9, 11, 12-14, 17 and 18) and 650bp (lanes 10, 15 and 16). (D) PCR products (PCR genotyping of repeat sequences at ORF75) of 10 colonies from cloning of PCR product contained 500bp, 600bp and 700bp in PGEMT easy vector that showed an individual band at approximately 500bp (lanes 2 and 4), 600bp (lanes 5 and 7) and 700bp (lanes 1, 3, 6 and 8-10).

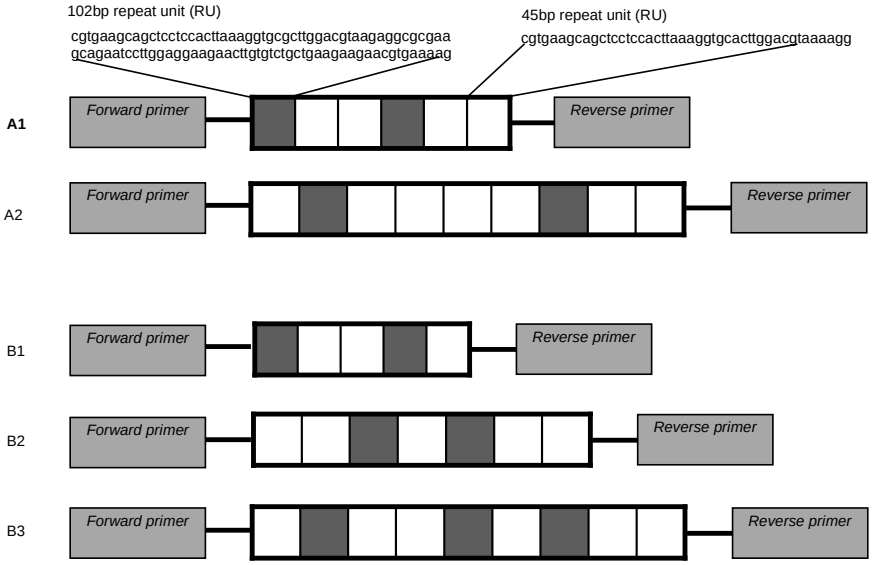

Figure 2. Schematic representation of differences in the number of compound RUs among clones A1 and A2 (derived from PCR product of sample A) and clones B1, B2 and B3 (derived from PCR product of sample B) in ORF75. Plasmid clones of sample A and B, containing inserts of the correct size, were subjected to sequencing using universal T7 and/or Sp6 primers.

The three WSSV VNTR loci were then analysed for a large number of virus isolates (573) collected in the Mekong Delta from 2006 to 2009 (Table 1). The results show that a number of single shrimp samples contained multiple WSSV genotypes. The number of samples in which mixed-genotype infections were detected was different for each molecular marker, and the highest for ORF125 (18.8%). For ORF125, we found three cases of infections with 4 WSSV genotypes detected in a single shrimp sample. WSSV mixed-genotype infections therefore not only occur in the field, they are even common at an overall prevalence of 25.7% (this is the prevalence of mixed-genotype infections for ORF75, ORF94 or ORF125, a mixed-genotype prevalence higher than that of any one VNTR locus). As the sensitivity of our assay varies depending on the VNTR locus and RU variants present, the actual frequency of mixed-genotype infections may be higher than our estimates. Others have reported evidence for the occurrence of mixed-genotype infections in the field (Hoa *et al.*, 2005; Pradeep *et al.*, 2008b), and mixed-genotype infections have been studied in laboratory settings (Marks *et al.*, 2005; Pradeep *et al.*, 2009). The work presented here provides the first confirmation – by cloning and sequence analysis – that mixed-genotype WSSV infections occur in the field.

Table 2. Number of shrimp with mixed-genotype WSSV infections, based on disease status of sampled ponds. The total number of shrimp sampled is given in parentheses, and the percentage of mixed infections is also given (% mixed).

Disease status	Locus							
	ORF75		ORF94		ORF125		All	
	Mixed (Total)	% mixed	Mixed (Total)	% mixed	Mixed (Total)	% mixed	Mixed (Total)	% mixed
non-outbreak	14 (321)	4.4	55 (321)	17.1	86 (321)	26.8	120 (321)	37.4
100% disease outbreak	2 (252)	0.8	6 (252)	2.4	22 (252)	8.7	27 (252)	10.7
Total	16 (573)	2.8	61 (573)	10.6	108 (573)	18.8	147 (573)	25.7

The disease status of ponds - either 'outbreak' or 'non-outbreak' - was determined during sample collection. Determination of a pond's disease status was done by means of examining shrimp from the (emergency) harvest, and by information from farmers. An outbreak pond is a pond in which virtually all shrimp are moribund or dead. In a non-outbreak pond, healthy shrimp are present and a high percentage of moribund or dead shrimp (> 50%) is not observed. Disease outbreaks are the result of interactions between the pathogen, the host and the environment. We found that individual shrimp in non-outbreak ponds were more likely to contain mixed-genotype WSSV infections. The frequency of mixed-genotype infections ranged from 0.8% (ORF75) to 8.7% (ORF125) in outbreak ponds, and from 4.4% (ORF75) to 26.8% (ORF125) in non-outbreak ponds (Table 2). There were significantly more mixed-genotype infections in non-outbreak ponds than in outbreak ponds ($\chi^2 = 51.254$, $P < 0.001$), as determined with a test of equal proportions (R2.7.0; The R Foundation, Vienna, Austria). The WSSV variants typically detected in outbreak ponds (i.e., ORF94: five, six, seven, eight and nine RUs; ORF125: four, five, six and seven RUs) were also regularly found in non-outbreak shrimp ponds. The genotypes found in single-genotype infections in outbreak ponds were also regularly found in mixed-genotype infections in non-outbreak ponds.

There was much variation in the frequency at which mixed-genotype infection was detected for each locus (Table 2). However, if one molecular marker indicated a mixed infection, then would the other two markers be more likely to indicate a mixed infection also?, i.e. are probabilities for detecting mixed-genotype infections independent for each marker? To test whether this was the case, we considered the observed rate of mixed infection at all three loci, $P_{obs}(A \cap B \cap C)$, and compared it to the predicted rate of mixed infection at all three loci under the assumption that the outcome of PCR genotyping (mixed-genotype infection or single-genotype infection) is independent: $P(A \cap B \cap C) = P(A) \cdot P(B) \cdot P(C)$. $P(A)$, $P(B)$ and $P(C)$ are the probabilities that a mixed infection is found for ORF75, ORF94 and ORF125, respectively, which we assume is the observed frequency. The observed rate of occurrence ($P_{obs}(A \cap B \cap C) = 0.256$) was significantly higher than the predicted

rate ($P(A \cap B \cap C) = 0.028 \times 0.106 \times 0.188 = 0.0008$), when compared with an exact binomial test ($P < 0.001$; R2.7.0) indicating that there is dependence between test outcomes and therefore supporting the idea that the mixed-infection results for the different markers overlap. However, there is no correlation among WSSV strains detected in a single sample (e.g., a sample containing ORF94-4 and ORF94-6 was detected in some cases, but a combination of ORF94-4 and ORF94-9 could be also isolated in other cases).

Experimental work has demonstrated that mixed-genotype virus populations are in many instances more virulent (i.e., causing lethal infection) than single-genotype populations (Hodgson *et al.*, 2004; Símon *et al.*, 2006; Vignuzzi *et al*., 2006). Even defective viruses, which often reduce the virulence of the virus population (e.g. Muñoz *et al.*, 1998; Zwart *et al*., 2008), in some particular instances increase the virulence of the population when co-infecting with a helper virus (Lopez-Ferber *et al.*, 2003; Lauzon *et al.*, 2005). Here, however, we have identified a case were genetic heterogeneity is inversely correlated with disease outbreaks. However, the underlying causal relationship between mixed-genotype infection and disease outbreaks still needs to be unravelled.

There are three such causal relationships between mixed-genotype infections and pond disease statuses possible. First, WSSV genotypic composition could determine outbreak status. Under this explanation, interference between different genotypes within the host (Chao *et al.*, 2000) may explain why the virus exhibits less virulence and there are fewer outbreaks for mixed-genotype infections. Host immune responses (Venegas *et al.*, 2000; Wu *et al.*, 2002) may be the mechanism mediating this interference. The fact that the same VNTR variants were found in both outbreak and non-outbreak ponds may contradict this explanation. On the other hand, linkage between the VNTR variants and virulence loci – if these exist in the WSSV genome – may be low, as VNTR sequences are highly variable (e.g., Pradeep *et al.*, 2008b; Dieu *et al.*, 2010). Possible virulence loci include the ORF14/15 and ORF23/24 variable regions, where deletion size may be inversely related to virulence (Marks *et al.*, 2005; Zwart *et al.*, 2010b). Second, outbreak status could determine WSSV genotypic composition. For example, there may be strong selection for certain WSSV genotypes during an outbreak, and this selection leads to the displacement of other genotypes from the virus population. Third, another factor determines both WSSV genotypic composition and outbreak status. Such a factor could be water quality, which could in principle lower the immunocompetence of shrimp, while reducing the probability of water-borne WSSV transmission and therefore the occurrence of mixed-genotype infections. Another factor that could influence both WSSV genotypic composition and outbreak status is the prevalence of other shrimp pathogens, such as species of *Vibrio* bacteria (Phuoc *et al*., 2008).

To better understand how mixed-genotype infections and outbreaks are causally related will require both epidemiological field work and experimental approaches. Longitudinal epidemiological studies could estimate at what time point – prior to an outbreak – mixed-genotype infections at the individual-shrimp level are lost, and what factors are associated with this loss (e.g., water quality, the presence of other pathogens). Experimental work could address what the biological properties of mixed-genotype WSSV infections are, and whether there is interference between WSSV genotypes within the host. Mixed-genotype WSSV infections have been studied in the laboratory (Marks *et al.*, 2005; Pradeep *et al.*, 2009). Quantitative analyses of the biological properties of mixed-genotype WSSV inoculums have not been reported (e.g., Símon *et al.*, 2006; Zwart *et al.*, 2010b), and such experiments could help if there is a relationship between mixed-genotypes and virulence.

This study shows the presence of multiple WSSV genotypes in single-infected shrimp, and provides evidence that mixed-genotype infections are correlated with low virulence, whereas single genotypes dominate in outbreak ponds. It is not clear how the transition from non-outbreak to outbreak ponds occurs and which environmental and viral factors influence this transition. Nevertheless, this observation provides an extra criterion to help establish the health status of a pond at an early stage and to take appropriate measures prior to an anticipated outbreak, such as boosting the defence of shrimp by using probiotics or using vaccines (Witteveldt *et al.*, 2004).

6
- CHAPTER -

Transmission of white spot syndrome virus in improved-extensive and semi-intensive shrimp production systems: A molecular epidemiology study

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ABSTRACT

Experimental evidence suggests that white spot syndrome virus (WSSV) can be transmitted horizontally through water, via carrier organisms and/or by cannibalism of infected shrimp, but also vertically through infected broodstock. However the mode(s) of WSSV transmission in shrimp farming systems and the epidemiological consequences are not well understood. In this study, molecular markers have been used to analyse the spread of WSSV within and between farms in Vietnam using two different farming systems: improved-extensive and semi-intensive. Samples were periodically obtained from twenty improved-extensive shrimp ponds and twenty-three semi-intensive shrimp ponds applying different sampling schemes. PCR-genotyping was employed using three WSSV molecular marker loci: the variable tandem repeat regions (VNTR) in ORF75, ORF94 and ORF125 (GenBank AF369029). An analysis on the combined results of all three VNTR marker loci made it possible to follow transmission pathways of WSSV in the respective shrimp farming systems. The transmission of WSSV infection on improved-extensive shrimp farms appeared mainly due to the recycling of WSSV over time in the same pond, whereas in semi-intensive shrimp farms transmission of WSSV was mainly from neighbouring ponds. These findings provide important information for field applications by suggesting pathway-specific control strategies.

INTRODUCTION

White spot syndrome virus (WSSV) is the most serious shrimp pathogen in shrimp farming regions worldwide, including Vietnam. In 1995, the disease caused by WSSV was detected in the Mekong Delta in Vietnam (de Graaf and Xuan, 1998). The disease severely impacted the production of cultured black tiger shrimp, and this situation has not change much since (Walker and Mohan, 2009). The estimated worldwide economic loss since 1993 has been estimated to be 10 billion USD (Stentiford *et al*., 2009). Since its first identification in northern Taiwan and southern China in 1992/1993, the virus has spread rapidly to all other key shrimp farming regions in the world (Nakano *et al.*, 1994; Flegel, 1997; Lightner *et al.*, 1997; Leu *et al*., 2008). An explanation for the quick spread of this devastating pathogen is found in its ability to infect a wide range of hosts encompassing at least 98 species, in its high virulence, the absence of major resistance in shrimp, the high volume of international shrimp trade and in its transmission characteristics (Escobedo-Bonilla *et al.*, 2008).

WSSV can be transmitted to healthy, susceptible shrimp, within or between shrimp farms, by (i) dead or moribund shrimp, (ii) via the water route (Chou et al., 1998) or (iii) from infected shrimp brooders or post larvae (Lo et al., 1997; Lo and Kou, 1998). A positive correlation between batches of WSSV infected post larvae on a commercial intensive farm and the subsequent infection of a neighbouring pond or nearby farm was reported (Withyachumnarnkul, 1999). Intermediate hosts, such as mud crabs (Kanchanaphum *et al*., 1998) and seabirds (Vanpatten *et al.,* 2004), have also been implied in the transmission of WSSV to shrimp ponds. Furthermore, krill *Acetes* sp. (Supamattaya *et al.*, 1998), Ephydridae insect larvae (Lo *et al.*, 1996), polychaete worms (Vijayan *et al.,* 2005), rotifers and their resting eggs (Yan *et al.*, 2004), marine molluscs (OIE, 2009) were suggested as possible sources for WSSV in shrimp ponds and surrounding environments. Finally, the use of crustaceans as feed (*Artemia*) was implied in the transmission of the disease (Sahul Hameed *et al*., 2002; Li *et al*., 2004; Zhang *et al*., 2010).

It has been previously shown that the transmission routes and the geographic spread of WSSV could be followed by the use of molecular markers (Dieu *et al*., 2004; Hoa *et al*., 2005; Pradeep *et al.*, 2008a). Despite the wide-spread occurrence and range of different hosts of WSSV, the approximately 300 kilo base pair genome has been shown to have little variation between isolates, with about 99% nucleotide sequence identity (Marks *et al.,* 2004). The major differences between isolates consist of multiple sequence repeats, three 'variable number tandem repeat' (VNTR) loci as part of ORF75, ORF94 and ORF125, and two insertion/deletion (Indel) regions of variable size: the region at the ORF14/15 junction and the region at the ORF23/24 junction (GenBank AF369029, Marks *et al.*, 2004; van Hulten *et al.*, 2001a). These variable regions have been evaluated and used in practice as molecular markers for identifying WSSV variants on different

spatiotemporal scales. The ORF14/15 and ORF23/24 variable regions have been used in tracking the migration of WSSV globally (Dieu *et al.*, 2004; Pradeep *et al.*, 2008a; Zwart *et al.,* 2010a) and within Vietnam (Dieu *et al.,* 2010), whereas VNTRs were suggested to be useful markers at smaller geographical scales (Hoa *et al*., 2005; Pradeep *et al.,* 2008b). Recently, Dieu *et al*. (2010) tested the suitability of these molecular markers to determine the spread of WSSV at a regional scale (spread within and between countries). The genomic deletion located in the ORF23/24 indel region was the only suitable genetic marker for determining the movement of WSSV at this intermediate spatiotemporal scale. VNTRs were suggested to be useful genetic markers for studying WSSV spread on much smaller small spatiotemporal scales (i.e. spread between ponds and farms or spread between clusters of shrimp farms and villages) because of the high levels of variation observed among WSSV isolates at these VNTR loci (Dieu *et al.,* 2010).

The development of molecular techniques for identification of viruses and discrimination of viral genotypes makes it possible to tackle the important, complementary task of quantifying transmission through different routes. Mathematical models have been found to be useful in the latter aspect (Lotz *et al*., 2001). Such mathematical models could be useful to the shrimp aquaculture industry by helping to rigorously test hypotheses, which may contribute to disease management strategies (Lotz and Soto, 2002). For example, the transmission rate of WSSV to *Litopenaeus vannamei* from different parts of infected shrimp (cephalothorax, abdomen, or whole shrimp cadaver) could be quantified based on a mathematical epidemiology model and bioassays (Soto *et al*., 2001). With the mathematical epidemiology model, Soto and Lotz (2001) showed that WSSV was more easily transmitted through cannibalism of infected shrimp cadaver than through cohabitation with infected hosts, and that *L. setiferus* was much more susceptible than *L. vannamei* to WSSV infection.

Epidemiological studies identified different risk factors which contributed to WSSV outbreaks in ponds, e.g. pond sites, non-optimal environmental factors as rainfall, drop in atmospheric temperature, rapid decrease in water temperature and salinity (Corsin *et al.,* 2001; 2005; Tendencia *et al.*, 2009; 2010). Bioassay and field investigations were also carried out and provided information on risk factors for WSSV entry to the shrimp ponds, e.g. stocking WSSV-positive post-larvae (Chanratchakool and Limsuwan, 1998; Withyachumnarnkul, 1999), and WSSV presence in crabs, wild shrimp or live feed (Lo *et al*., 1996; Kanchanaphum *et al.,* 1998; Maeda *et al.,* 1998; Yan *et al.,* 2004; Zhang *et al.*, 2010). However, how the virus is transmitted in real-life settings and the key parameters that determine the WSSV transmission rate in the farming system have not been studied well. This could now be approached by a combination of the use of suitable molecular markers for different viral genotypes and mathematical models to describe the transmission process.

This study now aims to apply molecular markers and mathematical models to estimate and explain WSSV transmission routes in two different farm settings: an improved-extensive shrimp farming system in Ca Mau Province and a semi-intensive shrimp farming system in Bac Lieu Province, both in the Mekong delta of Vietnam. This article more specifically addresses the following questions: (i) which transmission routes are the most important in each type of shrimp farming system? and (ii) which WSSV VNTR , or combination of VNTRs, is the most suitable molecular marker for WSSV epidemiological studies within and between ponds and farms?

MATERIALS AND METHODS

Study areas and pond descriptions

The study was carried out in two representative shrimp farming systems (improved-extensive and semi-intensive) in the Mekong delta, Vietnam (Figure 1a). The differences between these two systems are pond preparation, shrimp stocking density, feeding regimens and harvesting methods.

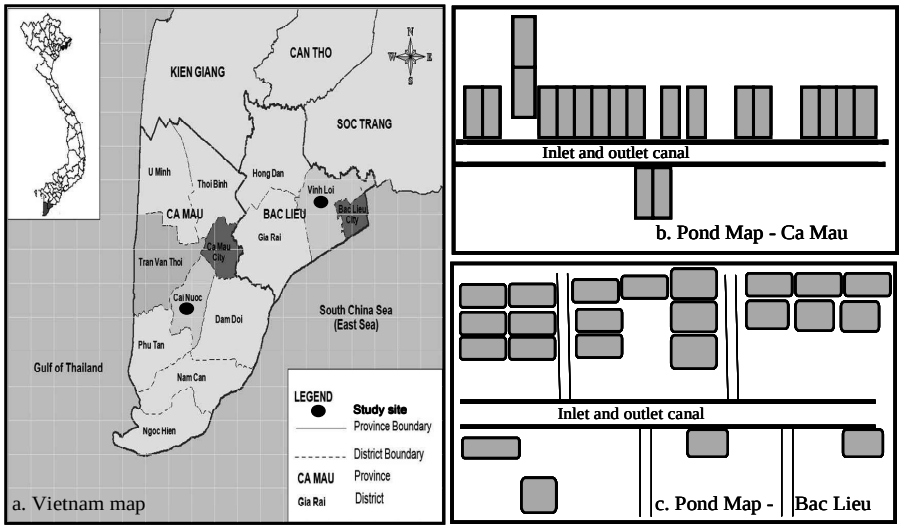

Figure 1. (a) Location of the study area in Bac Lieu and Ca Mau provinces, Vietnam; (b) map of the 20 improved-extensive shrimp ponds located in the study area, Ca Mau province; and (c) map of the 23 semi-intensive ponds located in the study area, Bac Lieu province.

The first experiment (Exp. 1) was conducted in an improved-extensive system in Ca Mau province (Cai Nuoc district) from 2008 to 2009. The twenty ponds used in the study were *P. monodon* ponds with pond sizes ranging from 0.9 to 1.4 ha (Fig. 1b). The ponds were stocked with un-screened post larvae (PL) 12-20 at a density of 0.5–2 shrimp/m^2/two months. PL batches were purchased from local hatcheries

and checked for health status by gross examination. Both stocking and harvesting took place continuously over the year, with partial harvesting being done at a spring tide by letting out part of the water at the low tide and refilling the pond at high tide. The shrimp were never fed with commercial pellets.

The second experiment (Exp. 2) was conducted in a semi-intensive shrimp system in Bac Lieu province (Vinh Loi district) from 2009 to 2010. The twenty-three ponds used in the study were stocked with *P. monodon* with pond sizes ranging from 0.22 to 0.68 ha in Vinh Loi district (Figure 1c). WSSV PCR negative post larvae (PL) 12–20 were stocked in these ponds at an average density of 12–20 shrimp/m^2. The PL quality assessment was done with different techniques i.e. gross examination, PCR screening and *Vibrio* testing. Stocking took place each at the beginning of a production period and all shrimp are harvested at the end of that period (all-in-all-out). The shrimp were fed with commercial pellets from stocking until harvest.

Study designs and shrimp sampling

Experiment 1. A longitudinal study was conducted in Ca Mau province, a representative area for improved-extensive farming systems, involving 20 shrimp ponds in Cai Nuoc district. A total of 276 whole shrimp samples were collected by sampling every two months (from 2-10 shrimp/pond) based on tidal schedule for the continuous crop (September 2008 to January 2009). Twenty-six wild crab samples were also collected from inside or outside of the sampled ponds. In addition, in case of disease outbreaks, moribund shrimp were sampled.

Experiment 2. In the semi-intensive system, sampling schedules were designed to collect shrimp in the sampling ponds from two contiguous production periods in Bac Lieu province from April 2009 to March 2010. The production cycle will be referred to as crop in this paper. In the first crop, twenty three of shrimp ponds (10 shrimp/pond) were sampled at the time of WSSV outbreak. In the subsequent crop, a longitudinal study was carried out in these twenty three ponds at particular times: (i) PLs were sampled for WSSV detection at each stocking event. (ii) Ten shrimp were collected for WSSV detection at the 30th, 60th and 90th days after stocking. (iii) The same number of shrimp were sampled at the end of a crop at harvest time. Moribund shrimp and additional wild crab samples were also taken in the event of a disease outbreak.

All samples were stored either in liquid nitrogen or absolute ethanol (Merck, Darmstadt, Germany) and preserved at –80^{o}C until further analysis.

WSSV detection

Viral DNA was isolated from gills of individual shrimp/crab using a CTAB-DTAB kit (GeneReach Biotechnology Corp., Taipei, Taiwan) and screened for WSSV infection by using a nested PCR with the IQ2000™ WSSV Detection and Prevention system from GeneReach Biotechnology Corp., Taipei, Taiwan. In total, 244 WSSV samples

were isolated from one longitudinal study in twenty improved-extensive ponds (Exp. 1) and another 327 WSSV samples collected from the longitudinal study of two crops in twenty three semi-intensive ponds (Exp. 2).

WSSV genotyping

Genotyping of WSSV-positive DNA from shrimp samples was done using a PCR-based method (Wongteerasupaya *et al*., 2003; Dieu *et al.*, 2004; Hoa *et al.,* 2011a). In detail, a PCR with specific primers and conditions (Table 1) was employed to amplify the VNTRs loci ORF94 (54 bp repeat unit - RU), ORF125 (69 bp RU), and ORF75 (compound RUs of 45bp and 102bp) (Genbank AF369029) (Figure 2). All PCR reactions were performed in an Applied Biosystems (Foster City, United State) thermal cycler using GoTaq Flexi DNA Polymerase (Promega; Madison, United State) following different annealing temperatures and elongation times for the amplification of different tandem repeat sequences (Table 1). The PCR products were stored at 4^{o}C until analysis by electrophoresis in 1% agarose gels. The size of the PCR product was estimated based on 1kb Plus Ladder as DNA markers (Invitrogen, Carlsbad, CA) and the number of repeats calculated.

Table 1. Primer sequences and conditions for polymerase chain reaction amplification of three tandem repeat regions of WSSV genome

Site	Primer name	Sequence (5'- 3')	Annealing temperature /Elongation time
ORF75	ORF75-flank-F	GAAGCAGTATCTCTAACAC	49^{0}C/80s
	ORF75-flank-R	CAACAGGTGCGTAAAAGAAG	
ORF94	ORF94 - F	TCTACTCGAGGAGGTGACGAC	52^{0}C/60s
	ORF94 - R	AGCAGGTGTGTACACATTTCATG	
ORF125	Geno125 - F	ACAGTGACCACACGATAATACCA	60^{0}C/60s
	Geno125 - R	TCGTTCACCATATCCATTGCCCT	

Statistical analysis

From the PCR-genotyping results, possible connections were established: these connections where finding the same genotype in earlier samples, for example those of the same pond, or of neighbouring ponds etc. Each such connection was then used in the statistical model as 0/1 variable to explain the infection (1) or absence of infection (0) in each of the ponds studied.

In the improved-extensive system, the predicted connections were based on four samples: (i) previous sample moment of shrimp in the same pond (fss); (ii) previous sample moment of crabs in same pond (fcs); (iii) previous sample moment of shrimp in a neighbouring pond (fsn) and (iv) previous sample moment of crabs in a neighbouring pond (fcn). In the semi-intensive system, the predicted connections were based on four samples: (i) shrimp sample of the previous crop in the same pond (fsp); (ii) shrimp sample in the same pond at a previous moment of the current crop (fsc); (iii) shrimp sample of the previous crop in a neighbouring

pond (fnp); (iv) shrimp sample in the neighbouring pond at a previous moment of current crop (fnc).

The epidemiological dataset was coded from genotyping information in an Excel sheet. Using a dummy variable, each observation of transmission route in the pond can either be 0 or 1, with 0 meaning no connection possible based on the genotype at measured time and 1 meaning connection is possible based on the presence of the genotype at measured time. A mathematical model was constructed to analyse the transmission of WSSV infection in the pertinent research areas. In this model we utilise the equation below to calculate the probability of susceptible individuals becoming infected.

Firstly, the rate of transmission (λ) was calculated based on the equation using the explanatory variables. In case of improved-extensive systems:

$$\lambda = C_1.fss + C_2.fcs + C_3.fsn + C_4.fcn$$

where C_1 through C_4 are constants that scale the effect of each transmission route to the overall rate of transmission in the improved-extensive system. In case of semi-intensive systems:

$$\lambda = C_5.fsp + C_6.fsn + C_7.fnp + C_8.fnc$$

where C_5 through C_8 are constants that scale the effect of each transmission route to the overall rate of transmission in the semi-intensive system.

Finally, the probability of infection of susceptible ponds (p) is:

$$p = 1 - Exp [- \lambda * \triangle t]$$

where Δt is measured time period (in months)

The 0/1 observed value for each pond follows a Bernoulli distribution with the probability of infection as given above. Thus the statistical analysis can be done by using generalized linear models (GLM) with a Bernoulli distribution for the dependent variable and a complementary log-log link function. The models accounted for potential transmission among observations in time series (two months interval for improved-extensive systems and monthly interval for semi-intensive systems within a crop). Transmission parameters to the shrimp in the recipient pond for the different connections were estimated and the best model was fitted by removing connections where the estimated coefficient is not statistically different from zero.

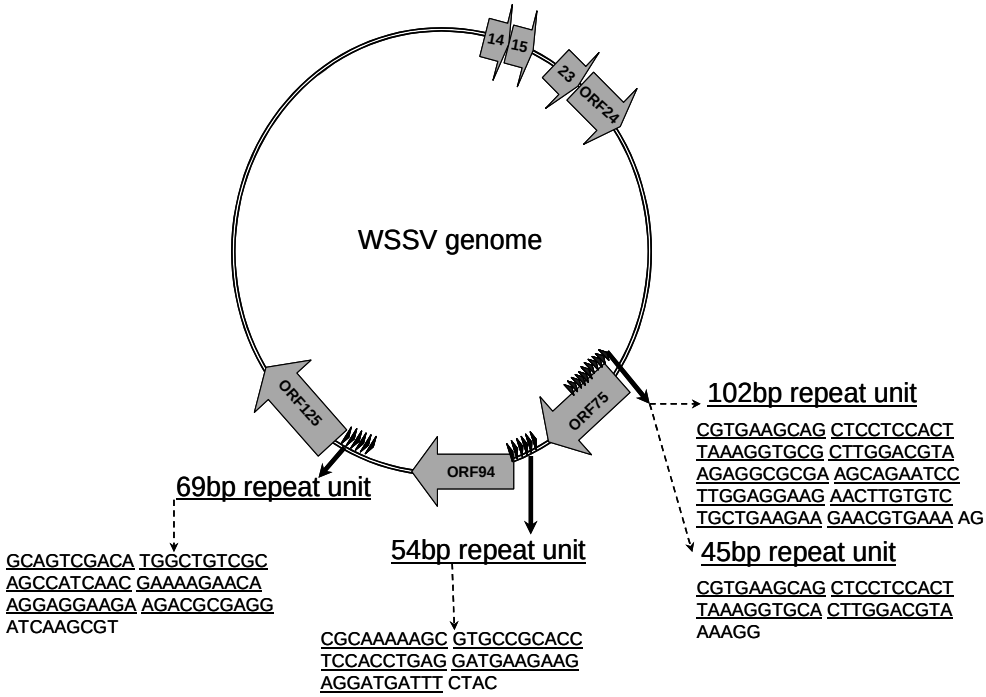

Figure 2. Schematic of three variable tandem repeat regions with repeat sequence used to genotype WSSV samples

RESULTS

Determination of WSSV transmission routes in two shrimp farming system using combined molecular marker model

The WSSV transmission parameters were estimated from twenty improved-extensive shrimp ponds and twenty three semi-intensive shrimp ponds by combining all three molecular marker loci. Fig. 3 and Table 2 show the number of detected connections leading to infection of the recipient pond for the two shrimp farming systems. Detected cases are recorded in case the genotype of infected shrimp matches that of the infected sources. In the semi-intensive systems, detection results revealed a somewhat similar number of detected cases in ORF75 (90 cases), ORF94 (89 cases) and ORF125 (91 cases) in the single molecular marker model compared to the combined molecular marker model (all three loci) with 62 cases. In the improved-extensive systems, large numbers of detected cases were found, with most of the cases detected in ORF125 (101 cases), ORF75 (82 cases), followed by ORF94 (62 cases). In contrast, using the combined molecular marker we observe only 11 cases.

Table 2. Rate of WSSV transmission in different shrimp farming system measured by combined molecular markers

Source		Detected cases*	Total investigation	Rate (95%CI)
Improved-extensive system		11	302	
Previous sample moment of shrimp in same pond	fss	6	52	0.25 (0.11-0.55)
Previous sample moment of shrimp in a neighbouring pond	fsn	4	208	0.04 (0.01-0.10)
Previous sample moment of crabs in same pond	fcs	1	16	0.13 (0.02-0.92)
Previous sample moment of crabs in a neighbouring pond	fcn	0	26	
Semi-intensive system		62	158	
Shrimp sample of previous crop in same pond	fsp	7	23	
Shrimp sample of previous crop in neighbouring pond	fnp	23	38	6.92 (1.51-31.8)
Shrimp sample in same pond at previous moment of current crop	fsc	3	52	0.00019 (0.00-0.01)
Shrimp sample in neighbouring pond at previous moment of current crop	fnc	29	45	27.89 (7.17-108.45)

Model has significant P-value ($P < 0.05$)

* Cases detected with genotype of infected shrimp in correlation to infected source

In these particular systems, genotype data from four molecular marker models (single model for ORF75, ORF94, ORF125, and a combined model using data from all three VNTRs) gave the same direction for the four predicted transmission routes. In the semi-intensive systems, most of the detected cases showed transmission with the largest number from neighbour pond within the current crop (fnc), followed by transmission from neighbour pond in the previous crop (fnp), transmission in same pond from the previous crop (fsp), and transmission from the same pond within the current crop (fsc) (Fig. 3).

In the improved-extensive shrimp farming system, the same predicted transmission routes were also recorded in single genotype models, but the outcome differed from analysis based on combined molecular markers. In detail, all single molecular marker analyses show that most of the detected cases showed transmission, with the largest number from shrimp of neighbour ponds (fsn), followed by transmission from shrimp in same pond (fss), transmission from crab of neighbour pond (fcn), and transmission from crab in same pond (fcs). However,

transmission route modelling based on combined markers give as the final estimation a model including fss, fsn, fcs, but not fcn (Table 3).

In general, the genotyping data of single molecular marker (ORF94 or ORF125) and combined molecular marker (ORF75, ORF94 and ORF125) showed that modelling with these two sets of markers could both detect virus transmission cases within and between shrimp ponds. However, the combined molecular marker model might give a better resolution of real transmission routes due to the combination of all three genotype patterns. The analysis of WSSV transmission pathways in the two shrimp farming systems is furthermore restricted to genotype patterns of the combined molecular markers. The combined molecular marker model is based on combined genotypic information from the three repeat regions of ORF75, ORF94 and ORF125.

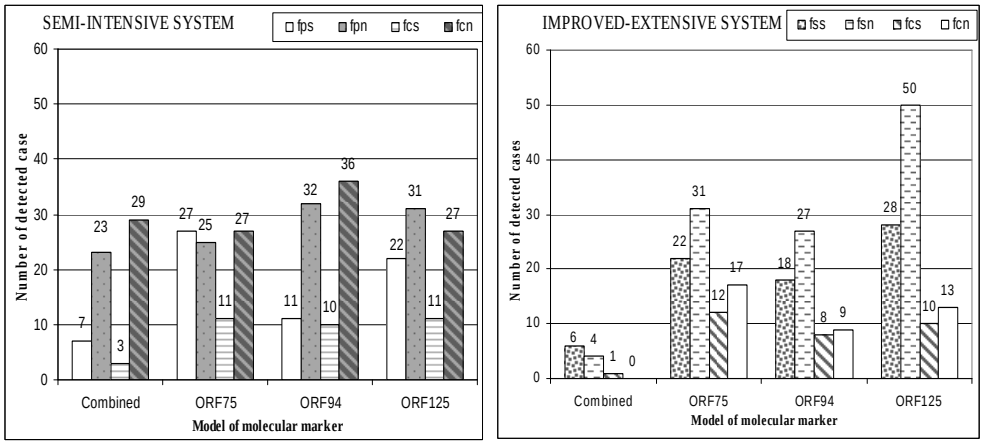

Figure 3. Number of detected cases of transmission in two shrimp farming systems

In the improved-extensive shrimp farming system, there were three detected routes with statistically significant transmission parameters using the combined genotype markers. In those cases, the rate of transmission of shrimp in the same pond (0.25 two-month^{-1}) is 6 times higher than that of transmission from of neighbouring ponds (0.04 two-month^{-1}). In addition, transmission from crab in the same pond is also found to be significant with value of 0.13 two-month^{-1}. In the improved extensive system, transmission was mostly from shrimp/crabs within ponds (Table 3).

In the semi-intensive shrimp farming system, the case of combined genotypes has been analysed (Table 4). For the best model, three constants have been found to give significant estimates: (1) the transmission of WSSV from neighbouring ponds at current crop had a rate of 27.89 month^{-1}; (2) the transmission of WSSV from neighbour pond in previous crop had the rate of 6.92 month^{-1}; and (3) the transmission rate of WSSV in the same pond at current crop was at 0.19 × 10^{-3}

month^{-1}. The combined genotyping data show that transmission from neighbouring ponds (from current crop or from previous crop) had the highest transmission rates and this suggested that infected neighbouring ponds are the main cause of WSSV transmission in the semi-intensive shrimp farming system.

Figure 4. Example agarose gel showing PCR amplification products of ORF125.
M: 1kb plus marker, 1 and 2: WSSV genotype ORF125-5 isolated from shrimp and 3: WSSV genotype ORF125-5 isolated from crab in same pond; 4 and 5: WSSV genotype ORF125-4 isolated from shrimp and 6: WSSV genotype ORF125-5 isolated from crab in same pond

Table 3. Rate of WSSV infection in improved-extensive system measured by molecular markers

Molecular markers	ORF75	ORF94	ORF125	Combined
Previous sample moment of shrimp in same pond	0.46* (0.18 -1.15)	0.43* (0.24 - 0.79)		0.25* (0.11-0.55)
Previous sample moment of shrimp in a neighbouring pond	0.37* (0.18 -0.74)	0.20* (0.12 - 0.33)	0.52* (0.35 - 0.78)	0.04* (0.01-0.10)
Previous sample moment of crabs in same pond				0.13* (0.02-0.92)
Previous sample moment of crabs in a neighbouring pond		0.25* (0.10 - 0.59)	0.21* (0.07-0.61)	

* Model has significant P-value ($p < 0.05$)

Table 4. Rate of WSSV infection in semi-intensive system measured by molecular markers

Molecular markers	ORF75	ORF94	ORF125	Combined
Shrimp sample of previous crop in same pond		0.10×10^{-2}* (0.10×10^{-3} – 0.01)	0.04* (0.01–0.24)	
Shrimp sample of previous crop in neighbouring pond		10.24* (2.49–42.06)		6.92* (1.51–31.8)
Shrimp sample in same pond at previous moment of current crop		0.04* (0.42×10^{-2} – 0.34)		0.19×10^{-3}* (0.35×10^{-8} – 0.01)
Shrimp sample in neighbouring pond at previous moment of current crop		22.15* (5.64–87.02)	15.18* (3.03–76.09)	27.89* (7.17 –108.45)

* Model has significant P-value ($P < 0.05$)

Determination of a suitable molecular marker model for WSSV transmission from pond to pond

Data of combined molecular markers are suitable for tracing the spread of WSSV within and between farms in semi-intensive system and the improved-extensive systems. However, it is important to explore the potential use of a single molecular marker in studying the spread of WSSV in small scale pond systems, rather than multiple markers, as this would reduce the labour and costs of epidemiological studies.

In the case of single molecular marker analyses, Table 3 shows estimates of WSSV transmission rates in the improved-extensive shrimp farming systems. From genotyping data of ORF75, two routes have been found to be statistically significant at $P<0.05$. In the first route, the transmission rate of WSSV from shrimp in same pond had a value of 0.46 $month^{-2}$. For shrimp of neighbouring ponds, the transmission rate had values 0.37 $month^{-2}$. For ORF94 and ORF125 genotyping data, there was a similar significant detection of transmission routes in the studied areas. Genotyping data of these two molecular markers all had transmission rates at significant numbers for 2 transmission routes including one from shrimp of neighbouring ponds and one from crabs of neighbouring ponds. However, there is an exception with 'transmission from shrimp in same pond' as this has only been found to be statistically significant in the case of ORF94. Among three individual molecular markers, ORF94 has been shown to quantify the transmission in more

cases than ORF75 and ORF125. Additionally, the outcome of single molecular model of ORF94 is similar to those found in combined molecular model.

The study was also carried out in the semi-intensive shrimp farming systems using single molecular marker models. Different results have been obtained when analysing data for different molecular markers (Table 4). From genotyping data of ORF75, there was no statistical significance of detected transmission rate with P-values ranging from 0.292 to 0.963. On the other hand, four transmission routes have been found to be statistically significant using genotyping data of ORF94. In these cases the transmission rate of WSSV in the same pond from previous crop was at 0.10×10^{-2} $month^{-1}$ while that from neighbour pond in previous crop, from WSSV in same pond at current crop, and from neighbour pond at current crop was at 10.24, 0.04 and 22.15 $month^{-1}$ (the highest rate), respectively. In the model of using the single molecular marker ORF94, transmission rate from neighbour ponds reached very high values for both crops (previous crop 10.24 and current crop 22.15 $month^{-1}$). For genotyping data of ORF125, there were only 2 transmission routes detected with statistical significance; the transmission rate of WSSV in the same pond from previous crop was at 0.04 $month^{-1}$ and the transmission rate of WSSV from neighbouring ponds at current crop was at 15.18 $month^{-1}$.

Therefore, the analysis based on combined molecular marker is the most suitable approach to study WSSV epidemiology in shrimp farming system. However, analysis based on the genotype pattern of ORF94 only could be used as a reasonable alternative model to study the WSSV transmission within both improved-extensive shrimp farming system as well as semi-intensive shrimp farming system

DISCUSSION

Four models incorporating information from the different molecular markers were employed for the quantification of WSSV spread in studied areas and shrimp farming systems. With regard to the number of detected transmission cases, there were more cases found in the semi-intensive systems (62 cases) compared to improved-extensive systems (11 cases) with the combined markers. This number of detected cases was calculated from the actual transmission routes (genotype identified from infected shrimp matched with genotype from the source). These data suggest that there may be more genetic variation in WSSV populations in improved-extensive systems, owing to the continuous production cycle and methods for stocking shrimp. If multiple genotypes are present in a continuous system such as the improved-extensive system, than recombination between genotypes will to lead to fewer chances to score the transmission route when using the combined markers. On the other hand, the high rate of within-pond transmission suggests continuity of the WSSV population in the pond. In semi-intensive systems, on the other hand, discontinuous and less variable WSSV

populations appear to be present. The suggestion that there are high levels of genetic variation within improved-extensive farms is also supported by other research (Hoa *et al.,* 2005). We also observed a high prevalence of mixed-genotype WSSV infections in single shrimp collected from improved-extensive systems (Hoa *et al.,* 2011a). In addition, many other species that are susceptible to WSSV infection (wild shrimp or mud crabs) (Maeda *et al.,* 1998; Kanchanaphum *et al.*, 1998) typically co-exist with shrimp in ponds in the improved-extensive system.

In the improved-extensive system, the estimated transmission rate was highest within the pond for all analyses. In contrast, the transmission from neighbouring ponds (at current crop or from previous crop) was the main route for WSSV transmission in the semi-intensive shrimp farming system. The most likely explanation of the differences in the relative importance of transmission routes in these two experiments is the farming systems used, because farming practice plays an important role in whether disease occurs (Joffre and Bosma, 2009) and whether viruses can persist in ponds (Dieu *et al.*, 2011). Improved-extensive farms have many sources for WSSV entrance, accumulation, and occupation in the system, e.g. stocking of unscreened WSSV PL, untreated inlet water, and the contiguous production cycle (Withyachumnarnkul, 1999; Mohan *et al*., 2008; Joffre and Bosma, 2009).

These same mechanisms, in contrast, are not operational in the semi-intensive farms. In these systems, the higher stocking density of semi-intensive systems can lead to more opportunities for horizontal transmission of the virus through cannibalism and the waterborne route (Wu *et al.*, 2001). An experiment conducted in India revealed that the removal of bottom sludge, the ploughing of the soil while it was still wet and liming had positive correlation with a reduced WSD. Before stocking, water filtration and water disinfection were also found to reduce the risk of WSSV outbreaks (MPEDA/NACA, 2003; Corsin *et al.,* 2005; Mohan *et al.,* 2008).

Farm practice therefore appears to influence the prevalence of WSSV, the occurrence of virulent disease outbreaks, and ultimately the dynamics and evolution of virus populations. Our observations on WSSV transmission between ponds have implications for improved disease prevention. For the semi-intensive system, blocking the transmission between neighbouring ponds appears to be a very logical step. Possible strategies include building fences between ponds to block the passage of crabs and other vectors. However, given that there are multiple routes by which WSSV could be transmitted between neighbouring ponds, it would be useful to identify the specific routes of WSSV transmission between adjacent ponds in semi-intensive systems. Moreover, the relative importance of each route may differ between ponds, aquaculture systems and geographic regions, because it is dependent on factors such as species composition, shrimp density, weather and stress (Tendencia *et al.,* 2009; Mohan *et al.*, 2008).

For the improved-extensive system, the impact of the apparent continuity of WSSV populations and high levels of genetic variability on farming are not clear. High levels of standing genetic variation in the virus population could allow for rapid adaptation when the farm environment is changed. This may make it difficult to implement measures to eradicate the virus. On the other hand, the continuous presence of WSSV in the improved-extensive system may make it more difficult for viruses from other ponds to invade these ponds, because of competitive exclusion. There is variation in virulence between WSSV isolates (Marks *et al.*, 2005; Zwart *et al.,* 2010a), and competitive exclusion may therefore be beneficial, depending on the virus genotypes circulating in the pond and the surroundings.

Due to high costs of analysis and to evaluate the potential for wider application, the single molecular markers were also used for further analysis. Our analysis shows that ORF94 could also be used as alternative to the combined marker model to study WSSV transmission within improved-extensive and semi-intensive shrimp farming systems. Hoa *et al*. (2005) and Pradeep *et al*. (2008b) also concluded that ORF94 is the most suitable molecular marker for general epidemiological studies, because of the high levels of variation typically observed. A total of 13 WSSV genotypes were observed for VNTR ORF94, followed by 11 types of repeat regions of ORF125. The compound repeat region of ORF 75 only displayed 6 different patterns of repeats (Pradeep *et al.,* 2008b). Our results are consistent with this observation: the genotyping data of ORF94 and ORF125 could detect more accurately virus transmission cases compared to other markers within and between shrimp ponds. Therefore, the fact that ORF75 gave the lowest number of transmission cases is very surprising, as with fewer genotypes one would expect more virus samples with the same variant by chance. In this particular case, we speculate that this observation may be due to differences in rates of recombination at the different loci, with the rate of recombination being highest at ORF75; although there are few variants, these variants constantly intermingle, making the tracking of the virus difficult.

CONCLUSION

In conclusion, the pond-to-pond transmission is more likely contributing to local spread of WSSV in the semi-intensive system while transmission within pond is the main source of WSSV spreading in the improved-extensive system. In addition, transmission pathways of WSSV in the shrimp farming system could be quantified by combined molecular marker model. VNTR sequences located in ORF94 are suitable as a stand-alone molecular markers for epidemiological studies at pond or farm scales, in both improved-extensive and semi-intensive shrimp farming systems.

7

- CHAPTER -

GENERAL DISCUSSION

THESIS FINDINGS

Molecular epidemiology techniques are powerful tools for elucidation of the links between virus infections as observed in the field and transmission routes, and can also provide novel and crucial insights into virus evolution over space, time, and within host species (Hoffmann *et al*., 2005). For example molecular markers like VNTRs have been used to demonstrate high levels of genetic variation between and within human cytomegalovirus populations (Davis *et al*., 1999), as well as strain typing in bacteria (Liu *et al*., 2003) and have been proposed as a standard method for typing *Mycobacterium tuberculosis* strains (van Soolingen *et al*., 2007). Genetic variation has also been related to virulence characteristics of pathogens (Perdue *et al*., 1997; Saundes *et al*., 2000). In aquaculture, molecular epidemiology is growing field with many potential applications. Molecular epidemiology is an applied form of molecular biology, using an epidemiological framework, that defines the distribution of pathogens to benefit our understanding of infectious diseases and the control thereof (Snow, 2011).

In terms of global WSSV epidemiological studies, molecular markers have been demonstrated to be a useful tool for WSSV strain differentiation (Wang *et al*., 2000; Chang *et al*., 2001; Wongteerasupaya *et al*., 2003) and for tracing virus origins and spread within and between countries (Dieu *et al*., 2004; Zwart *et al*., 2010a). Although WSSV molecular markers have been extensively studied, variation at these loci on small spatiotemporal scales and their utility as molecular markers for pond and farm level studies is not known.

Given this knowledge gap, the work presented in this thesis aims at obtaining more insight into the correlation of the genetic structure of WSSV strains and populations with disease outbreaks and farming systems. In addition to expanding our understanding of the epidemiology and routes/modes of WSSV transmission, this information may also elucidate the reasons for phenotypic differences between viruses, such as virulence. It may also provide a rationale for new, sustainable strategies in disease management.

A large number of WSSV strains were collected from the three main shrimp farming systems in the Ca Mau and Bac Lieu provinces in the Mekong Delta, Vietnam. PCR-based genotyping and sequencing were performed for five molecular markers in the WSSV genome, and the resulting data were subject to rigorous statistical analysis. The following questions were addressed for the thesis:

(i) To what extent can molecular markers be used to measure the diversity of WSSV genotypes within and between shrimp ponds?

(ii) Are molecular markers suited for WSSV epidemiology studies in relation to WSD outbreaks and shrimp farming systems?

(iii) Which molecular markers can best be used to study WSSV transmission within and between shrimp ponds?

(iv) Which are the most important routes of virus transmission in each type of shrimp farming system?

The thesis has demonstrated and verified the specific utilities of the five molecular markers in WSSV epidemiological studies at the farm-scale level.

Molecular markers to measure heterogeneity of WSSV genotypes from different hosts (broodstock, post-larvae, juvenile shrimp, crab and wild shrimp)

At the beginning of the study, PCR genotyping methods had already been developed and applied successfully to characterize WSSV strains. The methods, based on the VNTR region of ORF75, ORF94, ORF125 and indel regions of ORF14/15 (Indel-I) and ORF23/24 (Indel-II), had been identified as very promising tools for WSSV molecular epidemiological studies (Wongteerasupaya *et al*., 2003; Marks *et al*., 2004; Dieu *et al*., 2004). However, identifying the typing methods that are suitable for distinguishing WSSV genotypes at farm-scale level is still needed, in order to track viruses and determine the main transmission routes of WSSV.

In the current study, the discriminatory power of the VNTR loci and indel regions was evaluated for WSSV populations at the local and farm-scale levels. Based on the PCR-genotyping for the five molecular markers, the study could discriminate a large number of WSSV isolates, which existed both within shrimp ponds and the surrounding areas. It is clear from the findings that 18, 14, and 8 different genotype assemblies were found by the PCR-genotyping of ORF94, ORF125 and ORF75, respectively (Chapter 2 and Chapter 3). Four WSSV genotypes could be differentiated for Indel-II, while only two WSSV genotypes were found for Indel-I (Chapter 4) in the research areas. Additionally, mixed-genotype infections could be detected using all three VNTRs, although the VNTR of ORF125 appears to be best suited for this purpose (Chapter 5). At farm scale level, the VNTRs of ORF94 and ORF125 were found to have high discriminatory power among WSSV strains infecting different shrimp development stages (broodstock, post-larvae, juvenile shrimp) and in other infected hosts (crab and wild shrimp) (Chapter 2 and Chapter 3).

In shrimp ponds, the observed variation and discriminatory power were lower for Indel-I than for Indel-II and the VNTR regions. The Indel-I region is therefore not a useful marker for WSSV epidemiology at the farm-scale level. The obtained results are consistent with recent findings that VNTR regions appear to be highly variable over time compared to indel regions in shrimp farming systems (Dieu *et al*., 2011). Others have also reported detection of the whole range of repeat unit (RU) numbers in WSSV strains originating from a wide host range, including plankton,

wild crustaceans and farmed shrimp from other shrimp farming countries (Wongteerasupaya *et al*., 2003; Pradeep *et al*., 2008b; John *et al*., 2010; Walker *et al*., 2011b). The VNTR regions appeared to be useful markers for identification of WSSV strains in the field, particularly at small spatial scales due to their high variability (Wongteerasupaya *et al*., 2003; Pradeep *et al*., 2008b; Dieu *et al*., 2010).

From the specific PCR setups described (Wongteerasupaya *et al*., 2003; Dieu *et al*., 2004; Marks *et al*., 2005; Dieu *et al*., 2010), the PCR genotyping applied in this thesis provides further information on which primer pairs are appropriate for characterizing genetic differences in WSSV strains in the research areas. We concluded that VNTR typing is a useful tool: it can be used as a primary method for subtyping large numbers of WSSV strains and is extremely useful for epidemiological investigations at farm scale level.

The results provided in this thesis suggest that the VNTR region of ORF94, followed by ORF125, ORF75 and Indel-II region have high discriminatory power, which can be used as a tool to study the typology of WSSV structure population within and between clusters of shrimp farms.

WSSV genotypes and disease outbreaks

The existence of a trade-off between parasite transmission rates and parasite-induced host mortality is often postulated to explain the evolution of virulence (Ebert and Bull, 2003; Alison *et al.*, 2009). However, identifying ways to select against virulent forms of parasites and pathogens directly might be a more rewarding approach to managing the evolution of virulence (Ebert and Bull, 2003). Molecular typing methods can be used to measure and better understand the differences in virulence and transmissibility among pathogens (Comas *et al*., 2009). Molecular typing has been found to have wide application in identification of pathogenic bacteria (Klevytska *et al*., 2001; Peak *et al*., 1996) and their virulence factors (Saunders *et al*., 2000). The use of these methods for epidemiological investigation could aid the prevention of infectious diseases at local and global levels (Comas *et al*., 2009).

For WSD, molecular typing methods are even more important given that differences in pathogenicity exist among WSSV strains (Marks *et al.*, 2005; Zwart *et al.*, 2010a) and given the fact that there is no available treatment for this disease (Sánchez-Paz, 2010). A good understanding of the relationship between WSSV genotypes and the associated disease status of shrimp ponds may lead to novel management of WSSV. An important advance would be to find appropriate molecular markers that would allow for: (i) screening for virulent virus genotypes in shrimp brooders or shrimp seed, which would be cost-effective because shrimp infected with virulent WSSV genotypes could be discarded; and (ii) lead to informed decisions to perform continuous stocking or emergency harvest only

when potentially virulent virus genotypes are detected in shrimp ponds (Flegel *et al*., 2008).

In the thesis, particular WSSV genotypes were found to be positively associated with an increased risk of developing serious disease in culture ponds (Chapter 2, Chapter 3 and Chapter 4). For the WSSV VNTRs, the repeat structure of ORF94 was found to positively correlate with disease outbreaks (Chapter 2 and Chapter 3). The phenomenon of mixed-genotype infections in single shrimp is correlated with low virulence, whereas single genotypes dominate in outbreak ponds (Chapter 5). For the Indel-II region, the presence of small genomic deletions (i.e. a variant with a large Indel-II type) was found to correlate statistically with the occurrence of WSSV-induced disease (Chapter 4). Therefore, this thesis presents strong evidence for a correlation between WSSV genotypes and virulence.

In previous studies, the Indel-II region was reported to correlate with shrimp disease status (Lan *et al*., 2002; Marks *et al*., 2005). Zwart *et al*. (2010a) proposed that the WSSV genome has gradually become smaller over time and space, and that this decrease is positively correlated with enhanced virulence and fitness of the virus in aquaculture conditions. Inoculation of shrimp with a highly virulent WSSV isolate resulted in higher number of WSSV infected cells in different target tissues compared to low WSSV virulent isolate (Rahman *et al*., 2008). For field studies, variation in virulence (i.e., relative pathogenicity, shrimp mortality rate or infection level) has been observed at the local level among shrimp ponds (Sudha *et al*., 1998; Withyachumnarnkul, 1999; Tsai *et al.*, 1999, Walker *et al.*, 2011b) and among infected species (Chou *et al*., 1995; Lo *et al*., 1996; Chang *et al*., 1998). A Texas WSSV isolate was found to be more virulent than other WSSV strains obtained in China, Thailand, South Carolina and American National Park (Wang *et al*., 1999). Recently, shrimp with high levels of WSSV infection were observed in the absence of disease in shrimp ponds in India (Walker *et al*., 2011b).

Differences in virulence were also recorded for many other crustacean viruses. For instance, six genotypes of yellowhead-complex viruses have been detected in Asia. However, yellowhead virus - YHV (Type 1) is the only dangerous genotype (OIE, 2011). Similarly, Taura syndrome virus (TSV) was known to have different variants (Nielsen *et al*., 2005; Tang and Lightner, 2005), and differences in virulence exist among them (Erickson *et al*., 2002; Erickson *et al*., 2005; Tang and Lightner, 2005). In a similar vein, infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus monodon* from various geographical regions appears to differ in virulence for *Penaeus vannamei* (Tang *et al*., 2003). For the fish virus Infectious hematopoietic necrosis virus (IHNV), it was found that two genotypes differed in both virulence and fitness, and that these two properties were related (Wargo *et al*., 2010).

In recent years, various mechanisms for the maintenance of the WSD virulence have been suggested, including shrimp immune response (Flegel, 2007), trade-offs

between virus fitness components (Marks *et al*., 2005), differential selection for WSSV genotypes (Waikhom *et al*., 2006), interactions between pathogens in shrimp ponds (Phuoc *et al*., 2008; Dela Pena *et al*., 2003); trigger of environmental parameters (Tendencia *et al*., 2009), e.g. temperature (Rahman *et al*., 2006), salinity (Liu *et al*., 2006), and ammonia -N (Jiang *et al*., 2004). In theory, (management) factors that enhance pathogen transmission will lead to selection for increased virulence (Ewald, 1983).

Genetic basis for virulence

Genetic composition of WSSV populations may also contribute significantly to the occurrence of virulent WSD outbreaks. The mapped variable regions in the WSSV genome (Marks *et al*., 2004) may also be some of the key loci determining virulence (Marks *et al*., 2005; Waikhom *et al*., 2006; Zwart *et al.*, 2010a).

For the repeat region of ORF94, it is likely that the flanking regions might be involved in the determination of WSSV virulence, rather the repeat region itself. The repeat region of ORF94 is located between the genes for ribonucleotide reductase subunits 1 and 2 (van Hulten *et al*., 2000). Ribonucleotide reductase is an enzyme that catalyzes the formation of deoxyribonucleotide precursors, which are involved in DNA replication process (Elledge *et al*., 1992), and has been implied in the virulence of herpes viruses and pox viruses (Child *et al*., 1990; Lembo and Brune, 2009; Gammon *et al*., 2010). Given that these loci are adjacent we expect strong linkage, and that may explain why the repeat structure of ORF94 is correlated to WSSV virulence. However, this hypothesis needs further experimental support.

A link between WSSV Indel-II and virulence has been suggested previously based on laboratory experiments, although whether virulence decreases (Lan *et al*., 2002) or increases (Marks *et al*., 2005; Zwart *et al*., 2010a) with deletion size is contested. The significant correlation that was found between VNTR-ORF94 or Indel-II deletion size and disease outbreaks provides further evidence that such a link may exist, and that it may be relevant to disease outcomes under farm conditions. However, only a correlation was found, and care should be taken when inferring causal relationships. Nevertheless, our results suggest that larger deletions are associated with higher virulence, in line with the observations of Marks *et al*. (2005) and Zwart *et al*. (2010a). For the VNTR-ORF94, low RU numbers were reported to be associated with higher virulence (Pradeep *et al*., 2008a). A number of different experimental and field studies therefore provide strong support for the idea that smaller WSSV genomes may be more virulent, as originally suggested by Marks *et al*. (2005). However appealing this notion is, it could also be overly simplistic since different mechanisms may be operating for each locus, such as linkage of the molecular marker to immediately up- or downstream loci actually affecting virulence.

If virulence is not dependent on genome size but on the loci flanking the molecular markers, what possible mechanisms could be involved for the sequences neighbouring Indel-II? For all identified WSSV-isolates, the genomic deletion of Indel-II is located between wsv477 and wsv502 (WSSV-CN). The transmembrane protein (wsv479 of WSSV-CN) and putative protein wsv500 are not deleted in WSSV-VN, also in the 2006-2009 time periods (Chapter 4). The transmembrane protein gene wsv479 is located adjacent to the region of WSSV protein wsv477. Wsv477 is a viral early gene with a GTP binding domain (Han *et al*., 2007) and has been shown to interact with the shrimp actin by protein array (Chen, 2008). By interacting with actin, both of the structural proteins may help the viral nucleocapsid to move toward the host nucleus (Chen, 2008). In addition, wsv474 is a putative virus-specific attachment protein (Tsai *et al*., 2004). Mutations in this region might therefore result in greater virulence, which in turn may only correlate only with Indel-II size due to linkage. It is remarkable to note that Indel-II region is located within ORF23 and ORF24 (WSSV-TH), next to the region encoding the viral DNA polymerase, ORF27 (Yang *et al*., 2001; van Hulten *et al*., 2001a). This polymerase is an essential enzyme involved in the DNA replication process and its polymerase activity has been evaluated in preliminary studies (Chen *et al*., 2002; de-la-Re-Vega *et al*., 2011). Given that the Indel-II region is adjacent to the DNA polymerase, we expect strong linkage and this may also account for the link between Indel-II types and virulence. However, further experimental work is required to test this hypothesis.

The WSSV population structure for VNTR-ORF94 and the Indel-II region correlate with disease outbreaks. There have been several attempts to link a particular genotype to more severe WSD. However, conditions favouring WSSV outbreaks would also be an important factor determining disease outbreaks in shrimp ponds. For example, highly virulent strains might persist in the system without a chance for transmission and no disease outbreak occurs, or low-virulence strains are present under favourable conditions for transmission resulting in a disease outbreak. For instance, in the improved extensive systems with unfavourable transmission conditions, highly virulent WSSV strains could die out before transmission has occurred, while under semi-intensive system, low virulent isolates could become more virulent due to having a high transmission rate. The concept of the trade-off model between transmission and virulence of pathogens has been discussed in the paper of Ebert and Bull (2003). In detail, changing opportunities for pathogen transmission will affect virulence (Ebert and Bull, 2003). For mitigating the risk of virulence disease outbreaks, it is necessary to explore both WSSV population structure and shrimp farm management in parallel.

One more factor that may contribute to the virulence of WSD could be the presence of abundant WSSV-like sequences dispersed throughout the shrimp genome, comprising over 20% of the *P. monodon* genome in length (Huang *et al*., 2011). The

results of experiments using dsRNA-mediated RNA interference suggest that some of these WSSV-like sequences may be involved in WSSV pathogenesis (Lua *et al*., 2010). For instance, injection of dsRNAs specific to WSSV-like sequence reduced mortality of experimental kuruma shrimp upon WSSV challenge, when compared to the control treatment without dsRNA injection. Silencing of homologous WssvORFs by specific dsRNAs resulted in high shrimp survival (Lua *et al*., 2010).

WSSV genotypes and shrimp farming practices

Shrimp farming systems and regions appear to influence WSSV population structure as evidenced by the distribution of Indel-II regions of WSSV (Chapter 4). A possible explanation for the differences in WSSV genotypes may be found in the farming practices used. The higher stocking density of semi-intensive systems can lead to more opportunities for horizontal transmission via cannibalism and the waterborne route (Wu *et al*., 2001). In improved-extensive and rice-shrimp systems, the low stocking density offers fewer chances for direct contact among shrimp. Therefore, transmission rates and replication cycles are much lower than those in semi-intensive systems.

Shrimp farming practices, such as the farming system employed and farm management, play an important role in maintaining virulent WSD outbreaks, by selecting for virulent WSSV genotypes. In a shrimp pond, shrimp could be infected by WSSV from many sources and reservoirs (OIE, 2011) within the pond or the surrounding environment, depending on the farming system employed. Farming operations such as stocking (which determines the density of shrimp in the pond), feeding of shrimp with a particular diet (Tendencia *et al*., 2011), pond preparation with or without ploughing (Corsin *et al.*, 2005), filtration and disinfection of water (Corsin *et al.*, 2005) could have an affect on the occurrence of WSD. As a consequence, under favorable conditions for transmission (e.g. high stocking density) healthy shrimp are easily infected with WSSV by water-borne contact or ingestion of dead shrimp (Chou *et al*., 1998). WSSV infection of shrimp might be triggered by different physical water parameters as well (Tendencia *et al.*, 2010) which also promote severe infection levels and enhance outbreaks. These triggers include fluctuation of water temperature, pH, dissolved oxygen, salinity (Liu *et al*., 2006; Tendencia *et al*., 2010) and infection with other pathogens such as bacteria (Phuoc *et al*., 2008).

Besides the difference in genotype frequency, WSSV population structure was also different between the three farming systems in terms of genetic diversity. Higher genetic variation was maintained in the improved-extensive system despite the higher diversity of host ranges in the system and longer operation system (Chapter 3). In a recent epidemiological study in India (Walker *et al*., 2011a), a wide range of WSSV genotypes was also detected in plankton (pond water) and wild crustaceans, and found comparable to those genotypes in farmed shrimp in the improved-extensive systems. Another study proposed that the genetic diversity of *Panolis*

flammea nucleopolyhedrovirus (PaflNPV) population structure was preserved through the infection of different insect species (Hitchman *et al*., 2007). To some extent this explanation is corroborated by the finding that viral genotypic diversity could be maintained from the interaction of virus/host, e.g. hepatitis C virus and host (Manzin *et al*., 2000) and be specific to geographical distribution. For instance, genetic variation of 16 representative *Rice yellow mottle virus* isolates originated from the 14 countries of mainland Africa was linked to geographical distribution by analyses of full coat protein gene sequences (Traore *et al*., 2005). Due to farm practices, the improved-extensive system has more wild crustaceans living within the system. Therefore, shrimp farming practice (host species diversity, open farming system, farming techniques) could be proposed to be one of the reasons that result in differences in WSSV genotype population structure between the shrimp farming systems. This notion is compatible with the idea that farming practices play an important role in whether the disease occurs or not (Joffre and Bosma, 2009). Our observations and the interpretation thereof is also compatible with the findings of Dieu *et al.* (2011) that there is an effect of shrimp farming system on the genetic population structure of WSSV.

Molecular markers in WSSV transmission studies at farm scale

Molecular markers have been employed for studying transmission of different viruses. In particular, molecular tools have been applied in a novel manner to study the pandemic spread of influenza (Gatherer, 2009). Molecular markers, the proviral envelope and long terminal repeat sequences have been employed and have indicated the major mode of *ovine progressive pneumonia virus* transmission in a ewe flock as non-maternal transmission (Broughton-Neiswanger *et al*., 2010).

With the power of molecular markers in WSSV strain discrimination, the ability of VNTRs to measure the transmission rate and route within and between shrimp ponds was verified. For this purpose, using the VNTRs located at ORF75, ORF94, ORF125 as a single synthetic molecular marker could be the most appropriate approach (Chapter 6). In terms of effective and costly consideration, the single molecular marker in ORF94 could be also useful in such field transmission studies (Chapter 6). However, the variation in this VNTR is probably not neutral given its association to outbreaks, regardless of the mechanism linking this marker to virulence. Using these molecular markers, we measured different transmission routes and rates between the two shrimp farming systems: in improved-extensive systems, the main sources of WSSV infection were from inside the farm, whereas in semi-intensive system WSSV sources were mainly from outside farm system.

WSSV infections in a shrimp pond can originate from many sources. For example, WSSV can be transmitted through the water-borne route, via cannibalism of moribund shrimp (Chou *et al*., 1998; Wu *et al*., 2001), via mud crab/unscreened shrimp seeds (Kanchanaphum *et al.,* 1998; Peng *et al.,* 2001) or vertically via the trans-ovum route (Lo *et al*., 1997). All these transmission routes have been

demonstrated experimentally in the laboratory, but not (yet) in the field. Regarding the transmission routes within the shrimp farming system, the results are consistent with the assumption that transmission of WSSV in cultured shrimp production systems may occur from incoming infected shrimp, through the water and by cannibalism of moribund shrimps (Chou *et al*., 1998). An epidemiological study in India also suggested that WSSV transmission occurred directly through virus carriers or water exchange between adjacent farms at each location (Pradeep *et al*., 2008b). Our findings are in line with this finding, which also assumed that the combination of genotype patterns in ORF75, ORF94 and ORF125 could effectively be used to study WSD epidemiology in local production systems (Chapter 6).

WSSV transmission within and between shrimp ponds can be traced with the VNTR of ORF94 (Chapter 6). The combined molecular marker model provided a suitable marker to quantify the transmission rate of various modes of transmission among shrimp ponds (Chapter 6), which can be applied to predict transmission routes in the currently operating shrimp farming systems (e.g. rice-shrimp farming system or mangrove- shrimp farming system).

PRACTICAL IMPLICATIONS OF THE STUDY

The understanding gained from this research may serve as input to further WSSV molecular epidemiology studies at the farm-scale level.

Clearly, VNTRs and Indel markers have been demonstrated to be useful tool in studying the dynamics of WSSV infection in shrimp pond systems (Chapter 2, 3, 4 and 5). Each molecular marker has potential applications for different purposes in WSSV epidemiology studies in shrimp production systems. On this note, three implications could be inferred: (i) With the remarkable ability in WSSV strain discrimination and WSSV mixed-genotype detection from different hosts, VNTRs of ORF94 and ORF125 would be adequate markers for studies into WSSV genetic diversity and WSSV genotype surveillance at farm-scale level. (ii) The ability of ORF125 and ORF94 to distinguish WSSV genotypes in post-larval batches, individual shrimp or wild crustaceans allows the tracing of the origin of disease outbreaks in endemic and areas. (iii) VNTR-ORF94 and Indel-II region could be the starting point for future investigation in understanding virulence and pathogenesis *in vitro* and *in vivo*, which is important for tracing outbreaks and disease control.

With high power for tracking and identify transmission routes and transmission rates among shrimp ponds (Chapter 6), the combined molecular markers (VNTRs of ORF75, ORF94, ORF125), or only the ORF94 VNTR, will assist in the development of more effective strategies for WSSV disease management in the specific shrimp farming systems in the future.

FUTURE RESEARCH

The findings of the current research provide further input for the following prospective epidemiology studies, including:

1. Research into new molecular markers based on other repeat regions (homologous regions or VNTRs) dispersed throughout the WSSV genome in order to define more powerful and selectively neutral molecular markers for WSSV epidemiology studies.
2. Understanding the role of WSSV genotypes within an isolate and its association with diseases could lead to early intervention in the shrimp farming systems. Further research is therefore, conducted to explore the disease outcome of certain WSSV genotypes in shrimp pond settings.
3. WSSV population structure (degree of heterogeneity) in ponds related to disease.
4. VNTRs and indel markers should also be explored in other shrimp farming countries to determine whether virulent WSSV strains occur in other regions.
5. Understanding the genetic variation in different biological characteristics, such as virulence, by means of in vivo cloning of WSSV genotypes and the use of VNTRs as a marker.
6. Exploration of the application of these molecular markers in other stages of shrimp production cycles, including the hatchery stage to determine whether WSSV genotyping can help to track and trace vertical transmission.
7. Exploration of the application of these molecular markers in other existing shrimp farming systems to test the predictive value (for disease management) of the molecular marker technology.
8. Exploration of the application of these molecular markers to observe to what extent the molecular marker approach can be developed into high-throughput technology at a pond-side.

CONCLUDING REMARKS

The key observations and relevant conclusions of the thesis are as follow:

1. High genetic diversity of WSSV strains was present in small-scale shrimp farming operations in the Mekong Delta, Vietnam. The thesis also provides a historical record of circulating WSSV genotypes that may be useful in future studies.
2. Genetic population structure of WSSV is influenced by the farming system.
3. Genotyping methods based on VNTRs regions of ORF75, ORF94, ORF125 and Indel-II regions are useful tools for WSSV epidemiological studies at the farm level.
4. WSSV genotype structure could be a predictor of WSSV outbreaks in shrimp ponds (low numbers of repeat units in ORF94, mixed genotype infected in single shrimp, large deletion length of sequences in the Indel-II region).
5. Combined molecular marker models could quantify transmission pathways of WSSV in shrimp farming systems. However, VNTR sequences located in ORF94 are reliable enough to be used as a single molecular marker for epidemiological studies at the farm level (improved-extensive or semi-intensive shrimp farming systems).
6. Pond-to-pond transmission is more likely to contribute to local spread of WSSV in the semi-intensive system while within-pond transmission is the main source of WSSV spread in the improved-extensive system.

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LISTS OF ABBREVIATIONS

Ab	antibody
Ag	antigen
bp	base pair
CTAB	cetyltrimethylammonium bromide
DIG	digoxigenin
DTAB	dodecyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
dUTPase	dUTP pyrophosphate
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FAT	fluorescent antibody test
fg	femtogram
GAV	gill-associated virus
H&E	hematoxylin and eosin
HP	hepatopancreas
kDa	kilodalton
ICTV	International Committee on Taxonomy of Viruses
IF	immunofluorescence
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IHHNV	infectious hypodermal and haematopoietic necrosis virus
IHN(V)	infectious haematopoietic necrosis (virus)
IPN(V)	infectious pancreatic necrosis (virus)
ISH	*in situ* hybridization
LOS	lymphoid organ spheroids
LOV	lymphoid organ virus
LPS	lipopolysaccharide
MAb	monoclonal antibody
MBV	*Penaeus monodon*-type baculovirus
m.o.i.	multiplicity of infection
min	minutes
ml	millilitre
µg	microgram
µl	microlitre
µM	micromolar
mM	millimolar
NAb	neutralising antibody
NCBI	National Centre for Biotechnology Information
ng	nanogram
nM	nanomolar
nt	nucleotide
ORF	open reading frames
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

pg	picogram
PL	Postlarvae
RNA	ribonucleic acid
RR	Ribonucleotide reductase
RT-PCR	reverse-transcription polymerase chain reaction
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electophoresis
SPF	specific pathogen free
SEED	Shrimp Explosive Epidemic Disease
SEMBV	Systemic Ectodermal and Mesodermal Baculovirus
Taq	*Thermus aquaticus*
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
Tris	tris (hydroxymethyl) aminomethane
TSV	Taura syndrome virus
TK	Thymidine kinase
TMK	Thymidylate kinase
TSY	Thymidylate synthase
U	Units
UV	ultraviolet
YHD	Yellowhead disease
YHV	Yellowhead virus
WSBV	White spot disease baculovirus
WSD	White spot disease
WSSV	White spot syndrome virus
WSV	White spot virus

SUMMARY

White Spot Disease (WSD) has been the major scourge of shrimp culture for almost two decades. WSD caused by white spot syndrome virus (WSSV) was recorded from Japan in *Penaeus japonicus* in 1993. The causative agent, white spot syndrome virus (WSSV), is probably derived from China and has spread around the world through shrimp trade. Since its first appearance in Vietnam in 1994, WSD is seen as most devastating shrimp epidemic in the recorded history, also because of its socio-economic consequences. The lack of effective treatments stimulated the quest to find appropriate prevention or mitigation strategies to control the disease in shrimp. However, efforts to control this epidemic were challenged by the emergence of novel WSSV strains with different virulence and from various geographical origins. The use of molecular typing methods allowed epidemiological investigations of WSSV, a research avenue that aims at assisting in monitoring and prevention of infectious diseases at local and global levels. Moreover, correlations between genotype structure, farming system and outbreak status and longitudinal studies are often anecdotal and lack statistical support.

This study started with a characterization of the genetic variation of Vietnamese WSSV isolates from the presence of two major 'indel regions' found at ORF14/15 (Indel-I) and ORF23/24 (Indel-II) and three regions with variable numbers tandem repeats (VNTR) located in ORF75, ORF94 and ORF125, whereby the genome of WSSV-isolate TH (293 kilo base pairs) the reference was. In the next stage, PCR genotyping methods based on the VNTR regions of ORF75, ORF94, ORF125 and indel regions of ORF14/15 and ORF23/24 were developed in order to analyse these regions in more detail. These methods proved these Indel and VNTR regions to be very promising tools for WSSV molecular epidemiological studies at the local and regional level. However, the suitability of this analysis to understand disease outbreaks at the farm scale was not clear.

In the current study, the discriminatory power of the VNTR loci and indel regions was evaluated from WSSV isolates at the farm-scale level from the Me Kong delta. In Chapter 2 the VNTR-ORF94 was first explored to examine the extent of variation among WSSV isolates from *P. monodon* hatcheries and farms in different shrimp farming regions of the Mekong delta. This study would result in 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. High repeat number genotypes (i.e. ORF94-23 and -14), however, were not detected in cultured shrimp from the same pond. This implied that stocked postlarvae rather than invading wild crustaceans were the source of WSSV infection and WSD in the studied ponds. The findings showed that: (i) the molecular marker VNTR of ORF94 could be used to trace the origin of WSSV infection at certain shrimp ponds or

particular shrimp farming regions; (ii) genotype analysis via the VNTR of ORF94 region would be a useful indicator for tracking WSSV virulent isolates and for genotyping WSSV isolates at the farm-scale level.

From that knowledge base, a further investigation was carried out into the correlation (or not) between the number of repeat units (RUs) including the other VNTR regions (ORF75 and ORF125) and WSD outbreak status and/or shrimp farming practice (Chapter 3). A total of 662 WSSV samples derived from WSSV-infected *Penaeus monodon* were collected from 104 ponds in three shrimp farming systems (semi-intensive, improved-extensive and rice-shrimp farming system). Analysis of the WSSV samples with the PCR-genotyping of ORF94, ORF125 and ORF75 revealed 18, 14, and 8 different genotypes, respectively. Genotyping data in combination with statistical analysis showed that VNTR sequences of ORF94, ORF125 and ORF75 were useful markers for the purpose of studying WSSV genotype population structure. In addition, on the basis of specific VNTR of ORF94 and ORF125, the genotype population structure of WSSV also correlated with disease outbreaks in shrimp ponds: populations of WSSV with low repeat numbers correlated with disease, regardless the shrimp farming practice.

In Chapter 4, an examination was carried out into the nature of two indel regions (Indel-I and Indel-II) in 313 WSSV-infected shrimp collected between 2006-2009 from 76 shrimp ponds in the Mekong Delta region of Vietnam. Among the genotypes detected from the two regions of Indel-I and II, none of Indel-I types (size of deletions) showed a statistical correlation with the use of semi-intensive farming systems and the occurrence of disease. Indel-II detected genotype lengths, on the other hand, showed variation in terms of farm geographic location. More importantly, the presence of a specific Indel-II of 10,970 basepairs in length was found to correlate statistically with the occurrence of WSSV-induced disease and the use of semi-intensive farming systems. This suggests that Indel-II can be an informative molecular marker for predicting WSSV-induced disease.

In Chapter 5 it was shown using 'indel' and VNTR analysis that WSSV mixed-genotype infections correlate with lower outbreak incidence and that in contrast disease outbreaks correlate with single-genotype infections. The overall prevalence of mixed-genotype WSSV infections in the field was 25.7%. Non-outbreak ponds had a significantly higher frequency of mixed-genotype infections than outbreak ponds for all VNTR loci of WSSV, both at the individual shrimp as well as at the pond level. Since the genetic composition of WSSV populations appears to correlate with the health status of shrimp ponds, the causal relationship between genotypic diversity and disease outbreaks can now be experimentally approached.

In Chapter 6, molecular markers for the three VNTR regions were used to analyse the spread of WSSV within and between farms in Vietnam in two different farming systems: improved-extensive and semi-intensive. PCR-genotyping of the samples

taken from adjacent in a single region was employed using VNTRs in ORF75, ORF94 and ORF125, followed by statistical analysis on the combined results of all three VNTR marker loci. Results showed that the transmission of WSSV infection on improved-extensive shrimp farms appeared to be mainly due to the recycling of WSSV over time in the same pond, whereas in semi-intensive shrimp farms transmission of WSSV was mainly from neighbouring ponds.

In conclusion, the variable regions of the otherwise very conserved WSSV genome can be used to address epidemiological questions, such as the relationship between genotype structures of WSSV populations on the one hand and disease outcomes and farming system on the other. This thesis shows that there are differences in genetic structure of WSSV populations in shrimp culture areas, with more variation found in VNTR regions than in regions in which large deletions occur ('indels'). Furthermore, a correlation between WSSV population structure, disease outbreak status and pond farming system was observed. This provided evidence that the WSSV VNTR structure (in particular the number of repeat units in ORF94) statistically correlate with disease outbreaks and to a lesser extent to farming system. In addition mixed-genotype WSSV infections of shrimp are negatively correlated with disease outbreaks in ponds. Finally, it might be possible to use molecular markers (ORF94 and ORF125) to predict the outcome of WSSV infections in shrimp ponds in the future. For shrimp culture, these findings provide important information for the development of specific management strategies to control WSD and to advice farmers as to whether to harvest or not.

SAMENVATTING

'White Spot Disease' of WSD is al twintig jaar de belangrijkste ziekte in de garnalenteelt. De ziekte is voor het eerste gerapporteerd in Japan met een uitbraak in *Penaeus japonicus* in 1993. De veroorzaker, het 'white spot syndrome virus' (WSSV), is waarschijnlijk afkomstig uit China en heeft zich verspreid over de wereld via verhandelde garnalen. Sedert het eerste opduiken van de ziekte in Vietnam in 1994 wordt WSD beschouwd als de meest verwoestende epidemie in de jongste geschiedenis van de garnalenteelt, ook vanwege de sociaaleconomische gevolgen. Het ontbreken van effectieve behandelingen heeft de zoektocht naar methoden ter voorkoming of inperking van de ziekte in garnaal gestimuleerd. Echter, pogingen om de epidemie onder controle te brengen werden gedwarsboomd door het verschijnen van nieuwe WSSV-varianten met verschillend ziekteverwekkend vermogen, in diverse, geografisch verspreid liggende gebieden. Het gebruik van moleculaire typeringsmethoden stelde ons in staat om de epidemiologie van WSSV te bestuderen met als doel WSSV-epidemieën in kaart te brengen en te volgen, en wellicht de ziekte te voorkomen door de verspreiding op zowel lokaal als wereldniveau tegen te gaan. Ook is het niet duidelijk in hoeverre de genetische samenstelling van WSSV-isolaten verband houdt met het al of niet optreden van ziekte in garnalenvijvers, het management van diverse typen garnalenteelt en de voorgeschiedenis van de teelten. Veelal is de informatie anekdotisch van aard en mist deze een statistische onderbouwing om de correlatieclaims waar te maken.

In dit proefschrift wordt eerst de genetische variatie van Vietnamese WSSV-isolaten gekarakteriseerd op basis van twee zogeheten 'indels', gebieden waarin grote inserties/deleties optreden en die gelokaliseerd zijn rond open reading frame ORF14/15 (Indel-I) en ORF23/24 (Indel-II), waarbij het genoom van WSSV isolaat TH (293 kilobasenparen) de referentiestam was. Verder komt de variatie voor in drie gebieden met een variabel aantal zich herhalende nucleotidensequenties ('repeats'), ook wel VNTRs genaamd, en welke voorkomen rond ORF75, ORF94 en ORF125. Vervolgens werd een methode ontwikkeld (polymerasekettingreactie = PCR) om de ' indel' en VNTR-gebieden nauwkeurig te kunnen isoleren. Deze analyse bleek een goede manier te zijn om de epidemiologie van WSSV op lokaal en regionaal niveau te bestuderen. Het was echter niet duidelijk of deze techniek ook gebruikt kon worden om het ontstaan van ziekte in garnalenvijvers en teeltsystemen te begrijpen.

In de onderhavige studie werd het onderscheidend vermogen van bovengenoemde benadering via 'indels' en VNTR's getoetst met behulp van WSSV-isolaten van *P. monodon* uit de Mekong-delta. In hoofdstuk 2 werd eerst bestudeerd in welke mate de aantallen repeats van ORF94 varieerden in opkweekbedrijven van jonge garnalen ('hatcheries') en in garnalenbedrijven met postlarvae ('grow out ponds')

in de Mekong-delta. Op deze manier kon een beter inzicht worden verkregen in de epidemie-ontwikkeling bij garnalenteelt. Analyse van ca. 175 WSSV-isolaten liet overeenkomstige variaties in aantallen 'repeats' zien, waarbij enkele moederdieren meer dan één WSSV-variant (genotype) bevatten. Garnalen met hoge aantallen 'repeat' units (i.e. ORF94-23 en -14) werden evenwel in de garnalenvijvers niet gevonden. Dit hield in dat de postlarvae waarschijnlijk de bron van WSSV en ziekte zijn en in mindere mate de in het wild voorkomende kreeftachtigen. De resultaten gaven verder aan dat (i) de VNTR van ORF94 gebruikt kan worden als moleculaire merker om de herkomst van een WSSV-infectie te bepalen in bedrijven en gebieden met garnalenteelt, en dat (ii) genotypering van WSSV via de 'repeats' van ORF94 een bruikbare methode is om virulente stammen van WSSV op te pikken en te vervolgen, zelfs op bedrijfsniveau.

Op basis van deze resultaten werd verder onderzoek uitgevoerd om te zien of er een verband bestaat tussen het aantal 'repeat' units, inclusief de andere VNTR gebieden (ORF75 en ORF125), en ziekte-uitbraak enerzijds en garnalenkweeksysteem anderzijds (Hoofdstuk 3). Uit drie typen garnalenkweeksystemen (semi-intensief, verbeterd-extensief en 'rijst-garnaal'-systeem) en 104 vijvers werden in totaal 662 WSSV-monsters verkregen. Analyse van WSSV-monsters via PCR genotypering van ORF75, ORF94 en ORF125 wees uit dat er respectievelijk 18, 14 en 8 verschillende genotypen aanwezig waren. Deze analyse, samen met een statistische bewerking, liet zien dat de VNTR-sequenties van ORF75, ORF94 en ORF125 heel bruikbaar zijn voor het bepalen van de genotypische populatiestructuur van WSSV. Bovendien kon op basis van de specifieke VNTR-structuur van ORF94 en ORF125 vastgesteld worden dat er een positief verband bestaat tussen deze populatiestructuur en ziekte-uitbraak in garnalenvijvers: populaties van WSSV met weinig 'repeat' units vertoonden significant meer ziekte-uitbraak, onafhankelijk van het garnalenkweeksysteem.

Het onderzoek beschreven in Hoofdstuk 4 betreft de aard van de twee ' indel' -gebieden (Indel-I en Indel-II) van WSSV, aanwezig in 313 geïnfecteerde garnalen, die tussen 2006 en 2009 zijn verzameld in 76 garnalenvijvers in de Mekong-delta in Vietnam. Geen van de Indel-I type deletie) lieten statistisch een verband zien met het optreden van ziekte in semi-intensieve garnalenbedrijven. De omvang van de deleties in Indel-II daarentegen, vertoonde verschil afhankelijk van de geografische locatie van het garnalenbedrijf. Nog belangrijker was dat er een positief verband bestond tussen de aanwezigheid van een specifieke deletie in Indel-II van 10,970 basenparen enerzijds en het optreden van ziekte en met semi-intensieve garnalenteelt anderzijds. Dit doet vermoeden dat Indel-II een informatieve moleculaire merker kan zijn om uitbraak van WSSV te voorspellen.

In Hoofdstuk 5 werd via analyse van indels en VNTR's aangetoond dat infectie van garnalen met meer dan één WSSV genotype positief samenhing met een lage kans op ziekte-uitbraak en dat daarentegen ziekte-uitbraak positief gecorreleerd was

aan infecties met een WSSV-genotype. Het voorkomen van gemengde infecties in het veld was 25,7%. Bij WSSV uit garnalenvijvers zonder ziekte-uitbraak was de frequentie van gemengde infecties beduidend hoger dan in vijvers waar uiteindelijk de ziekte uitbrak. Dit gold voor alle VNTR-gebieden van WSSV, zowel voor individuele garnalen als voor garnalenvijvers. Omdat de genetische samenstelling van WSSV-populaties bepalend lijkt te zijn voor de gezondheidsstatus van garnalenvijvers, kunnen nu experimenten worden gedaan om een mogelijk oorzakelijk verband tussen variatie in genotypes en kans op uitbraak aan te tonen.

In het in Hoofdstuk 6 beschreven onderzoek werden de drie VNTR-gebieden gebruikt om te bestuderen hoe de verspreiding van WSSV binnen een garnalenbedrijf of tussen garnalenbedrijven (en wel voor twee teeltsystemen, semi-intensief en verbeterd-extensief) verloopt. Genotypering via PCR werd uitgevoerd met WSSV-monsters uit aangrenzende garnalenvijvers in een aaneengesloten garnalenteeltgebied. Dit werd gedaan aan de hand van de VNTR-gebieden in ORF75, ORF94 en ORF125, gevolgd door een statistische analyse van de gecombineerde VNTR-gebieden ten opzichte van de diverse garnalenteeltsystemen. De resultaten van deze analyse toonden aan dat de overdracht van WSSV in verbeterd-extensieve teeltsystemen vooral het gevolg was van recycling van WSSV in dezelfde garnalenvijver, terwijl in semi-intensieve teeltsystemen WSSV vooral afkomstig was van aangrenzende garnalenbedrijven. De variabele gebieden van de overwegend zeer geconserveerde WSSV-genomen kunnen dus worden gebruikt om epidemiologische vraagstellingen aan te pakken, zoals de vraag of er een mogelijke verband bestaat tussen genotypische structuur van WSSV-populaties enerzijds en de inrichting van garnalenteeltsystemen ten aanzien van het al of niet uitbreken van 'white spot disease' anderzijds. Onderzoek beschreven in dit proefschrift laat ook zien dat er verschillen zijn in genetische structuur van WSSV-populaties in garnalenteeltgebieden, waarbij meer variaties werden aangetroffen in de VNTR-gebieden dan in gebieden (indels) waar grote deleties kunnen voorkomen. Bovendien werden er positieve verbanden gevonden tussen de structuur van WSSV-populaties, ziekte-uitbraakstatus en, in mindere mate, garnalenteeltsysteem. Hiermee werd ook aangetoond dat de genetische structuur van VNTR's (in het bijzonder van 'repeat' units in ORF94) samenhangt met het al of niet uitbreken van ziekte en in mindere mate het garnalenteeltsysteem. Bovendien bleek er een negatief verband te bestaan tussen gemengde WSSV-infecties en ziekte-uitbraak in garnalenvijvers. Ten slotte is het nu mogelijk om in de toekomst moleculaire merkers, in het bijzonder die welke gelegen zijn in ORF94 en ORF 125 (VNTR), te gebruiken om voorspellingen te doen over het al of niet ontstaan van ziekte in garnalenvijvers. Voor de garnalenteelt bevat dit proefschrift belangrijke informatie voor het ontwikkelen van specifieke managementstrategieën om 'white spot disease' te voorkomen en garnalentelers te adviseren om al of niet te oogsten.

TÓM TẮT

Bệnh đốm trắng đã gây nhiều thiệt hại cho nghề nuôi tôm qua gần hai thập kỷ. White spot syndrome virus (WSSV) là tác nhân gây bệnh đốm trắng, được ghi nhận ở Nhật Bản vào năm 1993. Tuy nhiên, WSSV có khả năng xuất phát từ Trung Quốc và lây lan khắp thế giới thông qua hoạt động mua bán tôm. Năm 1994, bệnh đốm trắng xuất hiện lần đầu tiên ở Việt nam. Bệnh được đánh giá là dịch bệnh gây thiệt hại nặng nề nhất cho nghề nuôi tôm, do bởi các tác hại liên quan đến mặt kinh tế xã hội. Việc chưa có những biện pháp chữa trị hiệu quả đã thúc đẩy các nhà khoa học nghiên cứu các biện pháp phòng ngừa nhằm hạn chế dịch bệnh đốm trắng trên tôm. Tuy vậy, các nỗ lực này gặp nhiều khó khăn do sự xuất hiện của các dòng vi-rút đốm trắng khác nhau về độc lực, nguồn gốc địa lý. Việc sử dụng các phương pháp phân tử định kiểu gen đã tạo điều kiện thực hiện các nghiên cứu dịch tể học vi-rút gây bệnh đốm trắng, mở ra hướng nghiên cứu bệnh đốm trắng với mục tiêu giúp kiểm soát và ngăn ngừa dịch bệnh lây lan ở qui mô địa phương và toàn cầu. Ngoài ra, các nghiên cứu đoàn hệ về mối tương quan giữa kiểu gen, mô hình nuôi tôm với tình trạng bộc phát bệnh ở các ao tôm thì thường không mang tính chất qui mô và thiếu sự hỗ trợ của các phép phân tích thống kê.

Nghiên cứu này bắt đầu với việc tìm hiểu khả năng phân biệt các dòng vi rút đốm trắng thu tại Việt Nam bằng các chỉ thị phân tử: vùng gen thêm đoạn ở vùng mã hóa ORF14/15 (Indel-I), vùng gen mất đoạn ở vùng mã hóa ORF23/24 (Indel-II), và ba vị trí ở các vùng mã hóa ORF75, ORF94 và ORF125 có chứa các vùng ADN lặp lại liền kề (VNTR). Đây là các vùng gen được tham khảo từ trình tự gen của dòng vi-rút đốm trắng phân lập từ Thái Lan (WSSV-TH). Sau đó, các phương pháp PCR định kiểu gen (PCR-genotyping) được phát triển dựa trên các vùng VNTR của ORF75, ORF94, ORF125 và các vùng gen biến đổi của ORF14/15, ORF23/24. Các phương pháp định kiểu gen này đã cho thấy khả năng ứng dụng tốt trong các nghiên cứu dịch tể học vi-rút đốm trắng ở qui mô địa phương và toàn cầu. Tuy nhiên, khả năng ứng dụng của các phương pháp định kiểu gen này cho việc tìm hiểu sự bùng phát dịch bệnh giữa các ao trong vùng nuôi tôm thì vẫn còn chưa được sáng tỏ.

Trong nghiên cứu này, khả năng định kiểu gen của các chỉ thị phân tử thuộc ba vị trí VNTR và hai vùng gen biến đổi (indel) được đánh giá qua các dòng vi-rút đốm trắng thu từ các ao tôm nuôi ở Đồng bằng sông Cửu Long (ĐBSCL). Trong chương 2, VNTR-ORF94 được thử nghiệm đầu tiên để đánh giá mức độ khác biệt giữa các dòng WSSV thu từ các trại tôm sú giống, các ao tôm ở nhiều vùng nuôi khác nhau thuộc khu vực ĐBSCL. Kết quả nghiên cứu giúp hiểu rõ hơn về sự tiến triển của dịch bệnh ở các ao trong quá trình nuôi. Phân tích khoảng 157 dòng WSSV cho thấy sự khác biệt chung về số lượng vùng lặp lại, trong đó tôm bố mẹ thường nhiễm nhiều hơn một kiểu gen. Tuy nhiên, các kiểu gen có số vùng lặp lại cao ở ORF94 (ORF94 -23 và -14) lại không được phát hiện ở tôm nuôi trong cùng một ao. Điều này cho thấy tôm giống có thể là nguồn lây bệnh đốm trắng ở các ao nuôi trong nghiên cứu này, chứ không phải từ các loài giáp xác trong tự nhiên. Kết quả nghiên cứu này cho thấy: (i) chỉ thị phân tử

VNTR-ORF94 có thể sử dụng để truy tìm nguồn gốc sự lây lan bệnh đốm trắng tại ao tôm hay vùng nuôi tôm, và (ii) định loại kiểu gen thông qua chỉ thị VNTR-ORF94 sẽ giúp truy tìm các dòng WSSV có độc lực và định loại kiểu gen các dòng vi rút đốm trắng ở cấp độ hộ nuôi tôm.

Trên cơ sở hiểu biết ở trên, nghiên cứu tiếp theo được thực hiện nhằm tìm hiểu mối tương quan giữa các vùng VNTR thuộc ORF75 và ORF125 với tình trạng bùng phát dịch bệnh và/hoặc mô hình nuôi tôm (Chương 3). Tổng số 662 mẫu vi rút đốm trắng phân lập từ tôm sú nhiễm bệnh được thu thập từ 104 ao nuôi tôm thuộc ba hình thức nuôi là bán thâm canh, quảng canh cải tiến và tôm-lúa. Phân tích mẫu thu với phương pháp PCR định kiểu gen cho thấy 18, 14, và 8 kiểu gen tương ứng với các vùng VNTR của ORF94, ORF125 và ORF75. Dữ liệu phân tích kiểu gen kết hợp với phép phân tích thống kê cho thấy các vùng VNTR thuộc ORF94, ORF125 và ORF75 là những chỉ định hữu ích để nghiên cứu cấu trúc quần thể kiểu gen của vi-rút đốm trắng. Thêm nữa, trên cơ sở VNTR của ORF94 và ORF125 cho thấy mối tương quan giữa cấu trúc quần thể kiểu gen của vi-rút đốm trắng với tình trạng bộc phát bệnh ở các ao tôm: các quần thể vi-rút đốm trắng có số vùng ADN lặp lại liền kề thấp tỷ lệ thuận với tình trạng bộc phát bệnh, ứng với các mô hình tôm nuôi khác nhau.

Chương 4 trình bày nghiên cứu nhằm tìm hiểu khả năng ứng dụng của hai vùng gen biến đổi (Indel-I và Indel-II) trên 313 mẫu tôm nhiễm bệnh đốm trắng thu từ 76 ao tôm ở vùng ĐBSCL trong khoảng thời gian 2006 - 2009. Trong số các kiểu gen xác định được từ hai vùng Indel này, không có kiểu gen thuộc nhóm Indel-I nào cho thấy mối tương quan mang ý nghĩa thống kê với hình thức nuôi bán thâm canh và sự xuất hiện dịch bệnh. Mặt khác, vùng Indel-II thì lại cho thấy sự khác biệt theo vị trí địa lý của hộ nuôi tôm. Quan trọng hơn, kiểu gen Indel-II mất đoạn 10,970 bp có mối tương quan có ý nghĩa thống kê với sự xuất hiện dịch bệnh đốm trắng và hình thức nuôi bán thâm canh. Điều này cho thấy chỉ thị phân tử Indel-II có thể dùng để dự đoán bệnh đốm trắng.

Trong Chương 5, phân tích vùng gen biến đổi (Indel) và vùng ADN lặp lại liền kề (VNTR) cho thấy: hiện tượng đa nhiễm kiểu gen WSSV sẽ không có mối liên hệ với tình trạng bộc phát bệnh trong ao nuôi tôm. Ngược lại, các ao bộc phát bệnh thì chỉ nhiễm đơn một kiểu gen. Hiện tượng đa nhiễm chiếm tỉ lệ chung là 25,7% trên tổng số mẫu thu. Phân tích thống kê cho thấy, tỉ lệ cảm nhiễm này cao ở các ao tôm không bộc phát bệnh đốm trắng so với các ao có bộc phát bệnh. Kết quả này ứng với các trường hợp phân tích cho từng cá thể nhiễm bệnh hay cả quần thể tôm nhiễm bệnh trong cùng một ao. Do cấu trúc di truyền của quần thể vi-rút đốm trắng dường như có liên quan đến tình trạng bộc phát bệnh của ao nuôi tôm, cho nên mối liên hệ giữa tính đa dạng kiểu gen và tình trạng bộc phát bệnh của ao nuôi có thể được nghiên cứu thực nghiệm.

Trong Chương 6, các chỉ thị phân tử ở ba vùng VNTR được sử dụng để phân tích sự lan truyền của vi rút đốm trắng giữa các ao nuôi tôm ở Việt Nam thuộc hai hình thức nuôi quảng canh cải tiến và bán thâm canh. Phương pháp PCR định kiểu gen (ORF75, ORF94 và ORF125) được sử dụng để phân tích các mẫu thu từ hệ thống các ao tôm liền kề. Phân tích thống kê được sử dụng trên cơ sở kết hợp dữ liệu của cả ba vùng VNTR

của ORF75, ORF94 và ORF125. Kết quả cho thấy bệnh đốm trắng lây lan trong hệ thống nuôi quảng canh cải tiến chủ yếu là do nguồn vi-rút đốm trắng còn tồn lại trong ao từ vụ nuôi trước, trong khi đường lây lan bệnh đốm trắng trong hệ thống nuôi bán thâm canh chủ yếu là từ các ao lân cận.

Tóm lại, các vùng gen biến đổi của vi-rút đốm trắng có thể được sử dụng để giải thích các vấn đề dịch tễ học, ví dụ mối liên hệ giữa kiểu gen của quần thể vi-rút đốm trắng và tình trạng bộc phát bệnh của ao nuôi và các mô hình nuôi. Luận án này đã cho thấy có nhiều sự khác biệt về kiểu gen của quần thể vi rút đốm trắng ở các vùng nuôi tôm. Sự khác biệt về kiểu gen được phát hiện nhiều với các vùng ADN lặp lại liền kề VNTR hơn là những vùng gen biến đổi Indel. Ngoài ra, nghiên cứu cho thấy được mối tương quan giữa cấu trúc quần thể vi- rút đốm trắng với tình trạng bộc phát bệnh trong ao tôm và mô hình nuôi. Mối tương quan này là bằng chứng cho thấy cấu trúc các vùng VNTR của vi-rút đốm trắng, đặc biệt là VNTR-ORF94, tỷ lệ thuận có ý nghĩa thống kê với sự bùng phát dịch đốm trắng trong ao tôm và có tương quan ở mức độ thấp hơn với mô hình nuôi. Bên cạnh đó, hiện tượng đa nhiễm WSSV có mối liên hệ nghịch với tình trạng bộc phát bệnh đốm trắng trong ao nuôi. Tóm lại, chỉ thị phân tử (ORF94 và ORF125) có thể giúp dự đoán tình trạng bộc phát bệnh đốm trắng ở ao nuôi tôm. Đối với nghề nuôi tôm, kết quả nghiên cứu này: cung cấp thông tin quan trọng cho việc xây dựng các chiến lược quản lý để kiểm soát bệnh đốm trắng; là cơ sở giúp người nuôi đưa ra hướng xử lý phù hợp khi ao nuôi có bệnh đốm trắng.

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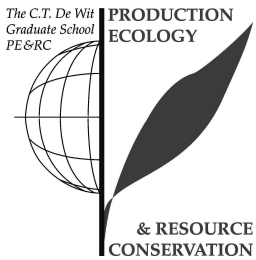
Tran Thi Tuyet Hoa

PUBLICATION ACCOUNT

1. Michele A Burford, Nigel J Preston, Truong Hoang Minh, **Tran Thi Tuyet Hoa,** Stuart EB and Vanessa MF (2004) Dominant sources of dietary carbon and nitrogen for shrimp reared in extensive rice-shrimp ponds. Aquaculture research 35: 195-203.
2. **Tran Thi Tuyet Hoa**, Richard AJ Hodgson, Dang TH Oanh, Nguyen T Phuong, Nigel J Preston and Peter J Walker (2005) Genotypic Variations in Tandem Repeat DNA Segments between Ribonucleotide Reductase Subunit Genes of White Spot Syndrome Virus (WSSV) Isolates from Vietnam. In P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). *Disease in Asian Aquaculture V*, p339-351.
3. **Tran Thi Tuyet Hoa,** Zwart MP, Nguyen T Phuong, Dang TH Oanh, de Jong MCM, Vlak JM (2011a) Mixed-genotype WSSV infections of shrimp are inversely correlated with disease outbreaks in ponds. Journal of General Virology 92: 675–680
4. **Tran Thi Tuyet Hoa,** Zwart MP, Nguyen T Phuong, Vlak JM, de Jong MCM (2011b) Transmission of white spot syndrome virus in improved-extensive and semi-intensive shrimp production systems: A molecular epidemiology study. Aquaculture 313: 7–14
5. **Tran Thi Tuyet Hoa,** Zwart MP, Nguyen T Phuong, Vlak JM, de Jong MCM (2012) Low numbers of repeat units in VNTR regions of White spot syndrome virus are correlated with disease outbreaks. Accepted to be published in the Journal of Fish Diseases
6. **Tran Thi Tuyet Hoa,** Zwart MP, Nguyen T Phuong, Dang TH Oanh, de Jong MCM, Vlak JM (2012) Indel-II region deletion sizes in the white spot syndrome virus genome correlate with shrimp disease outbreaks in southern Vietnam. Accepted to be published in Disease of Aquatic Organisms. DOI 10.3354/dao02463

PE&RC PhD Education Certificate



With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Molecular epidemiology and genome of white spot syndrome virus (WSSV) (2007)

Writing of project proposal (4.5 ECTS)

- Spatial spread and virulence development of White Spot Syndrome Virus (WSSV) in shrimp culture areas

Post-graduate courses (3 ECTS)

- Bioinformatics; University of Stuttgart, Germany (2006)
- Advanced statistics; Wageningen University (2010)

Laboratory training and working visits (4.5 ECTS)

- Working visit to study viral disease on aquatic organism; Yosu National University, Korea (2006)

Deficiency, refresh, brush-up courses (2.8 ECTS)

- Basic statistics (2009)
- Molecular Virology (2009)

Competence strengthening / skills courses (4.2 ECTS)

- Project and time management; Wageningen University (2007)
- Techniques for writing and presenting a scientific paper; Wageningen University (2007)
- Competencies for integrated agricultural research; Wageningen University (2007)
- Information literacy, including introduction Endnote; Wageningen University (2009)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC Day meeting (2007)
- PE&RC Day meeting (2009)
- PE&RC Weekend (2010)

Discussion groups / local seminars / other scientific meetings (4.5 ECTS)

- Rescopar conference with oral presentation- 2007 in Wageningen, 2008 in Vietnam, 2009 in Indonesia, 2010 in Vietnam (2007-2010)

International symposia, workshops and conferences (8.8 ECTS)

- 7th Symposium on disease in Asian Aquaculture-Taipei-Taiwan with presentation on: high variation of tandem repeat sequences in ORF94 among WSSV outbreak strains in the Mekong delta, Vietnam
- World Aquaculture Society-Asian Pacific chapter-Kuala Lumpur-Malaysia-with presentation on :detection of white spot syndrome virus in live foods used in shrimp hatcheries (2009)
- 9th Asian Fisheries & Aquaculture Forum-Shanghai-China with presentation on: variable number tandem repeats (VNTRs) and their application in WSSV epidemiological studies (2011)
- 8th Symposium on disease in Asian Aquaculture-Mangalore-India; with presentation on: variable tandem repeat structure of White spot syndrome virus genome populations correlates with shrimp disease status (2011)

Lecturing / supervision of practical' s /tutorials

- Molecular biology-aquaculture; 15 days / year (2007-2011)
- Infectious diseases of aquatic animal; 5 days / year (2007-2011)
- Epidemiology and aquatic animal disease management; 6 days / year (2007-2011)

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