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THESIS

TRADITIONAL AND MOLECULAR EPIDEMIOLOGY TO DETERMINE RISK FACTORS FOR OUTBREAKS OF SHRIMP WHITE SPOT DISEASE IN THAILAND

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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White spot disease (WSD) is a viral disease that causes acutely fatal and massive losses in most commercially cultivated marine shrimp species. A longitudinal retrospective study of the risk factors for WSD outbreaks was investigated based on management practices and environmental variables of a semi-closed farming system. Altogether 384 pond records of 8 continuous production cycles of a 70-pond farm from January 1998 to January 2002 were analyzed using multivariable logistic regression analysis and generalized estimating equations (GEE). WSD outbreak ponds were defined as those where shrimp gave a positive result for WSSV infection using a 1-step PCR method. The average time of shrimp culture in outbreak ponds was 80 days. While the average days of culture in normal ponds was 124 days. Forty-three percent of the ponds had WSD outbreaks throughout the study period. Season was the strongest factor that affected WSD outbreaks. Stocking shrimp during the rainy-winter season (June 16–December 31) dramatically increased the risk of disease (OR = 7.58). On the other hand, pond preparation durations longer than 17 days reduced the risk of WSD outbreaks (OR = 0.33).

A high value of the transmission coefficient (*ß)* in the rainy-winter season was found in the dynamic epidemiology study. It indicated that season is very important for epidemic outbreaks of WSD. Fluctuations in water salinity and low temperature probably had synergistic effects on the osmoregulation capacity of shrimp, increasing susceptibility to infection.

The number of tandem repeats in ORF94 and ORF125 VNTRs were highly variable among various Thai-WSSV isolates. When the 2 markers were used together, 18 WSSV genotypes were found out of 216 possible genotypes.

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Visanu Boonyawiwat

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LIST OF ABBREVIATIONS

agepl	=	age of postlarvae shrimp
bp	=	base pair
carricide	=	used carricide
CC	=	Chachoengsao
CI	=	confidence interval
cm	=	centrimeter
CP	=	Chumphon
disinfect	=	used disinfectant
DNA	=	deoxyribonucleic acid
dsDNA	=	double standed deoxyribonucleic acid
EtBr	=	ethidium bromide
ISH	=	*In situ* hybridization
IHC	=	Immunohistochemistry
kb	=	kilobase
kDa	=	kilodalton
M	=	molar
mM	=	milimolar
µg	=	microgram
µl	=	microliter
ml	=	milliliter
mg	=	milligram
nm	=	namometer
NP	=	Nakhon Pathom
NS	=	Nakhon Si Thammarat
obfmcrop	=	WSD outbreak in former crop
OR	=	odds-ratio
ORF	=	open reading frame
PCR	=	polymerase chain reaction
pdrestdu	=	duration of pond drying
PL	=	postlarvae

LIST OF ABBREVIATIONS (Continued)

pondsize	=	pond size
predurat	=	duration of pond preparation
RFLP	=	restriction fragment length polymorphrism
RNA	=	ribonucleic acid
rpm	=	round per minute
S.E.	=	standard error of mean
shrimpm^{-2}	=	shrimp per squaremeter
SNPs	=	single nucleotide polymorphrisms
ST	=	Surat Thani
stockden	=	stocking density
VNTR	=	variable number tandem repeat(s)
UV	=	ultraviolet
walevel	=	water depth

TRADITIONAL AND MOLECULAR EPIDEMIOLOGY TO DETERMINE RISK FACTORS FOR OUTBREAKS OF SHRIMP WHITE SPOT DISEASE IN THAILAND

INTRODUCTION

White spot disease (WSD) is a viral disease that affects many commercially cultivated marine shrimp species. WSD was initially recognized in Japan in 1993 and then spread rapidly throughout Asia. Currently, it is a widely distributed disease and the most serious viral pathogen of shrimp, with estimated losses of production amounting to hundred millions US$ annually. In Thailand the first outbreak of WSD was reported in1994 and it is still a major cause of economic loss for Thai shrimp farmers.

The principal clinical sign of the disease is the presence of white spots in the cuticle of infected shrimp. Affected individuals become lethargic and lose their appetite and mortality can reach up to 90-100% within 3-10 days after the first signs of disease. The causative agent of the disease is white spot syndrome virus (WSSV). It is a bacilliform, enveloped virus, with a double-stranded DNA genome that has an approximate length of 305 kbs. There are two major routes of transmission of WSSV, one is vertical transmission via infected broodstrock and the other is horizontal transmission via carriers. Several methods have been adopted to prevent WSSV outbreaks, but serious outbreaks still occur every year. During the past 3 years, several molecular marker assays have been developed for WSSV. Results from genomic DNA fragment analysis indicated extremely high DNA sequence homology for WSSV. However, variation in repetitive DNA fragment lengths allows researchers to distinguish among WSSV isolates.

This report describes (1) investigation into the risk factors for WSD outbreaks in intensive shrimp culture systems in Thailand, (2) evaluation the potential for

horizontal transmission of WSSV in different seasons, (3) attempts to develop new VNTR markers and to use 3 minisatellite markers (ORF94, ORF75 and ORF125) as epidemiological markers for genotyping WSSV from shrimp in Thailand. The knowledge from this study contributes to a better understanding of WSSV epidemiology in Thailand. Finally, a best fit preventive control program is described for distribution to Thai shrimp farmers.

OBJECTIVES

1. To study the risk factors associated with WSSV outbreaks in shrimp farms using a retrospective study.

2. To evaluate the potential for horizontal transmission of WSSV between ponds during difference seasons.

3. To establish new VNTR markers to be used with former markers in genotyping WSSV isolated from diseased shrimp in Thailand.

LITERATURE REVIEW

1. White spot disease (WSD)

The rapid expansion of intensive shrimp farming systems worldwide has been accompanied by the occurrence of many threatening diseases of shrimp. One of these is white spot disease (WSD) caused by white spot syndrome virus (WSSV). WSD has been formally recognized since it first occurred in 1993 in the northern part of the Asia. Thereafter, it spread rapidly throughout the Asian continent (Takahashi *et al.*, 1994; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995).

In 1992, a new virus appeared in shrimp farms in northern China causing disease and massive mortality (Chou *et al.*, 1995). In late 1993, the viral agent was first isolated from an outbreak in Japan (Inouye *et al.*, 1994) and within a few years this new pathogenic agent spread to several shrimp farming countries (Flegel, 1997). At first, it was thought that different viral agents had simultaneously appeared in different regions and each was assigned a specific name: rod-shaped nuclear virus of *Marsupenaeus japonicus* (RV-PJ) (Inouye *et al.*, 1994), *Penaeus monodon* non-occluded baculovirus (PmNOB III) (Wang *et al.*, 1995), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya *et al.*, 1995), white spot baculovirus (Chou *et al.*, 1995; Lightner, 1996), hypodermal and haematopoietic necrosis baculovirus (HHNBV) (Lightner, 1996) and penaeid rod-shaped DNA virus (Inouye *et al.*, 1994; Venegas *et al.*, 2000). Subsequently, it was recognized that a single viral agent was responsible for these reports. Finally, an informal consensus was reached to call it white spot syndrome virus (WSSV). Currently, WSSV is found in many shrimp cultivation countries and is considered to be one of the most serious diseases of shrimp aquaculture. The reports of WSSV outbreaks in various shrimp farming countries are shown in Table 1.

Table 1 Chronological order of white spot syndrome virus outbreaks in shrimp farming countries in Asia and America.

Year of occurrence	Country	Reference
1992	Taiwan	Chou *et al.*, 1995
1993	China, Japan, Korea	Zhan *et al.*, 1998; Inouye *et al.*, 1994; Park *et al.*, 1998
1994	Thailand, India, Bangladesh	Lo *et al.*, 1996a; Karunasagar *et al.*, 1997; Mazid and Banu, 2002
1995	USA	Lightner, 1996; Wang *et al.*, 1999a
1996	Indonesia, Malaysia, Sri Lanka	Durand *et al.*, 1996; Kasornchandra *et al.*, 1998; Rajan *et al.*, 2000
1997	Vietnam	Bondad-Reantaso *et al.*, 2001
1998	Peru	Rosenberry, 2001
1999	Philippines, Ecuador, Colombia, Panamá, Honduras, Nicaragua, Guatemala, Belice	Magbanua *et al.*, 2000; Bondad-Reantaso *et al.*, 2001; Hossain *et al.*, 2001; Wu *et al.*, 2001
1999–2000	Mexico	Bondad-Reantaso *et al.*, 2001
2002	France, Iran	Dieu *et al.*, 2004; Marks *et al.*, 2005
2005	Brasil	APHIS-USDA, 2005

Source: Escobedo-Bonilla *et al.*, 2008

Since its discovery in Taiwan in 1992, the virus spread quickly, causing serious losses to commercial shrimp farming in most countries where shrimp are cultivated (Flegel, 1997). In China, production losses of 80% of farmed shrimp were attributed to WSSV in 1992 (Zhan *et al.*, 1998). The WSD outbreak in Thailand was first reported in 1994 and it resulted in more than 500 million US dollars loss during 1996 (Flegel and Alday-Sanz, 1998). At present, the loss to Thai shrimp production

associated with WSD may be more than 20% of total production annually. In Asia, it is still the most serious viral pathogen of shrimp, with estimated losses in production amounting to hundreds millions of US$ per year (Flegel, 1997).

The spread of WSSV to other shrimp farming countries threatens the development of shrimp aquaculture. WSSV is able to infect all commercially cultivated marine shrimp species (Flegel, 2006). In 2002, WSSV was found in wild crustaceans of the French Mediterranean coast (Marks *et al.*, 2005). The presence of WSSV in the area may interfere with the development of shrimp aquaculture, especially in North African countries. The introduction of WSSV-infected organisms to areas where the pathogen was previously unknown may be possible through ballast water from cargo ships (Flegel and Fegan, 2002) or even frozen shrimp commodities if they are fed to cultivated shrimp (Durand *et al.*, 2000).

White spot disease affects all of the commercially cultivated marine shrimp species (Chou *et al.*, 1995; Flegel, 2006). It also has a broad host range including at least 18 cultured and/or wild penaeid shrimp (Wongteerasupaya *et al.*, 1996; Durand *et al.*, 1997; Lu *et al.*, 1997; Chou *et al.*, 1998; Lightner *et al.*, 1998; Park *et al.*, 1998), eight caridean species (Sahul Hameed *et al.*, 2000; Shi *et al.*, 2000; Pramod-Kiran *et al.*, 2002), seven species of lobster (Chang *et al.*, 1998a; Rajendran *et al.*, 1999), seven species of crayfish (Wang *et al.*, 1998b; Corbel *et al.*, 2001; Hossain *et al.*, 2001; Jiravanichpaisal *et al.*, 2001; Edgerton, 2004; Jiravanichpaisal *et al.*, 2004), 38 crab species (Lo *et al.*, 1996a; Kanchanaphum *et al.*, 1998 Kou *et al.*, 1998; Sahul Hameed *et al.*, 2001; Sahul Hameed *et al.*, 2003), six non-decapod crustacean species (Supamattaya *et al.*, 1998; Otta *et al.*, 1999; Hossain *et al.*, 2001), members of the phyla Chaetognata and Rotifera (Yan *et al.*, 2004, 2007), polychaete worms (Supak *et al.*, 2005; Vijayan *et al.*, 2005) and some aquatic insect larvae (Lo *et al.*, 1996a ; Flegel, 1997) that have been found to be WSSV-positive by PCR (Apppendex A). Although many of these species have been confirmed to support WSSV replication under experimental conditions, some other species collected from the wild have only been found WSSV-positive by PCR. This indicates that many such species are not necessarily WSSV natural hosts, but may only be mechanical carriers.

1.1 Clinical signs

A principal clinical sign of WSD is the presence of obvious white spots of 0.5–3.0 mm in diameter embedded in the cuticle of infected shrimp (Figure 1) (Lo *et al.*, 1996b; Kasornchandra *et al.*, 1998). The exact mechanism of white spot formation is not known. It is possible that the WSSV infection may induce dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots (Wang *et al.*, 1999b). Other signs of disease include reddish discolouration of the body and appendages because of the expansion of chromatophores (Lightner and Redman, 1998b). Affected individuals become lethargic and reduce their feed intake (Chou *et al.*, 1995; Flegel, 1997) and mortality can reach up to 90-100% within 2-7 days after the first appearance of gross signs of disease. In grow-out ponds, juvenile shrimp of all ages and sizes are susceptible to the disease but massive mortality usually occurs 1 or 2 months after stocking (Kasornchandra *et al.*, 1998). In addition, the persistence of infection in the shrimp (*P. monodon*) population for a very long time in the absence of massive mortality has also been demonstrated (Tsai *et al.*, 1999).

Figure 1 Presence of white spots in the carapace of farmed *Penaeus monodon* infected with white spot syndrome virus.

1.2 Pathology

Histopathological studies have demonstrated hypertrophied nuclei with eosinophilic inclusion and marginated basophilic chromatin in the cells of ectodermal and mesodermal origin in the early stage of infection (Wongteerasupaya *et al.*, 1995). These intranuclear inclusions are markedly distinct and bigger than the Cowdry A-type inclusions characteristic of infectious hypodermal and haematopoietic necrosis virus (Wongteerasupaya *et al.*, 1995). In the late stage of infection, infected nuclei become progressively more basophilic and enlarged (Figure 2). Karyorrhexis and cellular disintegration may also occur in necrotic areas characterized by vacuolization (Chang, 1996; Kasornchandra *et al.*, 1998; Wang *et al.*, 1999b).

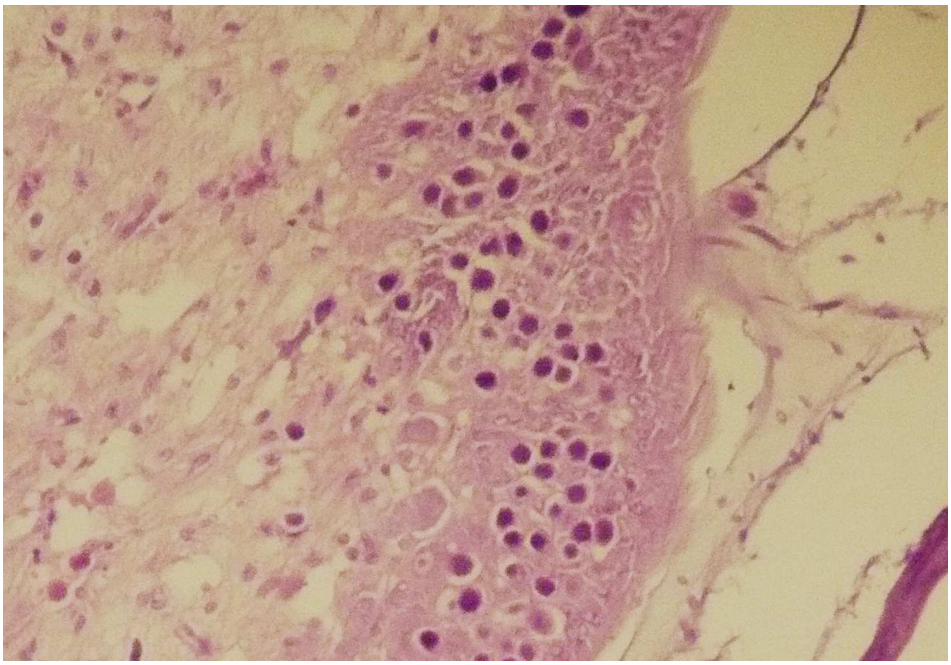

Figure 2 Histopathological lesions of white spot syndrome virus (WSSV) infection in cells of the stomach epithelium at the stage of WSSV infection showing hypertrophied nuclei with basophilic intranuclear inclusions (×400).

1.3 Pathogenesis

Simulated natural routes for experimental infection of WSSV have been developed. These inoculation methods incude waterborne challenge by immersing animals in water containing WSSV cell-free suspensions (Chou *et al.*, 1998) and by feeding them with WSSV-infected tissues (Lightner *et al.*, 1998). The ingestion route is considered to be the most important in natural and culture conditions (Chou *et al.*, 1998; Wu *et al.*, 2001; Lotz and Soto, 2002). According to experimental data on feeding shrimp with WSSV-infected tissues, the primary sites of WSSV replication in early juvenile *P. monodon* are the subcuticular epithelial cells of the stomach and cells in the gills, the integument and connective tissue of the hepatopancreas, as determined by in situ hybridization (ISH) (Chang, 1996). *P. monodon* inoculated by immersion showed many WSSV-positive cells in gills and only a few in the stomach epithelium. Recently, a standardized oral inoculation method was developed (Escobedo-Bonilla *et al.*, 2006). With the standardized inoculation technique, the primary sites of WSSV replication as determined by IHC were the epithelial cells in the anterior stomach chamber, cells in the gills, and only with a high dose (10 000 SID50), in cells of the antennal gland (Escobedo-Bonilla *et al.*, 2007).

The mechanism of viral spread from the primary replication sites to other target organs is still unclear. Several studies have indicated that WSSV infects shrimp haemocytes and travels throughout the body to the target organs (Wang *et al.*, 2002). Other studies have shown that WSSV might reach other target organs through haemolymph circulation in a cell-free form (Escobedo-Bonilla *et al.*, 2007). White spot syndrome virus targets cells of organs of ectodermal and mesodermal origin, including those of the epidermis, gills, stomach, hindgut (Wongteerasupaya *et al.*, 1995; Chang 1996), antennal gland, lymphoid organ (Durand *et al.*, 1996; Chang *et al.*, 1998a), muscles, eye-stalks, heart (Kou *et al.*, 1998), gonads (Lo *et al.*, 1997), haematopoietic cells and cells associated with the nervous system (Rajendran *et al.*, 1999; Wang *et al.*, 1999b). Epithelial cells of organs of endodermal origin such as the hepatopancreas, anterior and posterior midgut caeca and midgut trunk are refractory to WSSV infection (Sahul Hameed *et al.*, 1998). In the late stages of infection, the

epithelia of the stomach, gills and integument may become severely damaged (Chang 1996; Wang *et al.*, 1999b). This may cause multiple organ dysfunctions and it probably leads to death.

1.4 Transmission and Prevention

WSSV has two major routes of transmission, one is vertical transmission via an infectious brooder stocks. Accordingly, WSSVcan be found in the reproductive tissues of male and female *P. monodon* broodstock and in their postlarvae (PL) (Lo and Kou, 1998). Hence, infected PL are a major route of entry for WSSV into culture ponds (Limsuwam, 1997). The other is horizontal transmission via waterborne contact or oral ingestion from intake water and carriers. Many potential carriers of WSSV have been detected using PCR techniques. Results indicated that wild marine shrimp such as *Metapenaeus dobsoni, Parapenaeopsis stylifera, Solenocera indica, Squilla mantis*, small pest Palaemonid shrimp, copepods (subclass: Copepoda, *Schmackeria dubia*) and pupae of an Ephydridae insect were carriers of WSSV. Moreover, marine crabs such as *Charybdis annulata, C. cruciata, C. feriatus, Portunus pelagicus, P. sanguinolentus, Macrophthalmus sulcatus, Gelasimus marionis nitidus, Metopograpsus messor* and the pest crab *Helice tridens* also have the potential to be carriers of WSSV (Lo *et al.*, 1996a; Hossain *et al.*, 2001). Experimental infection studies have demonstrated that the freshwater crayfish (*Cherax quadricarinatus*), and the mud crab (*Scylla serrata*) (Chen *et al.*, 2000; Shi *et al.*, 2000) may also be carrieres. Experimental transmission has been investigated with *P. monodon* and *P. japonicus* via waterborne contact and oral ingestion (Chou *et al.*, 1998). In addition, transmission of WSSV from infected crabs (*Sesarma sp., Scylla serrata* and *Uca pugilator*) to shrimp *Penaeus monodon* has been achieved by cohabitation (Kanchanaphum *et al.*, 1998) (Appendex A).

WSD outbreaks can be prevented by eliminating potential vectors in intake water by filtering or chemical treatment of intake water and by screening PL for WSSV with the polymerase chain reaction (PCR) technique before stocking (Flegel, 1997). Although the PCR method has been used to screen for WSSV in PL

before stocking in many countries, the effectiveness has varied in different countries. Working in Thailand has shown that the 1- step PCR technique employing 50 PL may give 23- 43 % false-negative WSSV- PCR results (Withyachumnarnkul, 1999). This was supported by results from a comparative study done in India (Thakur *et al.*, 2002). Hence, the main reason of lack of success in PCR screening was false negative results (Chanratchakool and Limsuwan, 1998). For proposes of improving test sensitivity, it is now recommended that PL screening for WSSV be carried out using a nested PCR test with a sensitivity of less than 100 virions per PCR reaction vial and using a template DNA extract from samples of 300 PL adjusted to approximately 150 ng total DNA (never exceeding 300 ng) per reaction vial (Flegel, 2006). However, the MPEDA/NACA results suggest that the prevalence of WSSV among PL might be more important than the mere detection of the virus in the batch. Stocking density, for example, may play a critical role in the effect of WSSV in PL. Moreover, the sucessful screening of PL to decrease the risk for WSD outbreaks depended on the farming sytem (Corsin *et al*., 2005). No benefit of PCR screening was found in India and Vietnum where open and regular water exchange was routinely applied.

2. White spot syndrome virus (WSSV)

2.1 Morphology

White spot syndrome virus (WSSV), is a bacilliform, enveloped virus, with a double-stranded DNA genome (Wang *et al.*, 1995; Lightner, 1996). Intact enveloped virions range between 210 and 380 nm in length and 70–167 nm in width (Chang 1996). A tail-like appendage at one end of the WSSV virion can be observed in negatively stained electron micrographs (Wongteerasupaya *et al.*, 1995; Durand *et al.*, 1996) (Figure 3 a and b). The viral envelope is 6–7 nm thick and is a lipidic, trilaminar membranous structure with two electron-transparent layers divided by an electron-opaque layer (Wongteerasupaya *et al.*, 1995; Durand *et al.*, 1997). The nucleocapsid is located inside the envelope and is a stacked ring structure composed of globular protein subunits of 10 nm in diameter arranged in 14–15 vertical striations located every 22 nm along the long axis, giving it a cross-hatched appearance (Durand

et al., 1997; Nadala and Loh, 1998). When released from the envelope, the nucleocapsid increases in length indicating that it is tightly packed within the virion. The size of the nucleocapsid varies from isolate to isolate and ranges between 180 and 420 nm in length and 54–85 nm in width, with a 6-nm thick external wall (Kasornchandra *et al.*, 1998; Sahul Hameed *et al.*, 1998).

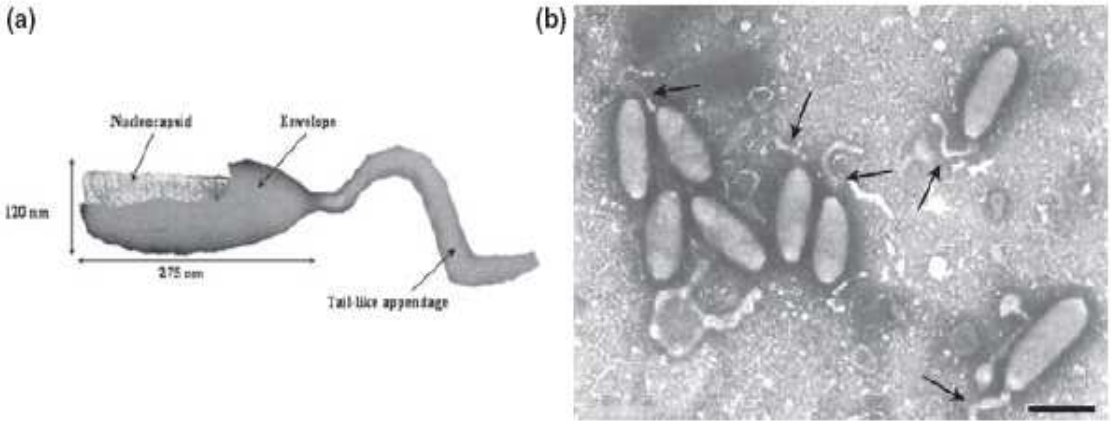

Figure 3 (a) Morphology of the white spot syndrome virus (WSSV) virion. (b) Electron micrograph showing WSSV virions with tail-like appendages (black arrows) (bar = 250 nm).

Source : Durand *et al.*, 1996

2.1 Genome and classification

The WSSV genome is a circular, ds DNA molecule with an A+T content of 59% homogeneously distributed. The genome size varies according to the viral isolate; 307 kb of Taiwan (WSSV-TW; AF332093; Wang *et al.*, 1995), 293 kb of Thailand (WSSV-TH; AF369029; van Hulten *et al.*, 2001b) (Figure 4) and 305 kb of China (WSSV-CN; AF440570; Yang *et al.*, 2001)

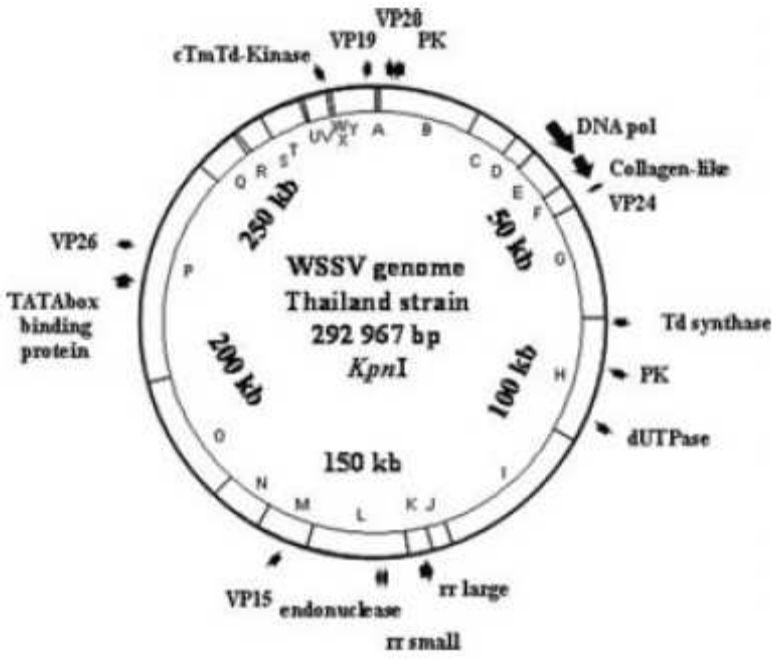

Figure 4 General structure of white spot syndrome virus genome Thai isolate (AF 369029).

Source : Escobedo-Bonilla *et al.*, 2008

Sequence analysis shows that the WSSV genome contains between 531 and 684 open reading frames (ORFs). Of these, 181–184 ORFs are likely to encode functional proteins with sizes between 51 and 6077 amino acids. These represent 92% of the genetic information contained in the genome (Van Hulten and Vlak, 2001; Yang *et al.*, 2001). About 21–29% of such ORFs have been shown to encode WSSV proteins or share identity with other known proteins. These proteins include enzymes involved in nucleic acid metabolism and DNA replication such as DNA polymerase (Chen *et al.*, 2002), a small and a large subunit of ribonucleotide reductase (van Hulten *et al.*, 2000b), thymidine kinase, thymidylate kinase and a chimeric thymidine–thymidylate kinase (Tsai *et al.*, 2000). At least 38 structural proteins have been located in the WSSV virion. These consist of, 21 envelope proteins, 10 nucleocapsid proteins and five tegument proteins (a putative structure located between the envelope and nucleocapsid) (Tsai *et al.*, 2004). A cell attachment motif that suggests a role in viral entry has been found in the envelope proteins VP31, VP110

and VP281 (Huang *et al.*, 2002; Tsai *et al.*, 2004; Li *et al.*, 2005; Xie and Yang, 2006), the tegument protein VP36A and the nucleocapsid protein VP664 (Tsai *et al.*, 2004; Leu *et al.*, 2005) and VP136A (Tsai *et al.*, 2004; Xie and Yang, 2006).

A recent report indicates deleted DNA at the ORF WSBV489, WSBV492, WSBV493, WSBV495 and WSBV479 can cause low virulence of WSSV (Lan *et al.*, 2002). ORF 151, 366 and 427 (Thai isolate) may encode putative proteins involved in WSSV latency (Khadijah *et al.*, 2003). Results from *in vivo* neutralization assays using antibodies against different structural proteins showed a significant delay of shrimp mortality, indicating that proteins such as VP28 (van Hulten *et al.*, 2001a), VP68, VP281, VP466 (Wu *et al.*, 2005) and VP24 (Xie and Yang, 2006), might have an important role in virus penetration. A 25-kDa membrane protein from shrimp haemocytes was found to bind to recombinant VP28 or WSSV virions. This protein has high homology to the small GTP-binding protein Rab7. *In vivo* neutralization assays with anti-Rab7 antibody inhibited the binding of WSSV virions to the cells and significantly reduced mortality upon WSSV challenge (Sritunyalucksana *et al.*, 2006). In crayfish, neutralization assays with the envelope proteins VP31, VP33 (also known as VP36B) and the tegument protein VP36A strongly inhibited WSSV replication, indicating that these proteins also have an important role in infection (Li *et al.*, 2006).

Sequence analysis of the DNA polymerase and the organization of several ORFs known to encode WSSV structural proteins were different from those of known baculoviruses, demonstrating that theWSSV is not closely related to this virus group (van Hulten *et al.*, 2000a). As WSSV is a distinct new virus, the International Committee on Taxonomy of Viruses (ICTV) approved the taxonomic position of the virus as a new viral family (*Nimaviridae*) and a new viral genus (*Whispovirus*) (Vlak *et al.*, 2005).

2.2 Genetic variability in WSSV strains

The various geographical isolates of WSSV identified so far are very similar in morphology and proteome. Limited differences in RFLP patterns have been

reported, suggesting a high degree of genomic stability (Lo *et al.*, 1999; Marks *et al.*, 2004). Little difference in virulence among various WSSV isolates has been reported (Wang *et al.*, 1999a). After complete sequencing of three different WSSV isolates, the major variable loci in the WSSV genome were mapped by alignment of those sequences (Marks *et al.*, 2004). All of the complete genome sequences have an overall identity of 99.32%. The five major differences that exist among the isolates include a large deletion region of ~13.2 kb in WSSV-TH and ~ 1.2 kb in WSSV-CN relative to WSSV-TW, a variable region prone to recombination, a transposase sequence present only in WSSV-TW, variation in the numbers of repeats in variable number tandem repeat (VNTR) regions, single nucleotide indels and single nucleotide polymorphrisms (SNP) (Dieu *et al.*, 2004; Shekar *et al.*, 2005). The variations associated with ORF 23/24 and ORF 14/15 within WSSV-TH are prone to deletion and recombination events, respectively, and are reported to be useful in identifying evolutionary changes in WSSV (Marks *et al.*, 2005).

3. Diagnosis of white spot disease

An efficient disease control programme must include the prompt reporting of outbreaks and rapid and accurate diagnosis. Since the shrimp immune system lacks immunoglobulins (Ig), T cell receptors (TCR) and the major histocompatibility complex (MHC), identification of the agent itself is the only way to diagnose WSD (Arala-Chaves and Sequeira, 2000).

3.1 Histology

The most commonly applied laboratory test is direct microscopic examination and routine histology and histochemistry. Histological findings typical of WSSV infection include enlarged nuclei in tissues of ectodermal and mesodermal origin. The most convenient tissue for diagnosis is the subcuticular epithelium. Usually, the subcuticular epithelium of the stomach provides excellent views revealing pathognomonic enlarged nuclei containing basophilic inclusions and surrounded by vacant cytoplasm. Nuclei at the early stage of infection show Cowdry

A-type inclusions (i.e., marginated chromatin separated from a central reddish inclusion by a ring of unstained nucleoplasm). In addition to tissue sections, it is also possible to prepare rapidly stained whole gill mounts and sub-cuticular epithelial tissue. These show the same histologocal signs seen in tissue sections but can be prepared much more conveniently and much faster than tissue sections (Alday de Graindorge and Flegel, 1999; Flegel, 2006). Other important techniques used less frequently are bioassay and enhancement that are employed for the detection of subclinical or carrer-states of infection (Lightner, 1996).

3.2 Virus isolation

Only primary shrimp cell cultures have been successfully prepared from the lymphoid organ, heart (Nadala *et al.*, 1993; Tong and Miao, 1996) and ovaries (Kasornchandra *et al.*, 1999). This limits the use of cell cultures to isolate and assay the virus.

3.3 Serological methods

Polyclonal antibodies against VP19 and VP26 structural proteins (Chaivisuthangkura *et al.*, 2006a, 2006b) and also a monoclonal antibody against VP28 structural protein (Chaivisuthangkura *et al.*, 2004) have been developed. Several serodiagnostic methods have been developed for use in shrimp disease diagnosis (Lightner and Redman, 1998a; Poulos *et al.*, 2001; Liu *et al.*, 2002; Okumura *et al.*, 2004). Assays utilizing HRP-conjugated virus-specific polyclonal antibodies have been developed for detection of the WSSV in gill homogenates of infected shrimp spotted onto nitrocellulose membranes (Nadala and Loh, 2000). Lateral flow chromatographic detection strips have also been described (Powell *et al.*, 2006; Sithigorngul *et al.*, 2006; Wang and Zhan, 2006).

3.4 Nucleic acid recognition methods

3.4.1 Polymerase chain reaction (PCR)

PCR has been documented for the diagnosis of WSSV. Numerous primer sets have been developed from different countries (Lo *et al.*, 1996b; Takahashi *et al.*, 1996; Boonyawiwat *et al.*, 2000; Hsu *et al.*, 2000; Kiatpathomchai *et al.*, 2001; Tsai *et al.*, 2002; Hossain *et al.*, 2004). The method described by Lo *et al.* (1996b) is the standard used by the International Organization for Animal Health, although a current publication suggests, this test may give false positive results with the Australian crayfish *Cherax quadricarinatus* (Claydon *et al.*, 2004). Methods for real-time PCR (Dhar *et al.*, 2001) and isothermal DNA amplification (Kono *et al.*, 2004) have also been described. PCR has been applied to pathogen detection, research for new hosts and pathogenicity studies of the WSSV.

3.4.2 In *situ* hybridization

Non-radioactive labeled DNA probes have been developed for investigating the presence of WSSV in tissue samples (Nunan and Lightner, 1997; Wang *et al.*, 1998a). The results from *in situ* hybridization examination showed positive signals in the following tissues and organs: pleopods, gills, the stomach, muscles, hemolymph, the midgut, the heart, pereiopods, the lymphoid organ, the integument, nervous tissue, the hepatopancreas, testes, ovaries, spermatophores, compound eyes and eye stalks (Lo *et al.*, 1997).

4. Epidemiological study of WSSV

During the period of initial WSD outbreaks, the scientific community hypothesized a number of potential risk factors for outbreaks based on information from other diseases, experimental tests, pathogenicity studies and circumstantial evidence. For example, natural carriers were found to be potential sources of disease transmission (Kanchanaphum *et al.*, 1998), as were infected postlarvae (Withyachumnarnkul, 1999) and contaminated water (Chou *et al.*, 1998). Stress (Sudha *et al.*, 1998) was also suggested to be a possible risk factor for WSD outbreaks. Lotz *et al.*, 2001 has suggested that two basic epidemiologic approaches

can be used for epidemiology studies in shrimp aquaculture disease control. One approach consists of statistical epidemiology that involves the application of statistical models to identify factors that are associated with the expression of disease. The other is dymanic epidemiology that involves attempts to understand epidemics from a cause and effect relationship, and focuses on the process of transmission and spread of pathgens. However, to date only a limited number of field studies have been conducted and, of those, only a few have used an epidemiological approach (Thompson *et al.*, 1997; Corsin *et al.*, 2005). Among those, the most extensive investigations were conducted in Vietnam (Corsin *et al*., 2001, 2002). The statistical epidemiology studies on the Vietnamese rice-shrimp farming system indicated that pond location, average weight at 1 month after stocking and earlier date of stocking consitituted risk factors for WSSV infection (Corsin *et al.*, 2001). However, variability in environment and management practices of shrimp culture may lead to differences in the risk factors associated with WSD outbreaks.

Prevalence of WSSV in PL of *P. monodon* has been studied in India. The results showed that the prevalence in hatchery tanks varied from 15 –92 % in different provinces, with sample sizes of 150 PL per batch. Moreover, the study showed that testing needed large sample sizes of PL in order to reduce errors of false negative results (Thakur *et al.*, 2002).

Dynamic epidemiology studies have been conducted to estimate the parameters (i.e. transmission rate, virulence rate and recovery rate) of WSSV infections in populations of *L. vannamei* and *L. setiferus* in the laboratory (Soto and Lotz, 2001). The results indicated that ingestion of cadavers of infected shrimp was a more important mode of transmission for WSSV than cohabitation. On the other hand, similar studies have never been performed using shrimp from production ponds. The S-I-R model introduced by De Jong (1995) has played a major role in mathematical /quantitative epidemiology. In the S-I-R model, a population is divided into three groups: the susceptible S, the infectives I, and the recovered R, indicated by symbols s, i, and r respectively. The total population is $n = s + i + r$. The susceptible are those

that are not infected and not immune, the infectives are those that are infected and able to transmit the disease, and the recovered those that have been infected but are immune (recovered).

Transmission of WSD between ponds can be expressed as the pond reproduction ratio, R_h, which is defined as the average number of outbreaks caused by one infectious pond. From the definition of R_h is >1, the virus will continue to spread. Only when the number of infected ponds is small, in the initial phase of an epidemic, may chance processes result in the extinction of an epidemic even though R_h is >1 (Metz, 1978). From the above, it follows that measures to eliminate WSSV must reduce R_h to below 1. Measures that reduce the transmission of a pathogen between ponds include decreases in one or more of the following aspects (adapted from Koopman and Longini, 1994): (1) the infectivity of infected ponds (the number of shrimp that excrete virus); (2) the susceptibility of non-infected ponds; (3) the amount of viable virus that is transferred during a contact; (4) the rate at which contacts occur; and (5) the number of different ponds that come into contact.

5. Study of molecular markers of white spot syndrome virus

Genomic analyses of WSSV indicated that conserved genes, often used in molecular epidemiological studies to unwind evolutionary relationships by phylogenetic analysis, are too homologous for this purpose (Marks *et al.*, 2004). For instance, the complete DNA polymerase gene of WSSV contains only three SNP and a 1 bp and 3 bp deletion when the three completely sequenced WSSV isolates were compared (Chen *et al.*, 2002; Marks *et al.*, 2004). Similar high homologies were found for other conserved WSSV genes (Chang *et al.*, 2001). Moreover, the major structural protein genes, which for some virus families show relatively large numbers of mutations due to antigenic drift or adaptation to different hosts, showed 99.5 - 100% nucleotide homology between several geographical WSSV isolates (Marks *et al.*, 2004). The restriction fragment length polymorphism (RFLP) patterns of isolates show only limited differences, suggesting a high degree of genomic stability (Nadala and Loh, 1998; Lo *et al.*, 1999; Wang *et al.*, 2000). These data indicate that the

WSSV isolates are very closely related and probably evolved recently from a common ancestor.

Study on the major variable region ORF23/24 and minor variable region RF14/15 showed various sizes of deletions among different geographical isolates. Based on gradually increasing deletions of both variable regions from a study in Vietnam (VN), it was suggested that the VN isolations and WSSV-TH had a common lineage that branched off from WSSV-TW and WSSV-CH early on, and that WSSV entered Vietnam by multiple introductions.

Tandem repeat loci exhibiting variability in their copy numbers are referred to as variable number tandem repeats (VNTR). VNTR of microsatellites (with repeat unit tracts ranging from 1-6 bp) and minisatellites (repeat unit tracts of 7-100 bp) occur in both prokaryotic and eukaryotic genomes. Within genomes, VNTR can be located in either protein-coding or non-coding regions. Studies on the inter-individual variability in copy numbers of VNTR have found application in DNA fingerprinting in human and other organisms. In bacteria, in addition to studying genotypic variation, VNTR serve as potential markers for the identification of pathogenic bacteria and for virulence factors associated with their pathogenicity.

A number of variable microsatellites, minisatellites and megasatellites have been reported as markers for WSSV. The variable number tandem repeats (VNTR) associated with the 3 minisatellites, ORF94, ORF75 and ORF125, have been suggested as potential markers for epidemiological studies (Dieu *et al.*, 2004; Marks *et al.*, 2004; Shekar *et al.*, 2005). The minisatellite in coding regions of ORF94, ORF125 and ORF75 consist of a 54, 69 bp uniform repeats and a compound repeat of 45 and 57 bp, respectively. Wongteerasupaya *et al*. (2003) first demonstrated a practical method of differentiating WSSV genotypes based on the VNTR associated with ORF94 located between genes encoding the large (RR1) and small (RR2) subunits of the ribonucleotide reductase gene. After that, the ORF94 locus was used for genotyping WSSV and for studing the distribution of genotypes in various locations (van Hulten *et al.*, 2000b; Wongteerasupaya *et al.*, 2003; Dieu *et al.*, 2004;

Hoa *et al.*, 2005; Pradeep *et al.*, 2008). Recently, three minisatellites have been used for variation studies in India and Vietnam (Dieu *et al.*, 2004; Pradeep *et al.*, 2008). The results suggested that an important source of infection was infected postlarvae (Wongteerasupaya *et al.*, 2003; Pradeep *et al.*, 2008)

MATERIALS AND METHODS

1. Study 1 of risk factors for WSD in black tiger shrimp

The retrospective study was conducted on a shrimp farm selected because of its good record system for risk factor analysis for WSD (i.e. environmental factors, management, post-larvae quality, disease prevention protocols, production data and WSSV outbreak data). Univariate and multivariate logistic regression were used to identify and quantify the risk factors. Commercial computer software was used for analysis.

1.1 Study site and general farm information

Data for this study were extracted from all the pond records produced at an intensive black tiger shrimp (*Penaeus monodon*) farm in the southern part of Thailand. The farm consisted of 70 culture ponds. The data from 8 continuous production cycles from January 1998 to January 2002 were used. Because of the irregular nature of the farm operation, the number of operated ponds varied over the 8 production cycles studied, and were 42, 46, 55, 47, 56, 26, 77 and 35 ponds. Thus, a total of 384 pond records were analyzed in the study. The farm was operated on a semi-closed intensive system in which the reservoir volume was around 30% of the pond volume used for shrimp culture. Two shrimp production cycles were completed in each year. The distance between the farm and the seaside was approximately 1 kilometer (km). The water for farming was supplied by the two canals located on the left and right hand sides of the farm. Several other nearby farms of medium (5-20 ponds) and small (less than 5 ponds) size were situated within a 5 km radius around the studied farm and some of these nearby farms also used water supplied from the same canals as the study farm (Figure 5). The salinity of the water changed over the period of a year. High salinity occurred during the summer period while low salinity occurred during the rainy season. Ponds were cleaned by the drying method (Chanratchakool *et al.*, 1998). During the pond preparation process, insecticides (e.g. trichlorfon) and disinfectants (e.g. calcium hypochlorite, povidone iodine,

benzalkonium chloride) were used to eradicate diseased carriers (e.g. wild shrimp, crabs, insects, etc.) and pathogenic microorganisms such as the WSSV.

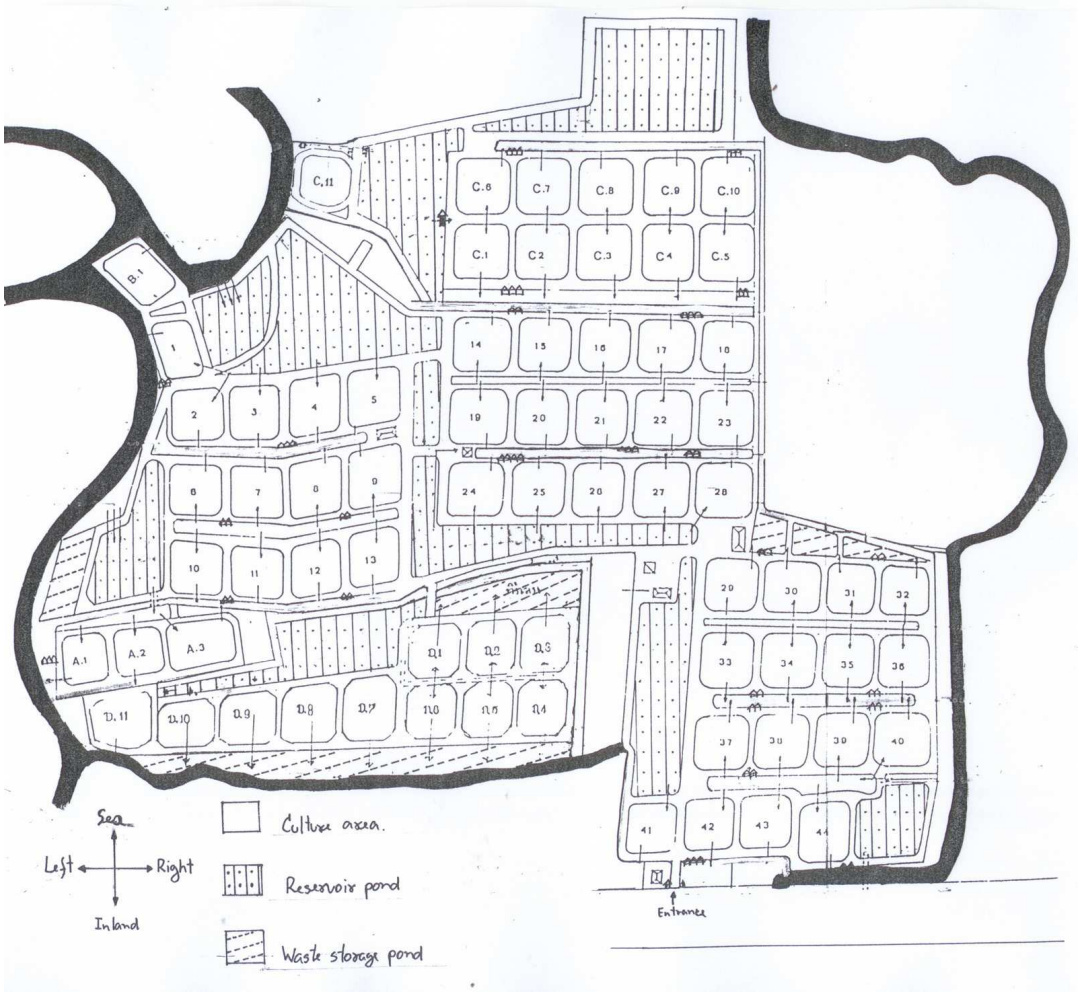

Figure 5 Sketch of the farm studied showing 70 culture ponds and reservoir ponds.

Postlarvae (PL) were obtained from many private hatcheries. All of the stocked postlarvae were negative for WSSV infection with 1-step PCR testing on pooled samples (50 post larvae). These shrimp also tested negative for monodon baculovirus (MBV) infection using the impression smear technique and also gave good scores using a PL quality assessment method (e.g. behavior, proportion of muscle to gut in the last abdominal segment, morphology, formalin stress test, etc.). The caretaker fed commercial pelleted feed on the first day of stocking at a rate of 0.5 to 2 kg/100,000 PL and then adjusted the feed amount by following the feed

manufacturer's instructions until 30 days post stocking. Thereafter, feed nets were used to control the feeding rate. Fresh feed was added to some of the ponds that experienced slow growth rates, especially when the production cycle was getting close to the end. No water was exchanged until at least 40 days post stocking and minimum water exchange was used to reduce the risk of disease transmission. Early harvesting was carried out soon after a pond experienced a WSD outbreak.

1.2 Description of variables

The clinical WSD outbreak pond was the only dependent variable considered in this study. The independent variables consisted of nine factors related to management practices and one environmental factor. All variables from each pond record used in the study are listed and briefly explained in Table 2.

1.2.1 Dependent variable

The clinical WSD outbreak pond (1 = yes, 0 = no) was the dependent variable. Any ponds that gave a positive WSSV infection result from testing by 1-step PCR in pooled samples of moribund shrimp was categorized as a WSD outbreak pond. The PCR testing was carried out on ponds with clinical signs of white spots in the cuticle and a drop of more than 15% in feed consumption rate compared to the preceding day. In case of negative PCR results, repeated testing was carried out during the following 5 to 7 days until the pond recovered (absence of clinical signs and increased feed consumption) or until it gave a positive result.

Table 2 Variables used in the study of risk factors for WSSV outbreaks (384 ponds records, Thailand, 1998-2002).

Variables	Units of interest	Description
Dependent		
Clinical WSD outbreak pond	pond (yes = 1; no = 0)	Whether or not shrimp in ponds showed white spots in the cuticle, reduced feed consumption more than 15% in a day and a positive result for WSSV by 1-step PCR
Independent		
Management practices		
Age of postlarvae (PL)	day (#)	Age of shrimp at stocking date
Duration of pond drying	day (#)	The duration from the harvest date of previous crop to the date of pumping water into the pond for the succeeding crop.
Duration of pond preparation	day (#)	The duration from the date of pumping water into the pond until the stocking date.
WSD outbreak in the former crop	pond (yes = 1; no = 0)	Whether or not a WSD outbreak occurred in the pond during the previous crop.
Stocking density	shrimpm^{-2} (#)	Stocking density of PL at the stocking date
Pond size	hectare (#)	Size of the pond
Used disinfectant	pond (yes = 1; no = 0)	Whether or not a disinfectant was applied to the water during the pond preparation periods
Used carricide	pond (yes = 1; no = 0)	Whether or not a carricide was applied to the water during the pond preparation periods
Water depth	cm (#)	Depth of water in the ponds
Environment		
Season (season)	pond (rainy-winter = 1; summer = 0)	Whether ponds were stocked during the rainy-winter season or summer season.

1.2.2 Independent variables

Ten independent variables were investigated. These were categorized into 2 types. The management practice variables consisted of 9 variables related to the production cycle. Of the 9 variables, 6 were continuous variables including age of postlarvae at stocking date (# of days), duration of pond drying (# of days), duration of pond preparation before stocking (# of days), stocking density (# shrimp m^{-2}), pond size (# hectares), water depth (# cm). The other 3 variables were categorical variables including WSD outbreaks during the previous crop and use of disinfectants and insecticides during pond preparation.

Season was considered as an environmental factor. After preliminary data analysis by plotting shrimp stocking day (365/year) against pond record number, we found that the 2 crops per year could be categorized into 2 crop operation seasons (Figure 6). Ponds categorized into the summer season were stocked during the interval January 1 to June 15 while those categorized in the rainy-winter were stocked at any other time of year.

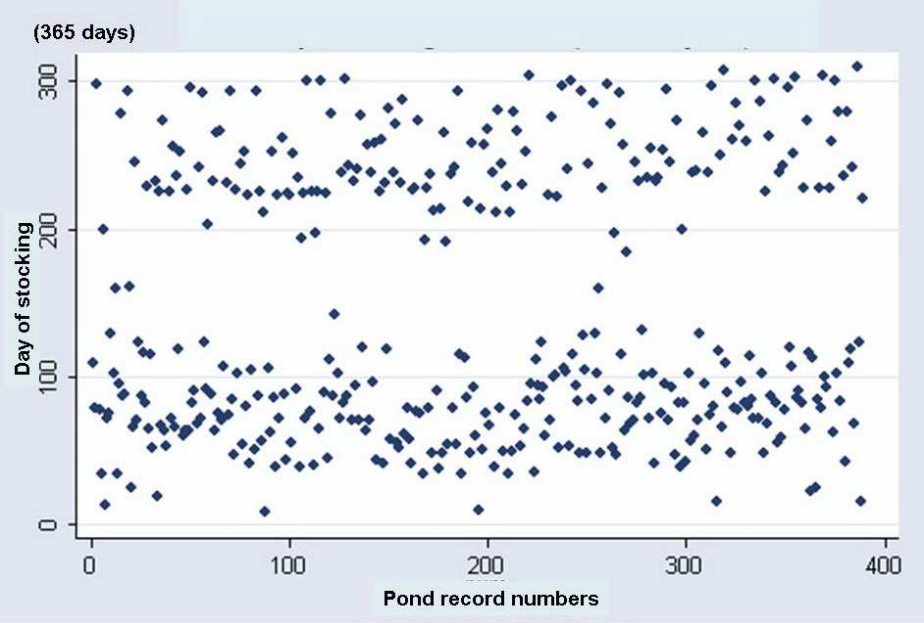

Figure 6 Plot of stocking day versus pond record number.

1.3 Statistical analysis

All of the descriptive statistics, data manipulation, logistic regression and generalized estimating equation (GEE) models were accomplished using the statistical software package STATA (Version 8.2, Stata Corp, College station, TX). The associations between predictor variables and an outcome variable were evaluated using a smoothed scatter plot of the log-odds of the outcome against the predictor variable prior to the logistic regression analysis. The logistic regressions were performed according to maximum-likelihood logit models. All the independent variables were initially run in a univariate regression analysis with WSD status as an outcome variable, and those giving *P*-values < 0.10 were selected for further analysis. To identify if predictors were highly correlated, simple (linear) correlations among factors were determined. The best-fit model was found by a manual backward selection process in which the likelihood-ratio test (LRT) was used to test the significance (*P*-value < 0.05) of subtracting one variable at a time from the models. The production cycle variable was included in the model to take an account of the hierarchical effects of repeated measures nested within a production cycle. Logistic regression model evaluations were performed if the model fit according to the Hosmer-Lemeshow goodness-of-fit test. Consequently, GEE models with an exchangeable correlation structure were used for building the final model to collect the potential hierarchical effects within a pond.

2. Study 2 on the potential for horizontal transmission of WSD between ponds

2.1 Model to quantify transmission of WSD between ponds.

The transmission of WSSV between ponds can be estimated from the relationship between the number of ponds newly infected per unit of time (that is the number of virus introductions per unit of time) and the number of infectious ponds present during the same unit of time. If there have been no new introductions from outside, every new infection must have been caused by one of the ponds that was infectious at the time of virus introduction. In this analysis, it was assumed that a

simple deterministic SIR model (De Jong, 1995) could describe the transmission of WSSV between ponds. In this model, *S* is the number of susceptible ponds, *I* is the number of infectious ponds and *R* is the number of recovered ponds. Because ponds were depopulated upon detection and there were no ponds in which a major outbreak had faded out before depopulation, $R = 0$ at all times in this study. In the model, the rate at which susceptible ponds become infected can be described as: $C = ß = SI/N$, in which *C* is defined as the number of virus introductions per unit of time into a susceptible pond and ß as the infection-rate parameter. In order to estimate *ß*, the parameters *C*, *S*, *I* and *N* have to be calculated from the data collected during an epidemic. Furthermore, infected ponds were depopulated at the rate of $D = \alpha \times I$, in which *D* is defined as the number of infected ponds depopulated per unit of time and α as the depopulation-rate parameter. The parameter α is the inverse of *T*, the average period that a pond is infectious. Finally, R_h can be estimated from (De Jong, 1995): $R_h = ß / \alpha$. To evaluate the potential for horizontal transmission between ponds during various seasons, it was necessary to estimate *ß* and R_h for the rainy-winter and summer seasons.

2.2 Data extraction and phase of study

The same data used in study 1 were transformed into date-based data. The 8 continuous production cycles from January 1998 to January 2002 were examined. Since WSSV can be transmitted either vertically or horizontally, the cases had to be divided accordingly as a production cycle that did not have an outbreak pond suspected to arise via vertical transmission, or a production cycle that had an index case (first outbreak pond of the crop) related to vertical transmission. The incubation period following stocking infected WSSV-PL into the ponds was 40-45 days (Withyachumnarnkul *et al*., 1999). Therefore, ponds where disease occurred prior to 50 days post stocking and in the absence of water exchange were categorised as cases of vertical transmission. Using these criteria, only the first to third (from January 1998 to August 1999) production cycles were included in the analysis. In study 2, we evaluated the transmission of WSSV during 3 phases of the epidemic. The first (15/1/1998 to 31/8/1998) and third (1/2/1999 to 31/8/1999) phases occurred in the

summer season. The second (1/9/1998 to 31/1/1999) phase occurred in the rainy-winter season.

2.3 Estimation of the model input parameters

Section 2.1 shows that to estimate *ß* for each unit of time, it was necessary to establish *S*, *C*, *I* and *N* in that unit of time. In subsequent estimation for R_h, we also had to calculate *T* (1/ α). In the following sub-sections, it is explained how estimations were done for *S*, *C*, *I* and *T* from the data collected during an epidemic using the formula $N = C+I+S$.

2.3.1 Number of susceptible ponds (*S*)

The operation ponds were increased by stocking of PL into empty ponds. Therefore, the number of susceptible ponds changed over time. During an epidemic, *S* decreased by emergency harvesting of the WSSV-infected ponds and by normal program harvesting (Table 3). Therefore, the outcome of the model changed considerably. This is because the ratio of the number of infectious ponds to the number of susceptible ponds at any given time changed extremely as a result of stocking rate and harvesting rate.

2.3.2 Number of newly infected ponds per unit period of time (*C*)

During an epidemic, it is usually unclear for most of the infected ponds when the virus was introduced (Withyachumnarnkul, 1999). However, it is possible to estimate this time from the incubation period. The incubation period in experimental infections of WSSV is 4 to 7 days (Pratanpipat *et al.*, 1996). Subsequently, to create the day that ponds changed from susceptible to newly infected ponds we randomly selected a number of incubation periods between 4 to 7 days for each infected pond.

Table 3 Estimates of the number of ponds newly infected (*C*), the number of infectious ponds (*I*), the number of susceptible ponds (*S*) and the infection rate parameter (*ß*) for each week of the epidemic.

Week	Phase	*C*	*I*	*S*	*ß*
98-18	1	0	0	41	0
98-19	1	0	0	40	0
98-20	1	0	0	40	0
98-21	1	0	0	40	0
98-22	1	4	0	36	0
98-23	1	2	2	30	1
98-24	1	1	1	26	2
98-25	1	0	0	23	0
98-26	1	1	0	21	0
98-27	1	0	1	19	0
98-28	1	0	0	16	0
98-29	1	0	0	14	0
98-30	1	0	0	13	0
98-31	1	0	0	15	0
98-32	1	0	0	18	0
98-33	1	0	0	25	0
98-34	1	0	0	30	0
98-35	1	0	0	36	0
98-36	2	0	0	39	0
98-37	2	0	0	42	0
98-38	2	0	0	44	0
98-39	2	0	0	43	0
98-40	2	0	0	44	0
98-41	2	1	0	44	0
98-42	2	0	1	44	0
98-43	2	1	0	43	7

Table 3 (Continued)

Week	Phase	*C*	*T*	*S*	*B*
98-44	2	3	1	42	3
98-45	2	4	1	40	4
98-46	2	6	3	34	3
98-47	2	3	5	28	1
98-48	2	2	3	24	1
98-49	2	2	2	21	1
98-50	2	3	0	19	8
98-51	2	4	2	14	2
98-52	2	1	2	13	1
99-01	2	4	1	10	4
99-02	2	0	1	6	0
99-03	2	2	0	4	20
99-04	2	0	1	1	0
99-05	2	0	1	0	0
99-06	3	0	0	0	0
99-07	3	0	0	4	0
99-08	3	0	0	12	0
99-09	3	0	0	16	0
99-10	3	0	0	25	0
99-11	3	0	0	37	0
99-12	3	0	0	46	0
99-13	3	0	0	55	0
99-14	3	0	0	56	0
99-15	3	0	0	56	0
99-16	3	0	0	56	0
99-17	3	0	0	56	0
99-18	3	0	0	56	0
99-19	3	0	0	55	0

Table 3 (Continued)

Week	Phase	*C*	*T*	*S*	β
99-20	3	0	0	55	0
99-21	3	1	0	54	0
99-22	3	0	0	54	0
99-23	3	2	0	53	0
99-24	3	1	1	52	1
99-25	3	0	1	51	0
99-26	3	2	1	48	2
99-27	3	1	1	44	2
99-28	3	1	0	37	0
99-29	3	2	2	31	3
99-30	3	0	0	27	0
99-31	3	0	0	21	0

2.3.3 Number of infectious ponds (*I*)

The infectious ponds are those that are infected and can transmit the disease. Usually, shrimp contacts between ponds are impossibile. The pattern of disease spread in farms suggests that the virus can be transferred among ponds by water borne vectors (Kanchanaphum *et al.*, 1998; Supamattaya *et al.*, 1998) such as crabs or dead shrimp dropped by birds during WSD outbreaks. For this analysis, a pond was considered infectious from the day the shrimp showed clinical signs. Then, the spread of virus to other ponds would begin. An infected pond was removed from the analysis on the day it was emergency harvested.

2.3.4 Average period that a pond is infectious (*T*)

The duration from the date that a pond became infectious until the date that it was emergency harvested is *T* in the model. The time between the

appearance of clinical signs and positive results for PCR analysis varied depending on the attention of the caretaker and the sensitivity of the test. Therefore, for each separate phase, a specific *T* had to be determined.

2.4 Analysis of data

Beginning with 15 January 1998, the number of newly infected ponds, the number of susceptible ponds and the number of infectious ponds were estimated for each week as described in Section 2.3. The numbers of susceptible and infectious ponds were corrected for the day in the week when the pond was infected or became infectious, respectively. For example, a pond that became infected on Friday of a week had infectivity of 3/7 during that week. Using the model described in Section 2.1, *ß* was estimated for each week. Subsequently, we estimated the average *ß* for each of the three phases (p1-p3) described in Section 2.2. The values of *ß* were compared non-parametrically. Using Kruskal±Wallis ANOVA, we first tested H0: *ß*1 = *ß*2 = *ß*3. The null hypothesis was rejected, if the *P*-value was less than 0.05. In that case, the distribution in a phase was compared with the distribution within the preceding phase by the Mann-Whitney U test. When performing two pair-wise comparisons according to Bonferroni inequality, these null-hypotheses are rejected if the *P*-values < 0.025 (0.05/2) (Miller, 1966).

Furthermore, *T* was estimated for each of the three phases as described in Section 2.3.4. The rankit plots and Wilk±Shapiro statistics showed that *T* did not have a normal distribution in all of the three phases. Using the Kruskal±Wallis ANOVA we first tested H0: *T*1 = *T*2 = *T*3. If H0 was rejected, then all *T*'s were compared pair-wise by the Mann-Whitney U method. Since there were two pair-wise comparisons, according to Bonferroni inequality, differences were considered significant if the P-value < 0.025 (0.05/2).

Finally, R_h was estimated from *ß* x *T* for each of the three phases. Under the assumption that *ß* and *T* are independent of each other within a phase, the standard deviation of R_h was estimated according to the following formula.

$$sd(R) = \sqrt{(\text{var } ß \text{ x var}T + ß^{-2} \text{ x var}T + T^{-2} \text{ x var } ß)}$$

However, because the distribution of R_h was not known, the different observations of this parameter could not be compared statistically. In contrast to *ß*, the values of R_h for the three phases could not be compared by a non-parametric statistical test either. This is because weekly estimates of R_h were not available (i.e. because we had no weekly estimates of T).

3. Study 3 on the molecular epidemiology of white spot syndrome virus

3.1 Analysis of complete WSSV gemones for the presence of VNTR loci

The three complete WSSV genomes in GenBank (accession number AF332093 (Taiwan), AF369029 (Thailand) and AF4450570 (China) were analyzed for the presence of tandem repeats using the Tandem Repeats Finder (TRF) program (Benson, 1999). The program was run with the parameters set to +2, -7, -7 (match, mismatch, indel), minimum score adjusted to 30 and the maximum period size 200 bps. Tandem repeats were thus obtained according to genomic location. For comparative analysis, the repeats obtained were compared for similarity regions and for copy variability within these regions using BLAST and ClustalW programs incorporated in 'BioEdit' Sequence Alignment Editor Program version 7.0.1. The presence of repeats within coding and non-coding regions was identified based on the annotation of the WSSV genomes in GenBank.

3.2 Design of primers and optimizing PCR conditions

The franking regions of the same tandem repeat loci of all of the complete WSSV genomes were aligned with ClustalW to find conserved regions. New primers were designed using PRIMER3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For ORF94 (Wongteerasupaya *et al.*, 2003), ORF75 (Dieu *et al.*, 2004) and ORF125 (Pradeep *et al.*, 2008) previously described primers

were used (Table 4). New primers were tested with WSSV DNA using thermal gradient PCR. The temperature range 52-58 ^{o}C was evaluated.

Table 4 Primers used for PCR analysis in WSSV genotyping

Primers specific for	Primer	Sequence (5'-3')	Source of Primers
WSSV	W201F	CAAGGACT(CT)TGCACTAGACAA	Boonyawiwat *et al.*, 2000
	W201R	GAGGAGGTACATCCACTGTT	
ORF66	ORF66F	ACCAATGGGAGTGCCAGTAA	This study
	ORF66R	TGGGAAGTGGGTTGGTATTC	
ORF75	ORF75 Flank(F)	GAAGCAGTATCTCTAACAC	Pradeep *et al.*, 2008
	ORF75 Flank(R)	CAACAGGTGCGTAAAAGAAG	
ORF76	ORF76F	TGGAGTATGGAAAGCACCAG	This study
	ORF76R	TGCTATGAGCAAAGAGCAAGTG	
ORF84	ORF84F	GGGAAATACTTGCCCAACAA	This study
	ORF84R	TTGGACGTGATTTCTGTACCC	
ORF94	ORF94-F	TCTACTCGAGGAGGTGACGAC	Wongteerasup aya *et al.*, 2003
	ORF94-R	AGCAGGTGTGTACACATTTCATG	
ORF116	ORF116F	TCGCATTGGAAGATTTCTTG	This study
	ORF116R	ACCCTTCTGCTGCAAGCAT	
ORF125	ORF125F	TGGAAACAGAGTGAGGGTCA	Pradeep *et al.*, 2008
	ORF125R	CATGTCGACTATACGTTGAATCC	

3.3 Shrimp specimens

Altogether, 49 WSSV DNA samples were prepared from shrimp specimens from ponds experiencing outbreaks of WSD from Chacheongsao, Surat Thani, Chumporn and Nakhonsrithumarat Province during the interval October 2000 to January 2002. These were kindly provided by Dr. Chinarong Wongteerasupaya. A further 15 specimens were obtained from Nakhonpathom province during October 1999 to January 2000 from ponds exhibiting clinical signs of WSD. Gills from moribound shrimp were clipped with cleaned scissors and placed directly in 95% ethanol for transport to the laboratory.

3.4 Nucleic acid purification

Viral DNA was extracted from clinical samples according to the protocol published by Lo *et al.* (1996b). In brief, approximately 100-200 mg of shrimp tissue were homogenized with a disposable stick in a 1.5 ml microfuge tube with 600 ul of lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH8, 25 mM EDTA (ethylene diamine tetra-acetic acid), 0.5% SLS (sodium N-Laurylsarcosinate) or 2% SDS (sodium dodecyl sulfate) and 0.5 mg/ml proteinase K (added just before use). After homogenization, samples were incubated at 65^{o}C for 1 hour before addition of 5 M NaCl to a final concentration of 0.7 M. Next, 1/10volume of N-cetyl N,N,N-trimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7M NaCl) was slowly added with thorough mixing. After incubatting at 65^{o}C for 10 minutes, and then at room temperature, an equal volume of chloroform/isoamyl alcohol (24/1) was added with gentle mixing. This was followed by centrifugation at 13,000 *g* for 5 minutes and transfer of the aqueous solution to a fresh 1.5 ml tube and the phenol extraction process was repeated 1 to 2 times. The final upper layer was collected to a new 1.5 ml tube and mixed gently with two volumes of chloroform/isoamyl alcohol (24/1) and centrifuged at 13,000 *g* for 5 minutes. The upper layer was transfered to a new 1.5 ml tube and the DNA was precipitated by adding two volumes of 95% or absolute ethanol followed by standing at –20 ^{o}C for 30 minutes or –80^{o}C for 15 minutes. After, centrifugation at 13,000 *g* for 30 minutes, the ethanol was discarded

and the DNA pellet was washed with 70% ethanol, dried and resuspended in 100 ul sterilized double-distilled water at 65 ^{o}C for 15 minutes. It was stored at –20 ^{o}C until used. A quantity of 1 ul of this DNA solution was used for one PCR reaction.

3.5 Diagnostic PCR for WSSV.

Samples containing WSSV DNA were identified using 1-step PCR using primers WSV201 F/R (Table 2). The reaction mixture contained 10Xbuffer (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 uM each of dATP, dTTP, dCTP and dGTP and 2.5U Fhusion TaqDNA polymerase (FINNZYMES®) (Appendix B). The mixture was incubated in the DNA Engine DYAD (MJ Research) thermo cycler using 35 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 30 s and 72 ^{o}C for 30 s, and a final elongation step at 72 ^{o}C for 10 min. PCR products were resolved by 1.5% agarose gel electrophoresis at 5 V cm^{-1}. Cycling conditions and expected product sizes are summarized in Table 5.

3.6 Analysis of variable number tandem repeats (VNTR).

For all WSSV positive samples, PCR was carried out for 7 minisatellite regions, consisting of ORF66, ORF75, ORF76, ORF84, ORF94, ORF116 and ORF125 (Table 4). The reaction mixture contained 10Xbuffer (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 uM each dATP, dTTP, dCTP and dGTP and 2.5U Fhusion TaqDNA polymerase (FINNZYMES®). Cycling conditions and expected product size are summarized in Table 5.

Table 5 VNTR primers, PCR cycling conditions and expected amplicon sizes.

Primers	Cycling conditions			No. of	Product
	Denaturation	Annealing	Extension	cycles	size (bp)
W201F/R	95^{o}C for 45 s	55^{o}C for 30 s	72 ^{o}C for 30 s	35	201
ORF66F/R	95^{o}C for 45 s	55^{o}C for 30 s	72 ^{o}C for 30 s	35	Variable
ORF75Flank(F/R)	95^{o}C for 80 s	49^{o}C for 80 s	72 ^{o}C for 80 s	30	Variable
ORF76F/R	95^{o}C for 45 s	55^{o}C for 30 s	72 ^{o}C for 30 s	35	Variable
ORF84F/R	95^{o}C for 45 s	55^{o}C for 30 s	72 ^{o}C for 30 s	35	Variable
ORF94-F/R	95^{o}C for 45 s	55^{o}C for 45 s	72 ^{o}C for 45 s	35	Variable
ORF116F/R	95^{o}C for 45 s	55^{o}C for 30 s	72 ^{o}C for 30 s	35	Variable
ORF125F/R	95^{o}C for 30 s	60^{o}C for 30 s	72 ^{o}C for 30 s	35	Variable

The PCR products were resolved by 1.5% agarose gel electrophoresis (Nusieve 3:1) at 5 V cm^{-1}, stained with EtBr and visualized using a gel document system (AlphaDigidoc®). The amplicon sizes were determined using AlphaEase®FC version 6.0 software. The numbers of repeat units in the amplicons obtained were calculated as shown in Table 6.

Table 6 Calculation of repeat unit numbers in each minisatellite locus.

Minisatellite loci	Calculate the number of repeat units
ORF66	[amplicon size - (52+64)]/36
ORF75	need DNA sequencing
ORF76	[amplicon size – (70 + 40)]/39
ORF84	[amplicon size – (69 + 70)]/33
ORF94	[amplicon size – (78 + 105)]/54
ORF116	[amplicon size – (105 + 150)]/42
ORF125	[amplicon size – (44 +14+3)]/69

3.7 Sequence analysis

PCR amplicons were purified from agarose gels using a Nucleospin quick Gel Extration Kit (Nucleospin). The direct sequence of PCR products was determined using ABI Prism ®BigDye ^{TM} Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystem). The sequencing reactions were analyzed at the Bioservice unit, Thai National Science and Technology Development Agency. The sequences obtained were analyzed for the presence of tandem repeats using Tandem Repeat Finder (TRF) program (Benson, 1999). Comparative analysis of repeats was performed using BLAST and ClustalW programs. The repeats were also compared with the 3 complete WSSV genomes at GenBank.

RESULTS AND DISCUSSION

Results

1. Study on risk factors for WSD in black tiger shrimp

1.1 Descriptive results and univariable analysis

Out of 384 ponds, 165 (43%) developed clinical WSD infections. The average duration from stocking in the growout ponds until start of the outbreaks was 80 days (range: 20 - 145 days). The average age at harvest for healthy ponds was 124 days (range: 20 - 162 days). The prevalence of WSD outbreaks from production cycles 1 to 8 was 19, 85, 18, 89, 18, 85, 14 and 66 %, respectively. The median number of operation times per pond during the study period was 5 with a range of 1-8. The descriptive statistics for all independent variables are presented in Table 7. Categorized variables were generated if the relationship between a continuous predictor and log odds of the outcome was not linear. The cut-offs were decided at the point of the most change in log odds when the independent variables changed and these were incorporated with biological reasons. These variables were transformed into categorized variables and recorded as dummy values prior to being included in the regression model (Table 7).

Table 7 Variables used in the study of risk factors for WSSV outbreaks (384 ponds recorded, Thailand, 1998 - 2002).

Variables	Description and Levels	Transformation
Dependent		
Clinical WSD outbreak pond	1 = yes, 0 = no, N=384	-
Independent		
Management practices		
Age of postlarvae shrimp (agepl)	range = 11 - 23, mean = 16.4, SD = 2.2, N = 348	Category (1 = < 19; 2 = ≥ 19)
Duration of pond drying (pdrestdu)	range = 0 - 309, mean = 126, SD = 105, N = 349	Category (1 = < 51; 2 ≥ 51)
Duration of pond preparation (predurat)	range = 1 - 395, mean = 34, SD = 30, N = 384	Category (1 = < 17; 2 = ≥ 17)
WSD outbreak in former crop (obfmcrop)	0 = no; 1 = yes, N = 336	-
Stocking density (stockden)	range = 29 - 74, mean = 54, SD = 7, N = 380	Category (1 = ≤ 48; 2 = > 48)
Pond size (pondsize)	range = 0.74 – 1.41, mean = 0.97, SD = 0.08, N = 384	Category (1 = ≤ 0.96; 2 = >0.96)
Used disinfectant (disinfect)	0 = no; 1 = yes, N = 384	-
Used carricide (carricide)	0 = no; 1 = yes, N = 384	-
Water depth (walevel)	range = 90 - 170, mean = 138, SD = 13, N = 384	Category (1 = ≤ 140; 2> 140)
Environment		
Season (season)	0 = summer, 1 = rainy-winter , N = 384	-

1.2 Significant variables

All independent variables of interest were associated with the presence of clinical WSD outbreaks at a significant level (*P*-value < 0.10) when the univariable regression analysis was performed with the WSD status as an outcome variable (Table 8).

Table 8 The univariate regression analysis results of risk factors for clinical WSD outbreaks in black tiger shrimp (Thailand, 1998-2002) for significant variables (*P*< 0.10). S.E.: standard error; CI: confidence interval

Risk factors (levels)	No. of records	odds-ratio (OR)	S.E. (OR)	95%CI (OR)	*P*-value
Age of postlarvae shrimp	348				
1	268	1	-	-	-
2	80	2.3	0.6	1.4 – 3.8	0.001
Duration of pond drying	349				
1	109	1	-	-	-
2	240	0.24	0.06	0.15 – 0.39	< 0.001
Duration of pond preparation	384				
1	55	1			
2	329	0.28	0.09	0.15 - 0.52	< 0.001
WSD outbreak in former crop	334				
Yes (1)	200	0.31	0.07	0.19 - 0.5	< 0.001
No (0)	136	1	-	-	-
Stocking density	380				
1	76	1	-	-	-
2	304	0.36	0.10	0.22 – 0.61	< 0.001
Pond size	384				
1	289	1	-	-	-
2	95	1.8	0.4	1.1 – 2.8	0.016

Table 8 (Continued)

Risk factors (levels)	No. of records	odds-ratio (OR)	S.E. (OR)	95%CI: (OR)	*P*-value
Use of disinfectants	384				
Yes (1)	283	1	-	-	-
No (0)	101	2.0	0.5	1.3 - 3.1	0.003
Used of carricides	384				
Yes (1)	85	0.39	0.10	0.24 - 0.63	< 0.001
No (0)	299	1	-	-	-
Water depth	384				
1	244	1	-	-	-
2	140	0.34	0.08	0.22 - 0.54	< 0.001
Season	384				
0	240	1	-	-	-
1	154	22	6	12 - 38	< 0.001

1.3 Correlations among significant independent factors

The correlations among significant independent factors were determined by the Pearson correlation method (Table 9). Most of the correlations among variables were small. Therefore, multi-collinearity was not a major concern for the regression analysis.

Table 9 The Pearson correlation coefficients among significant independent variables from the univariable regression analysis. Only statistically significant correlations ($P< 0.01$) were shown.

variables	agepl	pdrestdu	predurat	obfmcrop	stockden	pondsize	disinfec	carricide	walevel	season
agepl	1.00									
pdrestdu	-0.17	1.00								
predurat	-	-	1.00							
obfmcrop	-	-0.17	-	1.00						
stockden	-0.42	0.21	-	-	1.00					
pondsize	-	-	-	-	-0.13	1.00				
disinfec	-0.14	0.20	-	-0.14	0.27	-	1.00			
carricide	-	-	0.15	-	-0.21	-	-0.43	1.00		
walevel	-	-	-	0.20	-	-	-	-	1.00	
season	0.17	-0.32	-0.14	-0.40	-	-	0.19	-0.21	-0.35	1.00

1.4 Multivariable model

In the final model that included 2 predictive variables (e.g. season and predurat), age of shrimp at harvest date (aghav) as a confounding factor and production cycle number were considered as hierarchical effects on the data set. The other independent factors were dropped from the model because they had *P*-values larger than 0.05.

Shrimp ponds operated in the rainy-winter season were more likely to experience WSD outbreaks than in the summer. By contrast, ponds with preparation periods before stocking longer than 17 days showed a lower incidence of outbreaks. Interactions among the significant predictor variables were not present. The Logistic regression model fit well to the data using the Hosmer-Lemeshow goodness-of-fit test (*P*-value = 0.46). The GEE model with an exchangeable correlation structure gave slightly different parameter estimates and standard errors from the logistic regression model (Table 10). The estimated intra-class correlation coefficient (a measure for the strength of dependence between observations within a pond) was small (r = -0.0027).

Table 10 Parameter estimates (*b*) for two different statistical analyses of WSD data with the same risk factors.

	Logistic regression				Generalized estimating equation			
Variable	*b*	S.E.	95%CI:	*P*-value	*b*	S.E.	95%CI:	*P*-value
Season	2.03	0.86	0.35 - 3.71	0.018	2.03	0.86	0.35 - 3.71	0.018
Predurat	-1.1	0.55	-2.18 - -0.02	0.047	-1.1	0.55	-2.18 - -0.02	0.047
Aghav	- 0.065	0.008	-0.08 - -0.05	<0.001	- 0.065	0.008	-0.08 - -0.05	<0.001
Constant	5.37	1.79	1.86 - 8.89	0.003	5.37	1.79	1.85 - 8.88	0.003

The effect of the presence of all independent variables on the magnitude of the odds ratio of risk of WSD is shown in Table 11. The seasonal variable was the most powerful predictor variable for the model. The odds ratio for WSD outbreaks was increased by 7.58 times when the ponds were operating in the rainy-winter season. By contrast, the risk of WSD outbreak was decreased by a factor of 3.03 if pond preparation periods were longer than 17 days.

Table 11 Magnitude of change in odds ratio (OR) for clinical WSD outbreaks across reasonable intervals as used in the generalized estimating equation (GEE) model.

Variable	Magnitude of change (OR)	95%CI: for OR	*P*-value
Season			
- summer	1	-	-
- rainy-winter	7.58	1.41 – 40.68	0.018
Duration of pond preparation (days)			
- < 17	1	-	-
- ≥ 17	0.33	0.11 – 0.98	0.047

2. Study on the potential of horizontal transmission of WSD between ponds

In Table 3 the estimates of *C*, *I*, *S* and *ß* are listed for each week of the epidemic. Figure 7 shows how the distribution of newly infected ponds (*C*) is related to the distribution of the number of emergency harvested ponds (*I*) during the epidemic.

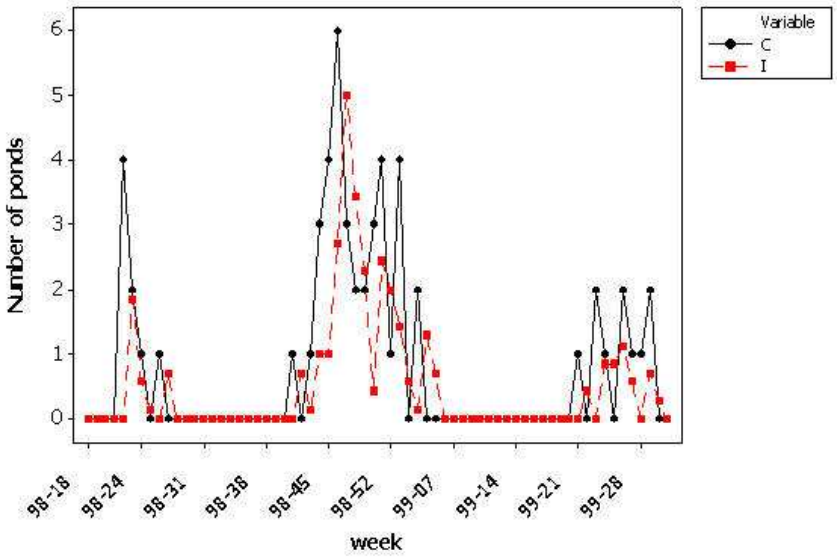

Figure 7 Estimated distribution of the number of virus introductions per week and distribution of the number of ponds emergency harvested during 1998-1999

The average estimates of *ß* for each phase are listed in Table 12. The result of the ANOVA test indicated that the probability distributions of *ß*‘s for the different phases were significant. The results of the pair-wise comparison of *ß*‘s showed that *ß* of phase 2 was significantly larger than that of phase 1 (*P*-value = 0.0043) and phase 3 (*P*-value = 0.0038). The difference between phases 1 and 3 was not significant (*P*-value = 0.7039)

The average values of *T* and their standard deviations are shown in Table 12. The result of the Kruskal-Wallis ANOVA showed the T’s of all phases had the same probability distribution (*P*-value = 0.512). The R_h values for the three phases, and their accompanying standard deviations are also shown in Table 12. The highest R_h was observed in phase 2 whereas the R_h of phase 1 was lower than that of other phases.

Table 12 Characteristics of transmission of WSSV between ponds during three consecutive phases of the 1998-1999 epidemics in the study farm.

Phase	Number of weeks	Number of case	ß[i]	*T*	R_h[ii]
1	18	8	0.17(±0.51)[a]	0.41(±0.4)[a]	0.07(±0.3)
2	22	36	2.47(±4.54)[b]	0.63(±0.75)[a]	1.57(±4.82)
3	26	10	0.3(±0.77)[a]	0.61(±0.6)[a]	0.18(±0.68)

[i]Average number of introductions per infectious pond per week.

[ii]Average number of cases caused by one infectious pond.

Data in the same column having different letters are significantly different (*P*-value < 0.025).

3. Molecular epidemiology study of white spot syndrome virus

3.1 Analysis of the complete WSSV gemone for the presence of VNTR loci

The repeat unit size and the total number of occurrences of those repeats within all three complete WSSV genomes is shown in Figure 8. The output of TRF software analysis showed that there was a relatively a large number of repeat regions ranging between sizes 1-90 bp within the 3 WSSV genomes. A similarity in the pattern of repeat occurrence, size and copy number was observed. Additionally, the short repeats occured more frequently than larger repeats.

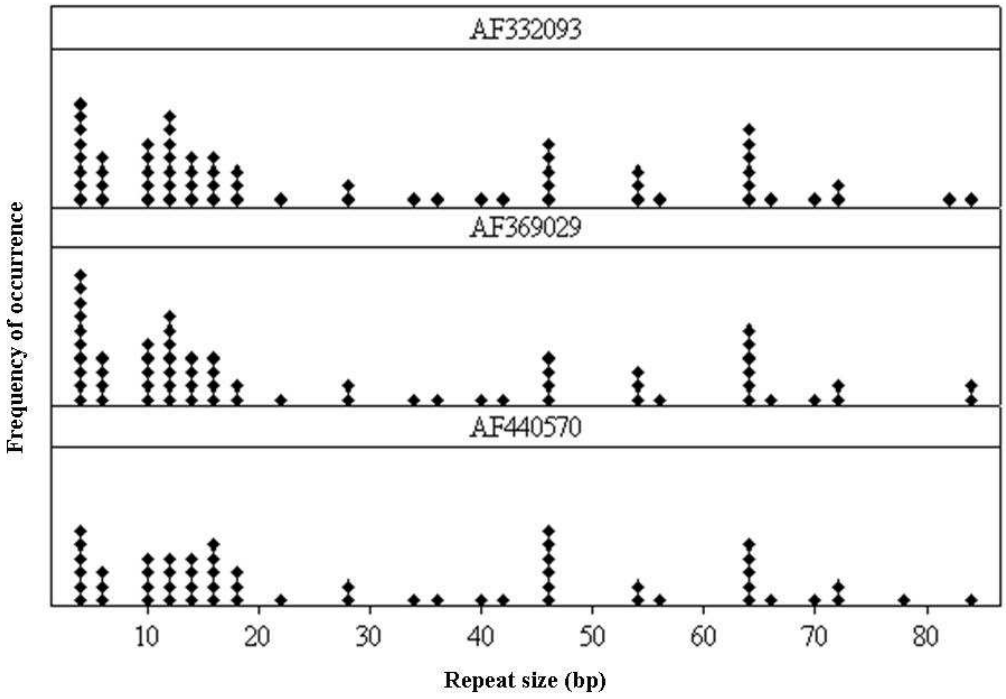

Figure 8 Comparative occurrence of various repeat sizes within the three complete WSSV genomes (AF332093 (Taiwan), AF36029 (Thailand) and AF440570 (The republic of China).

Computer analysis also revealed the presence of 7 minisatellite copies with 3 polymorphic VNTR loci of varying repeat sizes in the WSSV genomes. Table 13 summarizes the repeat sizes, genomic positions, copy numbers, classification of repeats, presence within coding or non-coding regions and associated open reading frames (ORFs) for minisatellite VNTR within the three genomes. The repeat loci were classified as “perfect” or “compound” based on whether they occured as a single unit or in combination with two or more repetitive units. Four loci (ORF66, ORF75, ORF84 and ORF116 based on accession number AF369069) showed no variation in copy numbers among three complete WSSV genomes. However, three polymorphic VNTR loci (ORF75, ORF94 and ORF125) were found. VNTR ORF75 was a compound repeat with a 45 bp repeat unit interspersed with a 57 bp nucleotide sequence. The 45 bp unit was a perfect repeat varying only in copy number. The copy

numbers for the 45 bp unit for the 3 complete WSSV genomes (AF332093, AF369029 and AF440570) were 15, 11 and 21 copies, respectively. While the copy numbers for the 57 bp unit were 4, 3 and 5 copies, respectively. The VNTR ORF94 and ORF125 were classified as perfect repeats with 54 and 69 bp, respectively. The repeat units of ORF94 among the complete WSSV genomes AF332093, AF369029 and AF440570 were 12, 6 and 6 copies, respectively. The repeat units of ORF125 among the complete WSSV genomes AF332093, AF369029 and AF440570 were 8, 6 and 6 copies, respectively. While those of ORF125 were 8, 6 and 6 copies, respectively.

Table 13 Minisatellite copy numbers for variable and non-variable repeat regions within similar genomic loci of three complete WSSV genomes.

Repeat unit (bp)	Genome position		Copy numbers	Classification	Associated ORF
36	AF332093	47052-47137	$(36)_2$	Perfect	wsv103
	AF369029	96004-96089	$(36)_2$	Perfect	ORF66
	AF440570	80633-80718	$(36)_2$	Perfect	wssv159
45 and 57	AF332093	59013-59926	$(45)_2 57 (45)_5 57 (45)_3 57 (45)_3 57 (45)_2$	Compound	wsv128
	AF369029	107964-108686	$(45)_2 57 (45)_5 57 (45)_3 57 (45)$	Compound	ORF75
	AF440570	92594-93835	$(45)_2 57 (45)_5 57 (45)_4 57 (45)_3 57 (45)_5 57 (45)_2$	Compound	wssv183
39	AF332093	60066-60149	$(39)_2$	Perfect	non-coding
	AF369029	108826-108909	$(39)_2$	Perfect	ORF76
	AF440570	93945-94058	$(39)_2$	Perfect	non-coding
33	AF332093	70619-70689	$(33)_2$	Perfect	wsv143
	AF369029	119382-119452	$(33)_2$	Perfect	ORF84
	AF440570	104525-104579	$(33)_2$	Perfect	wssv198

Table 13 (Continued)

Repeat unit (bp)	Genome position		Copy numbers	Classification	Associated ORF
54	AF332093	93475-94134	$(54)_{12}$	Perfect	wsv178
	AF369029	142744-143079	$(54)_6$	Perfect	ORF94
	AF440570	127388-127723	$(54)_6$	Perfect	wssv234
42	AF332093	128025-128135	$(42)_2$	Perfect	non-coding
	AF369029	176986-177096	$(42)_2$	Perfect	ORF116
	AF440570	161628-161738	$(42)_2$	Perfect	non-coding
69	AF332093	138949-139487	$(69)_6$	Perfect	wsv249
	AF369029	187912-188294	$(69)_8$	Perfect	ORF125
	AF440570	172565-173085	$(69)_6$	Perfect	wssv304

3.2 Primer design and PCR optimization

The previously described primers for ORF94 (Wongteerasupaya *et al.*, 2003), ORF75 (Dieu *et al.*, 2004) and ORF125 (Pradeep *et al.*, 2008) were used in this study. New primers for 4 other minisatellite loci were designed based on the conserved sequence of the franking regions of each tandem repeat locus. The tandem repeat sequences and flanking regions with primer positions for ORF66, ORF76, ORF84 and ORF116 are shown in Figures 9, 10, 11 and 12, respectively.

ACCAATGGGAGTGCCAGTAAGAAGAGGCGTCTCACGCCTGACACTAGTAAT
ATGGGAACAAGCACTGATGTGCAAGAATTCCAAACG
ATGGGAACAAATACTGATATGCAAGAATTCCAATCA
ATGGGAACAAATAC
CAACCCCATAGAGACTTCATCAGTGGGTGTGAATACCAACCCACTTCCCA
(186 bp)

Figure 9 Representative ORF66 region showing the tandem repeat sequence of 36 bp units, flanking regions and primer positions.

TGGAGTATGGAAAGCACCAGAACCTACAGGTTAAAATGTGGCTG
AACATCACATATACAAGTTAATTGGA
  TAATTGAAACAATTTGACTTTTTAGTACTTAATGTTTAT
  TAATTGAAACAATTGGACTCTTTCCTACCTAAGGTTTAT
TAATTGGTACATTGTTAACACTTGCTCTTTGCTCATAGCA
(188 bp)

Figure 10 Representative ORF76 region showing the tandem repeat sequence of 39 bp units, flanking regions and primer positions.

GGGAAATACTTGCCCAACAACTTCAAGAAATGAAAGA
ACAAATGCGCATAAAGGAAGAGGAGAGGCGGA
  AAGAACTAGCAGATAAGGAGGAAGAAAAACGTC
  GAGAACTAGCAGCCAAGGAGGAAGAAAAGCGTC
AAGAAATATTAGCTAAAGAAGAGCAACTTGAAAAATT
GAATTTCCAGTTGGGTACAGAAATCACGTCCAA
(205 bp)

Figure 11 Representative ORF84 region showing the tandem repeat sequence of 33 bp units, flanking regions and primer positions.

TCGCATTGGAAGATTTCTTGTGTACATTTCTGAGAGATTCTTTT
TGTTCCTCAGAGACTTTGAAAAGTTGTTCAGTTTCGATATACGA
GTTTTTGTACTCCTCTT
  TCTTTACTACTTGTGTTTCTAGTTCCTTTTCAACTTTTGTGG
  TCTTTACTACTTGTGTTTCTAGTTCCTTTTCAACTTTTGTGG
TCTTTTCGACCTGGTTTTCTAGTTCCTCTTTTATTTGTAAATTT
TTCCTAGCCAACTCTTCGCGTATCTGGCGTTGCTTTTCATCACT
GTATAAGATAACAGCGTAGGCTAGTGTCGAAAGGTACATTTCAA
TGCTTGCAGCAGAAGGGT (339 bp)

Figure 12 Representative ORF116 region showing the tandem repeat sequence of 42 bp units, flanking regions and primer positions.

The news primers were tested with genomic DNA extracted from WSSV infected shrimp by thermal gradient PCR in the temperature range 52-58^{o}C. PCR products on 1.5% agarose gel electrophoresis from primers ORF66, ORF76, ORF84 and ORF116 are shown in Figures 13, 14, 15 and 16, respectively.

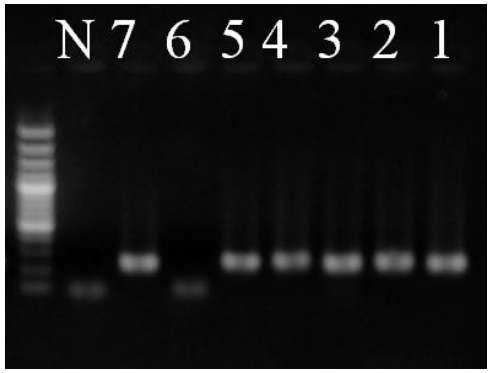

Figure 13 Agarose gel showing PCR amplification products of primers for ORF66 by thermal gradient PCR, Marker = 1 Kb Plus DNA Ladder (Invitrogen®), N = negative control. For lanes 1-7 annealing temperatures were 52, 53, 54.6, 55.6, 56.6, 57.6 and 58 ^{o}C, respectively.

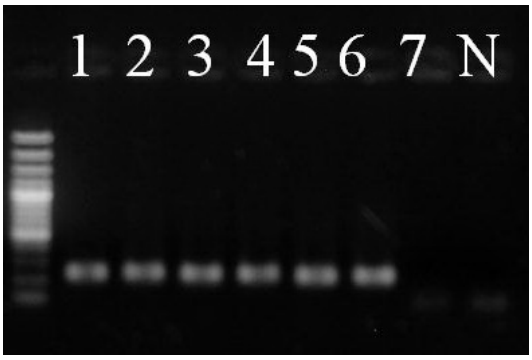

Figure 14 Agarose gel showing PCR amplification products of primers for ORF76 by thermal gradient PCR, Marker = 1 Kb Plus DNA Ladder (Invitrogen®), N = negative control. For lanes 1-7 annealing temperatures were 52, 53, 54.6, 55.6, 56.6, 57.6 and 58 ^{o}C, respectively.

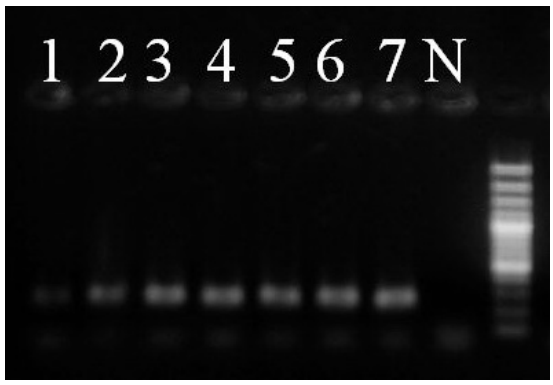

Figure 15 Agarose gel showing PCR amplification products of primers for ORF84 by thermal gradient PCR, Marker = 1 Kb Plus DNA Ladder (Invitrogen®), N = negative control. For lanes 1-7 annealing temperatures were 52, 53, 54.6, 55.6, 56.6, 57.6 and 58 ^{o}C, respectively.

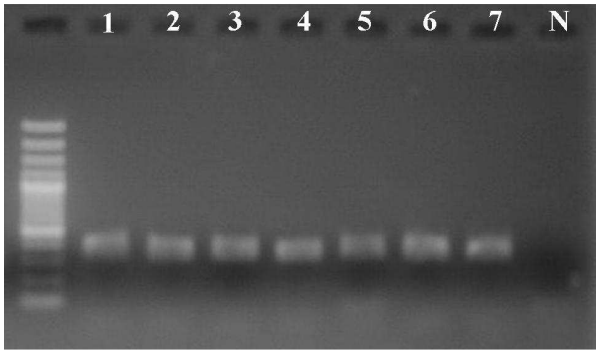

Figure 16 Agarose gel showing PCR amplification products of primers for ORF116 by thermal gradient PCR, Marker = 1 Kb Plus DNA Ladder (Invitrogen®), N = negative control. For lanes 1-7 annealing temperatures were 52, 53, 54.6, 55.6, 56.6, 57.6 and 58 ^{o}C, respectively.

Results from thermal gradient PCR revealed that all the new primers gave good amplicon yields when annealing temperatures were between 54 – 56 ^{o}C. Therefore, the annealing temperature of all new primers was set at 55 ^{o}C. The PCR

cycling conditions of all the primers used in this study are shown in the Table 5 (materials and methods).

3.3 Shrimp specimens and diagnostic PCR for WSSV

Of the 64 samples analyzed, 41 samples (64.1%) were positive for WSSV. All positive samples were used for the study of variation in number of tandem repeats in each minisatellite locus with the primer sets indicated in Table 14. The numbers of samples used for VNTR study by year of sampling (1999, 2000 and 2002) were 7, 16 and 18, respectively. The numbers of samples categorized by location as central or southern of Thailand were 21 and 20, respectively.

Table 14 Location, date and number of samples collected for the study.

Province	Location	Date	No. of Samples	No. of samples positive to WSSV primers
Chacheongsao	Central Thailand	2000	12	0
	near Bangkok	2002	13	13
Chumphon	Mid-SW coast,	2000	4	2
	Gulf of Thailand	2002	1	1
Nakhon Pathom	Central Thailand	1999	14	7
	near Bangkok	2000	1	1
Nakhon Si Thammarat	Lower SW coast,	2000	2	2
	Gulf of Thailand			
Surat Thani	Lower SW coast,	2000	11	11
	Gulf of Thailand	2002	6	4
Total			64	41

3.4 Analysis of variable number tandem repeat (VNTR) regions.

3.4.1 PCR for repeat unit examination in ORF66, ORF76, ORF84 and ORF116

The percent of samples that successfully yielded amplicons with primers specific to minisatellite loci ORF66, ORF76, ORF84 and ORF116 were 95.12, 82.93, 90.24 and 58.54%, respectively. No variation in repeat unit (RUs) numbers were found for minisatellite loci ORF66, ORF76, ORF84 and ORF116 (Table 15).

Table 15 The number of RU present in the regions encoding ORF66, ORF76, ORF84 and ORF116. DF= detection failure

VNTR loci	No. of RU	Amplicon size (bp)	No. of samples	Frequency (%)
ORF66 (36 bp RU)	2	186	39	95.12
	DF	-	2	4.88
ORF76 (39 bp RU)	2	188	34	82.93
	DF	-	7	17.07
ORF84 (33 bp RU)	2	205	37	90.24
	DF	-	4	9.76
ORF116 (42 bp RU)	2	339	24	58.54
	DF	-	17	41.46

3.4.2 PCR for repeat unit examination in ORF94 and ORF125

Out of 41 samples, 34 (82.93%) successfully yielded amplicons with primers specific to minisatellite locus ORF94. Eleven different repeat types ranging from 5 to 20 RUs were found in ORF94. The GenBank records for WSSV-TH and WSSV-TW (6 repeats) and WSSV-CH (12 repeats) fall within this range. The most frequent repeat type had 8 RUs (19.51%) while types with 11, 13, 14, 16 and 18 RUs were not observed (Table 16). For the benefit of explanation, we numbered these repeat regions ORF94-6 to ORF94-20. As an example a gel representing the fragments of 5, 7, 9, 10, 12 and 15 repeats is shown in Figure 17.

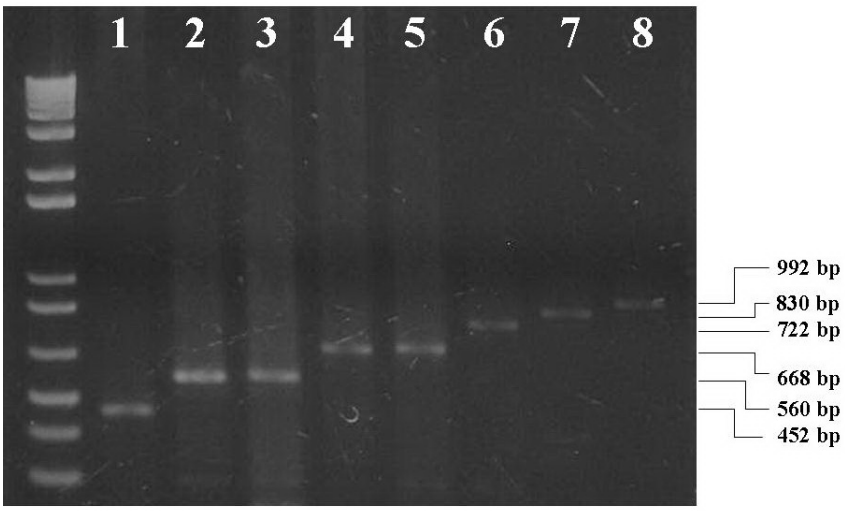

Figure 17 An example of agarose gel showing PCR amplification products of minisatellite locus ORF94. Marker = 1 Kb Plus DNA Ladder (Invitrogen®); Lanes 1 to 8 show amplicons for 5, 7, 7, 9, 9, 10, 12 and 14 RUs respectively.

Out of 41 samples, 32 (78.05%) successfully yielded amplicons with primers specific to minisatellite locus ORF125. Eight different repeat types ranging from 5 to 14 RUs were found in the ORF125 locus. The GenBank records for

WSSV-TH and WSSV-CH (6 repeats) and WSSV-TW (8 repeats) fall within this range. The most frequent repeat type had 6 RUs (34.15%) while types with 12 and 13 RUs were not observed (Table 16). For the benefit of explanation, we numbered these repeats regions ORF125-5 to ORF125-14. An example of gel fragments representing 3, 4, 5, 8 and 9 repeats is shown in Figure 18.

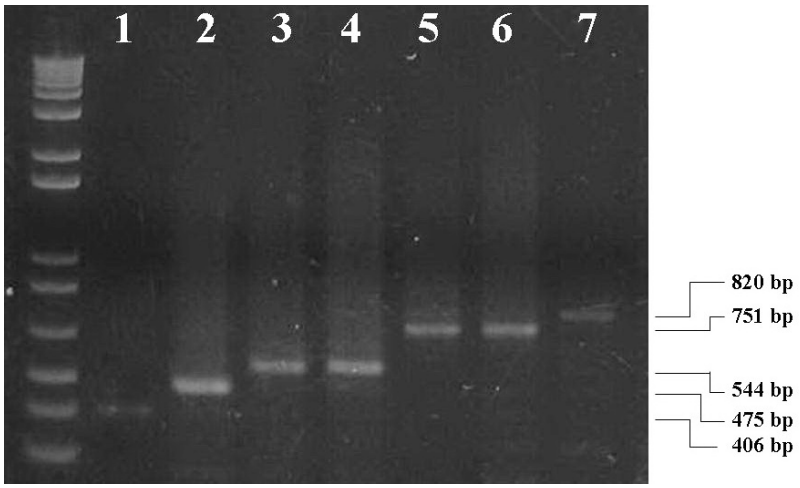

Figure 18 Example of an agarose gel showing PCR amplification products of minisatellite locus ORF125. Marker = 1 Kb Plus DNA Ladder (Invitrogen®); Lanes 1 to 7 show amplicons for 3, 4, 5, 5, 8, 8 and 9 RUs respectively,

Table 16 The number of RUs present in the region encoding ORF94 and ORF125. Result studied from India and Thailand (Pradeep *et al*., 2008, Wongteerasupaya et al., 2003) is in parenthesis, respectively.

No. of RU	ORF94 (54 bp RU)		ORF125 (69 bp RU)	
	Amplicon size (bp)	Frequency (%)	Amplicon size (bp)	Frequency (%)
2	290	0 (8.5, 0)	199	0 (2.8, -)
3	344	0 (6.6,0)	268	0 (1.9, -)
4	398	0 (2.8, 0)	337	0 (47.2, -)
5	452	2.44 (0.9, 0)	406	12.2 (15.1, -)
6	506	12.2 (5.7, 13.9)	475	34.15 (0, -)
7	560	7.32 (11.3, 12.3)	544	9.76 (4.7, -)
8	614	19.51 (8.5, 29.3)	613	2.44 (16.0, -)
9	668	17.07 (2.8, 15.4)	682	9.76 (0.9, -)
10	722	7.32 (6.6, 7.6)	751	4.88 (1.9, -)
11	776	0 (0, 1.5)	820	2.44 (0.9, -)
12	830	7.32 (2.8, 6.2)	889	0 (0.9, -)
13	884	0 (0.9, 0)	958	0 (0, -)
14	938	0 (0.9, 3.1)	1027	2.44 (0.9, -)
15	992	2.44 (2.8, 1.5)		
16	1046	0 (2.8, 0)		
17	1100	2.44 (0, 3.1)		
19	1208	2.44 (0, 4.6)		
20	1262	2.44 (0, 1.5)		
	DF	17.07 (38.7, 0)	DF	21.95 (6.6, -)

When comparing the number of ORF94 repeats found in Thai WSSV isolates, there was no particular pattern in the repeats for farms located in various farming areas (Table 17). However, ORF94-6 and 9 were found to be dominant in Surat Thani in 2000 but not found in 2002. The fragments ORF94-6 to ORF94-15 were found mainly in Chacheongsao in 2002. The large numbers of repeats (i.e. ORF94-17 to 20) were found especially in Nakhon Pathom province during 1999 -2000.

Table 17 Pattern of ORF94 repeat groups for WSSV outbreak ponds sampled during 1999 – 2002. NP=Nakhon Pathom, CP=Chumphon, NS=Nakhon Si Thammarat, ST=Surat Thani, CC=Chacheongsao, DF = detection fail

No of	1999	2000				2002			Total
repeats	NP	CP	NP	NS	ST	CC	CP	ST	
5	1								1
6					3	2			5
7						2		1	3
8		1			2	3		2	8
9	2				3	1	1		7
10				2		1			3
12	1					2			3
15						1			1
17			1						1
19	1								1
20	1								1
DF	1	1			3	1		1	7
Total	7	2	1	2	11	13	1	4	41

When comparing the number of ORF125 repeats found in Thai WSSV isolates from various shrimp farming areas (Table 18), there was no particular pattern, except that ORF125-6 was found to be dominant in Surat Thani and Nakhon

Si Thammarat in 2000 and in Chacheongsao in 2002. ORF125-7 dominanted in Nakhon Pathom province.

Table 18 Pattern of ORF125 repeat groups for WSSV outbreak ponds sampled during 1999 – 2002. NP=Nakhon Pathom, CP=Chumphon, NS=Nakhon Si Thammarat, ST=Surat Thani, CC=Chacheongsao, DF = detection fail

No of repeats	1999	2000				2002			Total
	NP	CP	NP	NS	ST	CC	CP	ST	
5					1	3		1	5
6				2	5	6	1		14
7	3				1				4
8		1							1
9					1	2		1	4
10					1	1			2
11	1							1	1
14						1			1
DF	4	1	1		2			1	9
Total	7	2	1	2	11	13	1	4	41

3.4.3 PCR for repeat unit examination in ORF75

Out of 41 samples, only 17 (41.42%) successfully yielded amplicons with the primers specific to minisatellite locus ORF75. Three different amplicon sizes were found. The most frequent amplicon size was 656 bp (24.4%) (Table 19). When comparing the number of ORF75 repeats found in Thai WSSV isolates from various shrimp farming areas, there was no particular pattern (Table 20).

Table 19 Repeat units (RU) present in the region encoding ORF75. Result studied from India (Pradeep *et al*., 2008) is in parenthesis. DF= detection failure

ORF75 (45 and 57 bp RUs)		
Amplicon size (bp)	No. of samples	Frequency (%)
320	0 (1)	0 (0.9)
427	0 (1)	0 (0.9)
525	0 (25)	0 (23.6)
610	0 (15)	0 (14.2)
656	10 (0)	24.4 (0)
778	0 (3)	0 (2.8)
1028	6 (0)	14.64 (0)
1355	1 (0)	2.44 (0)
DF	24 (56)	58.28 (52.8)

Table 20 Pattern of ORF75 repeat groups for WSSV outbreak ponds sampled during 1999 – 2002. NP=Nakhon Pathom, CP=Chumphon, NS=Nakhon Si Thammarat, ST=Surat Thani, CC=Chacheongsao, DF = detection fail

Size	1999	2000				2002			Total
(bp)	NP	CP	NP	NS	ST	CC	CP	ST	
656		1	1	1	3	3	1		10
1028				1	2	2		1	6
1355	1								1
DF	6	1			6	8		3	24
Total	7	2	1	2	11	13	1	4	41

3.5 Sequence analysis

3.5.1 Sequence analysis on ORF66, ORF76, ORF84 and ORF116

Selected amplicons for repeat groups of WSSV obtained using ORF66 (F/R), ORF76 (F/R), ORF84 (F/R) and ORF116 (F/R) primers were sequenced and the results are presented in Table 21. A comparative analysis of the sequences showed 100% identity with those of the three WSSV complete genome sequences published.

Table 21 Results of Megablast nucleotide analyses of our sequenced minisatellite samples with those of the three complete WSSV genome sequences on ORF66, 76, 84 and 116 locus. ST=Surat Thani, CC=Chacheongsao

Loci	Isolate	size (bp)	accession number	maximum identity (%)
ORF66	ST#1-1	150	AF332093	100
			AF369029	100
			AF440570	100
	ST#2-3	150	AF332093	100
			AF369029	100
			AF440570	100
	CC#2-3	150	AF332093	100
			AF369029	100
			AF440570	100
ORF76	ST#2-3	142	AF332093	100
			AF369029	100
			AF440570	100
	CC#2-3	142	AF332093	100
			AF369029	100
			AF440570	100

Table 21 (Continued)

Loci	Isolate	size (bp)	accession number	maximum identity (%)
ORF84	ST#1-1	163	AF332093	100
			AF369029	100
			AF440570	100
	ST#2-3	163	AF332093	100
			AF369029	100
			AF440570	100
	CC#2-3	163	AF332093	100
			AF369029	100
			AF440570	100
ORF116	ST#1-1	273	AF332093	100
			AF369029	100
			AF440570	100
	CC#2-10	273	AF332093	100
			AF369029	100
			AF440570	100

No variations in tandem repeat sequence numbers were observed for all isolates tested with ORF66 (F/R), ORF76 (F/R), ORF84 (F/R) and ORF116 (F/R) primers. The amplicons showed repeat patterns of 36 x 2, 39 x 2, 33 x 2 and 42 x 2, respectively.

The nucleotide sequence of selected amplicons obtained using ORF75 FLANK (F/R) primers showed high but not complete identity to the three WSSV complete genome sequences (Table 22). Sequencing of selected fragments confirmed the presence of 45 and 57 bp tandem repeats identical to those in WSSV-TH, WSSV-TW and WSSV-CH at GenBank.

Table 22 Results of Megablast nucleotide analyses of our sequenced minisatellite samples with those of the three complete WSSV genome sequences for ORF75 locus. NS = Nakhon Si Thammarat, CC = Chacheongsao

Isolate	size (bp)	Accession number	Maximum identity (%)
CC#2-2	589	AF332093	97
		AF369029	98
		AF440570	97
NS#1	723	AF332093	97
		AF369029	97
		AF440570	98

The sequence of a 656 bp fragment obtained from ORF75 FLANK (F/R) primers revealed a compound repeat pattern of $(45)_2 57 (45)_3 57 (45)_3 57$ (Figure 19) that differed from $(45)_2 57 (45)_5 57 (45)_3 57 (45)_2$ for the ORF75 region of WSSV-TH (AF369029). Sequencing of a larger fragment of 1028 bp showed a repeat pattern of $(45)_2 57 (45)_5 57 (45)_3 57 (45)_3 57 (45)_2$. Comparing all 45 bp RUs within one isolate, they contained SNPs at positions 4, 16, 31, 43 and 45 bp, while the 57 bp RUs showed an extra SNP at position 39 bp. Each of the RUs could be recognized by its specific SNPs.

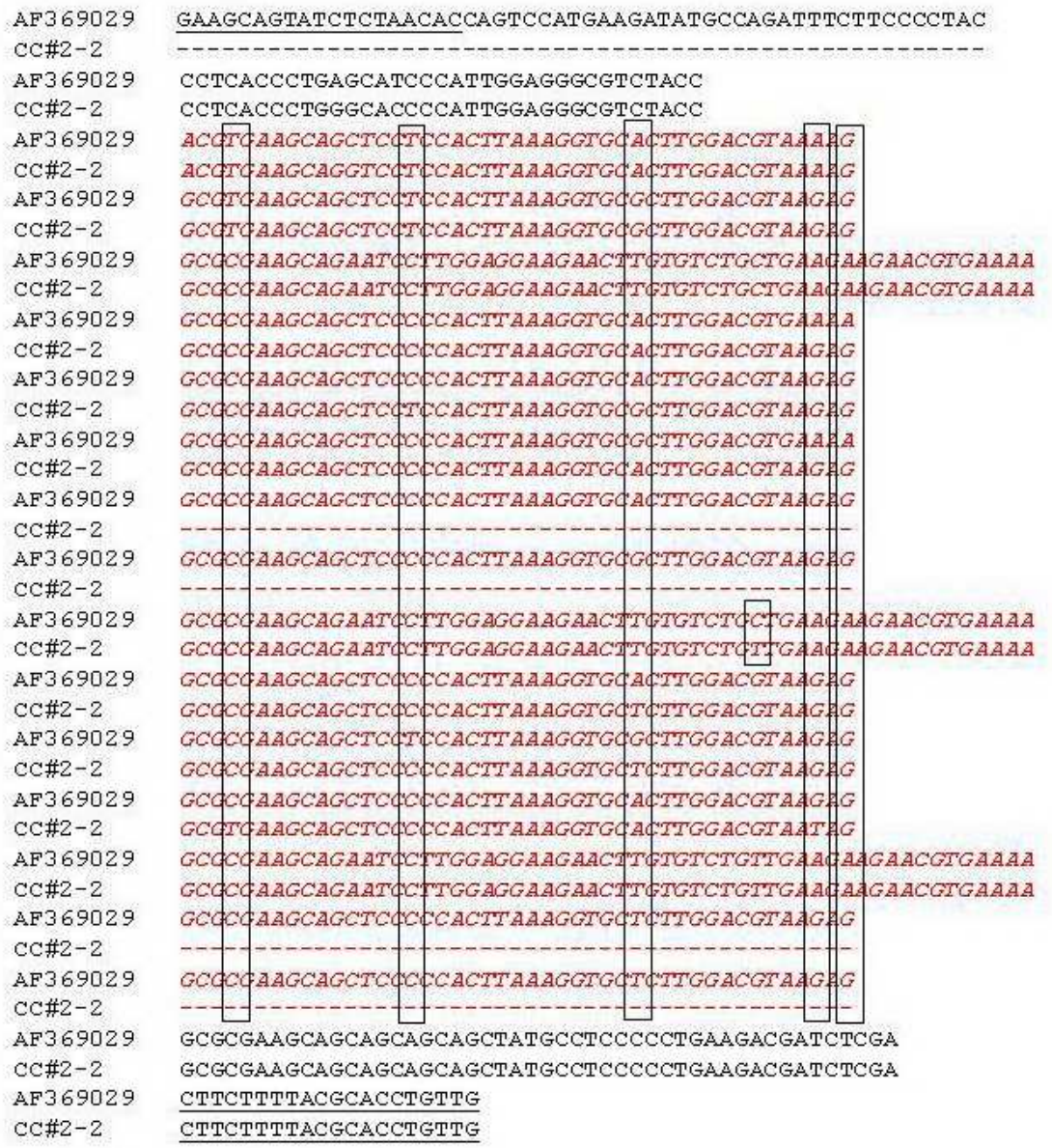

Figure 19 Comparison of nucleotide sequences of our sequenced minisatellite samples from ORF75 with those of WSSV-TH (AF369069). The 45 bp SNPs positions are indicated by long blocks of outlined text while a 57 bp SNPs position is indicated by a small block. Red and italic letters indicate repeat sequences. Primer binding sites are underlined. CC = Chacheongsao

3.5.3 Sequence analysis on ORF94

The nucleotide sequences of PCR products obtained using ORF94 (F/R) primers showed high but not complete identity to the three WSSV complete genome sequence (Table 23). Sequencing of selected fragments confirmed the presence of 54 bp tandem repeats of identical length to those in WSSV-TH, WSSV-TW and WSSV-CH at GenBank.

Table 23 Results of Megablast nucleotide analyses of our sequenced minisatellite samples with those of three complete WSSV genome sequences at ORF94 locus. NS = Nakhon Si Thammarat, CC = Chacheongsao

Isolate	size (bp)	accession number	maximum identity (%)
NS#2	695	AF332093	96
		AF369029	96
		AF440570	98
CC#2-4	518	AF332093	99
		AF369029	98
		AF440570	98

The fragment of 560 bp revealed a repeat pattern of 54 x 7 copies that differed from 54 x 6 copies for the ORF94 region of WSSV-TW (AF440570). The single nucleotide polymorphism (SNP) at the 48^{th} base of $1^{st} – 6^{th}$ RUs of WSSV-TW were T, T, T, G, T and T, respectively. While the SNP at the 48^{th} base of $1^{st} – 7^{th}$ RUs of a sample taken from Chacheongsao province in 2002 were G, G, T, T, G, T and T, respectively (Figure 20).

Figure 20 Comparison of nucleotide sequence of our sample with WSSV-TW (AF440570) at ORF94 locus. The SNP positions are indicated by a block of outlined text. Red and italic letters indicates the repeat sequences. Primer binding sites are underlined. CC=Chacheongsao

3.5.4 Sequence analysis on ORF125

The sequence of PCR amplicons obtained using ORF125 (F/R) primers showed high but not complete identity to the three WSSV complete genome sequences (Table 24). Sequencing of selected fragments confirmed the presence of a 125 bp tandem repeat identical in length to those in WSSV-TH, WSSV-TW and WSSV-CH at GenBank.

Table 24 Results of Megablast nucleotide analyses of our sequenced minisatellite samples with those of three complete WSSV genome sequences at ORF125. NP = Nakhon Pathom, ST = Surat Thani

Isolate	size (bp)	accession number	maximum identity (%)
NP#1	493	AF332093	95
		AF369029	96
		AF440570	96
NP#8	493	AF332093	96
		AF369029	96
		AF440570	96
ST#1-5	333	AF332093	96
		AF369029	96
		AF440570	96

A fragment of 406 bp and two fragments of 544 bp revealed 69 bp repeat units of 5 and 7 copies, respectively (Figure 21). The copy number differed from 6 copies for the ORF125 region of WSSV-TH (AF369029). Sequencing of the first repeat unit reveled SNPs at positions 29, 37, 40, 43, 46, 48, 50, 51, 53, 54, 61, 64 and 65, and in the last repeat unit at positions 36, 37, 39, 41, 45, 48, 50, 53, 55 – 59, 61, 62, 64, 65 and 66. By examining variation of SNPs in the inner repeat unit, the single nucleotide polymorphisms that had the potential for variation studies were at position 2, 9, 50, 53 and 61. A summary of SNPs found in sequenced ORF125 of selected amplicons compared to GenBank references for Taiwan, Thailand and China is shown in Table 25.

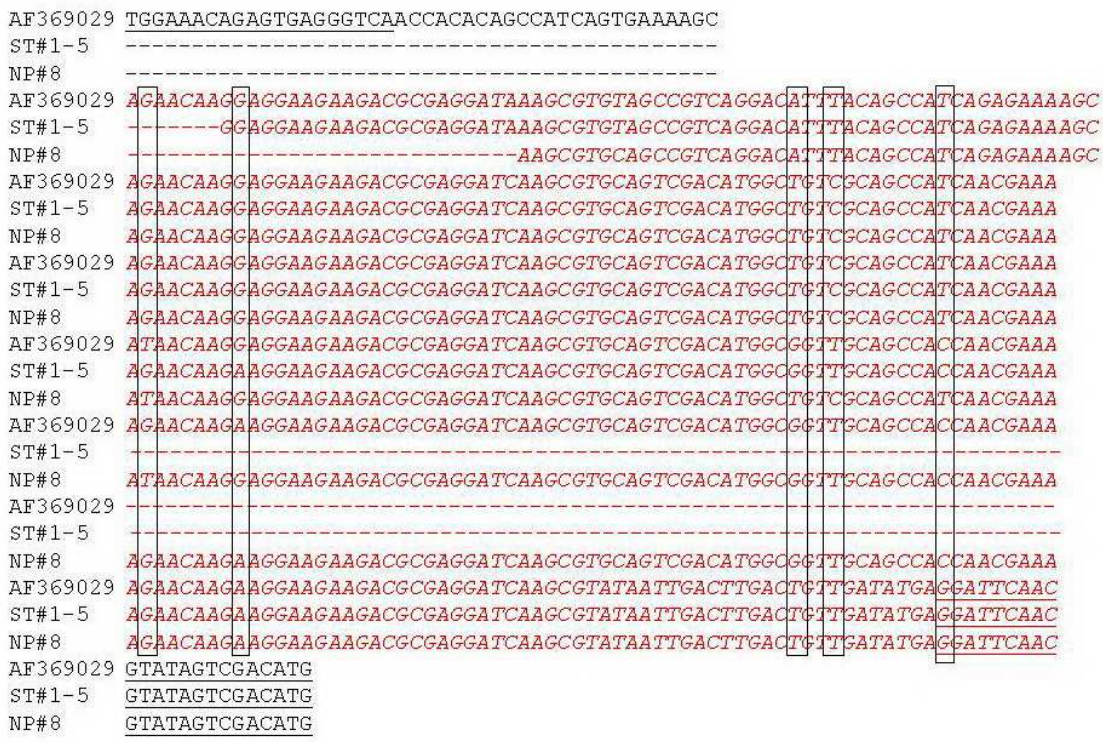

Figure 21 Comparison of nucleotide sequences of our samples with WSSV-TH (AF369069) at ORF125. The SNP positions are indicated by blocks. Red and italic letters indicate repeat sequences. Primer binding sites are underlined. CC = Chacheongsao, ST = Surat Thani

The SNPs at position 2, showed an alternate T or G. The G nucleotide dominated overall, while T was found to be dominant in ORF125-4. At position 9, variation in alternate G or A was observed. G was found to dominate in this position. At position 50, three bases (A, T and G) were found, but T was dominant. However A and G were dominant in ORF125-1 and ORF125-6, respectively. At position 53, a variation in C and T was found. C was found mainly in early repeats while T was found mainly in late repeats. At the last position (61), three bases T, C and G were found. T was found to be dominant in early repeates while C was found mainly in late repeats (Table 25).

Table 25 Summary of substitutions found in sequenced tandem repeat regions of ORF125 for selected amplicons of Thai shrimp samples from Nakhon Pathom (NP) and Surat Thani (ST), compared to GenBank references for white spot syndrome virus (WSSV) in Taiwan (AF440570) and China (AF332093) (8 repeats) and Thailand (AF369029) (6 repeats). DF = detection failure

Position	Isolation	ORF125 repeat number							
		1	2	3	4	5	6	7	8
2	ST#1-5	DF	G	G	G	G	-	-	
	AF369029	G	G	G	T	G	G	-	
	NP#1	DF	G	T	T	T	G	G	
	NP#8	DF	G	G	T	T	G	G	
	AF332093	G	G	G	T	G	T	T	G
	AF440570	G	G	G	G	G	T	G	G
9	ST#1-5	G	G	G	A	A	-	-	
	AF369029	G	G	G	G	A	A	-	
	NP#1	DF	G	G	G	G	A	A	
	NP#8	G	G	G	G	G	A	A	
	AF332093	G	G	G	G	G	G	G	A
	AF440570	G	G	G	G	G	G	A	A
50	ST#1-5	A	T	T	G	T	-	-	
	AF369029	A	T	T	G	G	T	-	
	NP#1	A	T	T	T	G	G	T	
	NP#8	A	T	T	T	G	G	T	
	AF332093	A	T	T	G	T	G	G	T
	AF440570	A	T	T	T	T	G	G	T

Table 25 (Continued)

Position	Isolation	ORF125 repeat number							
		1	2	3	4	5	6	7	8
53	ST#1-5	T	C	C	T	T	-	-	-
	AF369029	T	C	C	T	T	T	-	-
	NP#1	T	C	C	C	T	T	T	-
	NP#8	T	C	C	C	T	T	T	-
	AF332093	T	C	C	T	C	T	T	T
	AF440570	T	C	C	C	C	T	T	T
61	ST#1-5	T	T	T	C	G	-	-	-
	AF369029	T	T	T	C	C	G	-	-
	NP#1	T	T	T	T	C	C	G	-
	NP#8	T	T	T	T	C	C	G	-
	AF332093	T	T	T	T	T	T	C	G
	AF440570	T	T	T	T	T	C	C	G

Discussion

1. Study 1 on risk factors for WSD in black tiger shrimp cultivation

1.1 Significant risk factors

The season of shrimp cultivation was the most powerful variable associated with the WSD outbreaks in this study. Abrupt fluctuations in temperature and salinity were documented during the rainy-winter periods. Temperatures lower than the optimum level (e.g. 28 - 32 ^{o}C) increased mortality in shrimp experimentally infected with WSSV (Vidal *et al.*, 2001; Rahman *et al.*, 2007). Also, frequent changes in water temperature have been demonstrated to increase stress and susceptibility to diseases of cultured shrimp (Kaustsky, 2000). Salinity changes have an effect on shrimp immune responses and are also associated with increased susceptibility to WSD (Liu *et al.*, 2006).

A seasonal variation of WSSV in *P. monodon* broodstock and PL was demonstrated by infected seed that has been reported to occur at higher frequency in the winter season (Withyachumnarnkul *et al.*, 2003). Moreover, the 1-step PCR protocol that was used to screen for WSSV infection in PL probably led to a high frequency false negative test results (Thakur *et al.*, 2002). Therefore, the seasonal variation in WSSV might be associated with differences in the WSSV status of PL used for stocking ponds.

The purpose of pond preparation is to provide the shrimp of subsequent crops with a sanitary environment, high quality water, and also a pond that is free from predators, disease carriers and pathogenic organisms. Pond preparation requires a lot of work that has to be carried out between each shrimp production batch. The pond bottom sediment can be removed or decomposed by any suitable means (e.g. dry or wet methods) to reduce organic material accumulated by intensive culture. WSSV is able to infect not only cultivated penaeid shrimp, but also a wide range of other decapods, including crabs, wild shrimp, copepods and possibly aquatic insect larvae

(Flegel, 2006). Thus, filtration, insecticides or disinfectants can be applied to avoid contamination by potential WSSV carriers in the water. Water quality adjustment (e.g. pH, alkalinity) and plankton stimulation are the last two steps taken before stocking the PL. The plankton provides oxygen during day hours and is a natural food resource. It also reduces shrimp stress by providing a dark environment and controlling water temperature. Hence, pond preparation is a very important managerial practice that can help to prevent disease outbreaks, and pond bottom preparation and water management prior to stocking are main strategies in the better management practices (BMPs). A BMP program implemented with small-scale shrimp farmers in southern India helped to avoid negative impacts of WSD (Subasinghe, 2005). Following this program, production performances of demonstration ponds improved significantly when compared to non-demonstration ponds. Although, pond bottom and water treatments are effective measures, the whole process requires at least two weeks to be completed. A short period between each production cycle indicates lack of an appropriate pond preparation and it increases the chance of a WSD outbreak.

1.2 Confounding factors

Recording of pond observations was stopped when ponds experienced an outbreak. Thus, ponds that stayed in the study for longer periods could have bridged seasons. Therefore, the age of shrimp at harvest date (aghav) was investigated as a potential confounder. A significant difference between the crude odds ratio and the adjusted odds ratio for seasonal factors was observed when the age at harvest date was put into the model. Thus, the confounding effect of age at harvest was considered, even though it was not considered to be an interesting predictor.

1.3 Comparison of statistical approaches

Data with small correlation coefficients and large numbers of cluster members are best analyzed using the GEE approach (Tsou, 2000). In contrast, loss of accuracy can be quite significant for large cluster size data. Hence, GEE analysis was

suitable for our data given that it had a very small intra-class correlation (r = -0.0027) and data sets for large numbers of ponds (70). It can be seen that the standard error of the estimated parameters for the GEE analysis was close to that obtained by ordinary logistic regression analysis. However, results of the two logistic-type multifactorial statistical analyses gave the overall impression that the estimates and standard errors were agreeable. The results indicated that ordinary logistic regression was robust enough to handle the longitudinal data that comprised many pond data sets and only 2 levels of data structure.

2. Study 2 on the potential for horizontal transmission of WSD between ponds

In this study, we describe a method to quantify the horizontal transmission of WSSV between ponds from data collected during an outbreak. With this method, we estimated and compared the transmission of WSSV during 3 phases of the 1998 – 1999 epidemics in the study farm. With no difference in *T* value among the three study phases, the horizontal transmission of WSSV was highest in the second phase during the rainy-winter season. The observed value of R_h exceeded 1 and, consequently, the epidemic continued to grow. However, the transmission rate was very low in phases 1 and 3 with R_h value <1. Therefore, the epidemic faded in the summer season.

The high value of *ß* in phase 2 indicated that season was very important for epidemic outbreaks of WSD. Usually, sudden fluctuations in temperature and salinity were documented during the rainy and winter period in the area of the study. Changes in ambient salinity may disrupt the osmotic balance in shrimp. In order to readjust the osmotic balance, shrimp may have to use a considerable quantity of energy (Chen and Lin, 1998). Moreover, change in ambient temperature can also effect to the osmotic characteristics of fluids, particularly in living systems by influencing water movements across cell membranes and the uptake and loss of ions (Vernberg and Silverthorn, 1979). A previous study in *Penaeus chinensis* juveniles, concluded that the osmolality of the hemolymph increased with an increase in salinity, and decreased with an increase in temperature (Chen *et al.*, 1995). Therefore, fluctuations in water

salinity and low temperature could have a synergistic effect on osmoregulation capacity in shrimp. Stress induced by extreme changes in temperature and salinity can make shrimp more susceptible to infection (Kaustsky, 2000; Liu *et al.*, 2006).

Several previous reports have indicated a relationship between pathogenesis of WSSV infection and ambient temperature. The favourable temperature range for WSSV replication was 23 to 28 ^{o}C. This temperature gave the highest WSSV replication rate, the shortest LT50 and the most serious infections with *Penaeus japonicus* (Guan *et al.*, 2003). During infection with WSSV, the total hemocyte count (THC) decreases in severely infected shrimp. This would most likely be due to a very large number of haemocytes being infected by WSSV and dropping out of circulation. Further, the haemopoietic tissue is infected so that haemopoiesis is prevented. Crustacean haemocytes play an important role in the host immune responses including recognition, phagocytosis, melanization and cytotoxicity. Decrease of THC can affect crustacean resistance to pathogens (Persson *et al.*, 1987). In crayfish, mortalities reached 100% when the animals were at 22±2 ^{o}C while it could be delayed after transfer to a temperature below 16 ^{o}C (Jiravanichpaisal *et al.*, 2004).

The relationship between environment and WSSV viability has been reported previously. The virus is viable for at least 30 days at 30°C in sterile seawater under laboratory conditions (Momoyama *et al.*, 1998). It remains infective for 10, 7 and 5 days at 4, 25 and 28 °C, respectively, when suspended in sea water at a low concentration (Maeda *et al.*, 2000). It is viable in ponds for at least 3-4 days at ambient temperature (Chang *et al.*, 1998b; Maeda *et al.*, 1998; Nakano *et al.*, 1998). The previous information showed relatively similar periods for WSSV infectivity at shrimp cultivation temperature.

Usually, the transmission potential of a pathogen between ponds increases if one or more of the following aspects increase: amount of viable virus that is transferred during a contact increases, the susceptibility of non-infected ponds increases, the rate at which contact occurs increases and the number of different ponds that make contact increases (Koopman and Longini, 1994). Much evidence indicates

that the risk of WSSV infection increases for shrimp cultured in the rainy-winter season due to increased stress, decreased immunological response and more virulence at low temperature. This study confirmed the importance of the environment (i.e. temperature and salinity) in triggering WSSV outbreaks.

3. Study 3 on the molecular epidemiology of white spot syndrome virus

Genotyping of WSSV is based mainly on the variable number tamdem repeats (VNTR) associated with DNA minisatellites in the WSSV genome and on SNPs associated with these repeats. Of the 3 minisatellites in the WSSV genome, ORF94 with a 54 bp repeat region has been most commonly used for previous attempts in genotyping (van Hulten *et al.*, 2000b; Wongteerasupaya *et al.*, 2003; Dieu *et al.*, 2004; Hoa *et al.*, 2005; Musthaq *et al.*, 2006). In this study, the 3 minisatellite regions from ORF75, 94 and 125 plus other tandem repeat loci analyzed using novel primer sets were used for genotyping WSSV isolates in Thailand.

The novel primer sets designed for the flanking regions of ORF66, ORF76, ORF 84 and ORF116 failed to detect variation in repeat unit loci. However, the amplification success for ORF66 (95.1%) and ORF84 (90.2%) were rather high when compared to success for primer sets for ORF76 and ORF116. The low amplification performance of primer sets hinders their usefulness for epidemiological analysis. However, the results from this study showed that these loci were conserved regions that could not be referred to as variable number tandem repeats (VNTR).

The primers ORF75FLANK (F/R) had poor amplification capability (41.5%) with WSSV isolates in Thailand. Those results correlated with the observations of Pradeep *et al*. (2008) for Indian isolates (47.2%). The failure in PCR could not be due to low levels of WSSV or the poor quality of DNA template since good amplification results were obtained using the same template with other primer sets [i.e. ORF94 (82.9%)]. It is possible that variation in primer target sequence was sufficient to result in reduced primer binding and false negative assay results, particularly for very low copies of viral DNA (Kiatpathomchai *et al.*, 2005; Pradeep *et al.*, 2008). The lower

rate of failure in the case of ORF94 PCRs suggests the primer binding region flanking the RU is more stable and less prone to genetic variation. Due to the fact that we used unpurified WSSV virus for DNA extraction, the high proportion of host DNA present also could have affected the efficiency of the genotyping PCR. The ladder effect sometimes seen as bands smaller than the expected product has also been recorded as an amplification artifact. The ladder effect can be resolved by dilution of the sample template while the expected product remains (Hoa *et al.*, 2005).

The ORF75 VNTR is a compound repeat of 45 and 57 bp repeat units. This is in agreement with the previous observation of Shekar *et al*. (2005), but is in contrast with those reported by Marks *et al*. (2004) and Dieu *et al*. (2004) who found RUs of 102 and 45 bp. Compared to the other two VNTR (i.e. ORF94 and ORF125), the repeats in ORF75 seem to be rather conserved within and between each sampling location in Thailand. This result correlated with a study of ORF75 in samples collected from different areas in Vietnam (Dieu *et al.*, 2004) and India (Pradeep *et al.*, 2008). ORF75 gave 6 genotypes with Indian isolates and only 3 genotypes with Vietnam isolates (Dieu *et al.*, 2004). The amplicon sizes of WSSV-IND (India isolate) were mainly 525-540 bp while those for the WSSV-VN (Vietnam isolates) were mainly 527-532 bp and the largest fragment was 1046 bp. The Thai isolate gave larger fragments than those found in both these other countries. Because this locus included a compound repeat unit (i.e. 45 and 57 bp), DNA sequencing was necessary to know the exact numbers of each RUs. Thus, it is more convenient to use to the other two minisatellites for genotyping WSSV. The low amplification success was also a problem for this locus. Perhaps the high incidence of failure could be reduced by design of different primers.

The largest genomic variation among the Thai isolates was observed for the tandem repeats located in ORF94. In the present study, samples collected from Nakhon Pathom, central of Thailand supplemented information reported in a pervious study (Wongteerasupaya *et al.*, 2003). No specific pattern was observed with the Nakhon pathom isolates, except that they showed large fragments (i.e. 17 to 20 repeat units) when compared to isolates from other locations. In the previous study, large

amplicon sizes were found mainly in the samples isolated from Chachoengsao province in 2000. These samples from both locations were obtained from outbreak ponds over the interval 1999 – 2000. However, the distance between the two provinces is approximately 150 km. The results suggested that these outbreaks were caused by the same type of WSSV, dispite separation of more than 100 kilometers and different water sources used. This being the case, it is unlikely that the ponds became infected by cross contamination (i.e. horizontal transmission). On the other hand, Chachoengsao is an important postlarvae (PL) supplier for inland shrimp culture areas in central Thailand. The PL stocked in Nakhon Pathom and nearby areas are mainly supplied from nurseries in Chachoengsao and the single–step PCR assay gives large numbers of negative results (Kiatpathomchai *et al.*, 2005). It is also considered that WSSV infected PL are the main source of WSD outbreaks in Thailand (Withyachumnarnkul, 1999). Unfortunately, we did not have isolates of Chachoengsao in 2000 for DNA sequencing and SNP study. Additional studies would be needed to prove our hypothesis regarding vertical transmission of WSSV in Thailand.

In Thailand during 2000 to 2002, RU of 6 to 9 accounted for 70% of samples from outbreak ponds (Wongteerasupaya *et al.*, 2003). In Vietnam during 2001 to 2002, repeats of 7 and 9 were dominant and the 8 repeat genotype was absent in outbreak ponds (Hoa *et al.*, 2005). In India during 2002 to 2004, genotypes with 6, 7 or 8 RUs accounted for 57% of outbreak samples (Musthaq *et al.*, 2006). In general it appears that WSSV genotypes with less than 9 repeats in ORF94 are dominant in outbreak ponds (Pradeep *et al.*, 2008).

As with ORF94, there were also variations in the number of 69 bp RUs in ORF125 (Dieu *et al.*, 2004; Pradeep *et al.*, 2008). Those authors reported three RU genotypes ranging from 5 to 7 in Vietnam while eleven RU genotypes were reported from India. The main genotype found in India was 3 RU (47.2% prevalence). We found that number repeats varied from 5 to 14 and that the 6 RU type was dominant (34.15% prevalence). Also, we observed that the second to the penultimate RU contained SNP at positions 2, 9, 50, 53 and 61 bp, and this contrasted with the report

from Vietnam (Dieu *et al.*, 2004), Thailand (Marks *et al.*, 2004) and India (Pradeep *et al.*, 2008).

Previous studies indicated that the ORF94 repeat region had potential for use in epidemiological studies of WSSV outbreaks and in tracing the origin and spread of infections in farming areas. In this study, the ORF125 and ORF75 were also tested for genotyping of WSSV. ORF125 was highly variable in number of repeats as with ORF94 although this result contrasted with the study in Vietnam by Dieu *et al*. (2004). ORF75 seemed to be more stable. Thus, ORF75 might be suitable for study of WSSV spread on a more regional scale.

Using ORF94 and ORF 125 together might give more benefit than using either one alone for genotyping WSSV among outbreak ponds in the same farm or for tracing the sources of the pathogen (i.e., PL, biological carriers, vector, e.t.c.). The result indicated 18 actual WSSV genotypes out of a possible total of 216 (ORF94 *ORF125 : 18 * 12) if we use both two markers together (Figure 22).

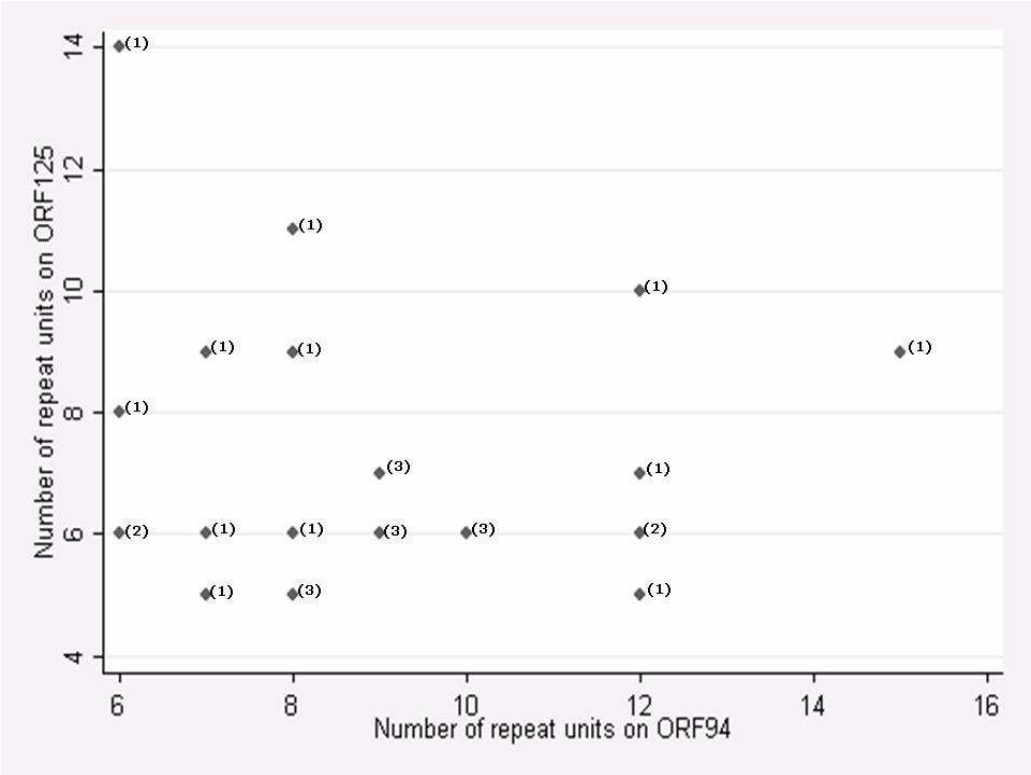

Figure 22 Scatter plot of the distribution of ORF94 repeat units against ORF125 repeat units. The plot indicated 18 actual WSSV genotypes. Letters in parentheses indicated numbers of samples found for each pattern.

CONCLUSION

Traditional epidemiology was conducted to determine the risk factors and possibility of horizontal transmission of white spot syndrome virus between ponds. Genetic markers (VNTR) were investigated to develop a better system for tracing the origin of the pathogen.

The field study on risk factor identification was conducted to identify and demonstrate the strength of association between predictive variables and WSD outbreak ponds. Shrimp seemed to be more sensitive to WSD outbreaks during the rainy-winter season. Therefore, seasonal culture (e.g. stocking shrimp during the summer, mid January to early June) would be the most important preventive measure against WSSV infection. Good pond preparation with appropriate duration of fallow would also be a strategy to avoid WSD outbreaks.

The potential for horizontal transmission of WSD between ponds during the rainy-winter season was very high when compared to the summer season. This circumstance could result from 2 factors. First, the shrimp may be more susceptible to infection with WSSV due to the synergistic effect of fluctuations in salinity and low temperature stress in the rainy-winter season. Secondly, WSSV may survive longer in the environment at low temperature. Therefore, the amount of viable virus that is transferred during a contact might be increased and this could increase the transmission potential between ponds. Although, seasonal culture would be the best method to avoid disease occurrence, improved culture during the rainy-winter season may focus on factors that alter osmoregulation in the shrimp. Under pond production conditions, temperature is difficult to control but salinity monitoring and adjustment to maintain stable conditions may be possible.

This study showed potential for the use of genetic markers to study WSSV epidemiology. Using the two VNTR (ORF94 and ORF 125) together might be more beneficial than using either one alone, to trace the sources of WSSV during outbreaks.

Unfortunately, during studies 1 and 2, WSSV samples were not collected and genetic markers to analyse them were not developed until a later date.

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APPENDICES

Appendix A

White spot syndrome virus host range

Appendix Table A1 White spot syndrome virus host range

Source: Escobedo-Bonilla et al., 2008

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
Penaeid shrimp	*Farfantepenaeus aztecus*		X	Histo	USA[10]
	F. duorarum		X	Histo	USA[10]
	Fenneropenaeus chinensis	X	X	Histo, ISH, PCR	China, Korea, Thailand[13, 25, 26]
	F. indicus	X	X	Histo, PCR, TEM	India, Indonesia, Thailand[14, 15]
	F. merguiensis	X	X	Histo, PCR, IIF	Malaysia, Thailand[3, 4, 18, 19]
	Litopenaeus setiferus		X	Histo	USA[10]
	L. stylirostris	X	X	Histo	USA, Latin America[10, 13]
	L. vannamei	X	X	Histo, ISH, TEM	USA, Latin America[10, 13]
	Marsupenaeus japonicus	X	X	Histo, PCR, TEM	China, Japan, India[11, 13, 23, 24, 26]
	Metapenaeus dobsonii	X	X	Histo, PCR, TEM	India[4, 16]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
	M. ensis	X	X	ISH, PCR	Taiwan[1, 11, 23, 24]
	M. monoceros		X	PCR	India[16]
	Penaeus monodon	X	X	Histo, ISH, PCR	At least eight Asian countries[1, 11, 14, 15, 23, 24, 26]
	P.penicillatus	X		ISH, PCR	Taiwan[11, 23]
	P. semisulcatus	X	X	ISH, PCR	India, Taiwan[11, 15, 23]
	Parapenaeopsis stylifera	X		PCR	India[4]
	Solenocera indica	X		PCR	India[4]
	Trachypenaeus curvirostris	X	X	ISH, PCR	Taiwan[23, 24]
Caridean shrimp	*Alpheus sp.*		X	PCR	Thailand[11]
	Callianassa sp.		X	PCR	Thailand[11]
	Exopalaemon orientalis		X	ISH, PCR	Taiwan[23, 24]
	Palaemon sp.	X		ISH, PCR	Taiwan[11]
	P. adspersus		X	TEM, ISH, PCR, dot-blot	France[2]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
	Macrobrachium idella		X	Histo, WB	India [5]
	M. lamerrae		X	Histo, WB	India[17]
	M. rosenbergii	X	X	Histo, ISH, PCR	India, Taiwan[4, 11, 15, 23, 24]
Lobster	*Panulirus homarus*		X	Histo	India[15]
	P. longipes	X	X	ISH, PCR	Taiwan[24]
	P. ornatus	X	X	Histo, ISH, PCR	India, Taiwan[15, 23]
	P. penicillatus		X	ISH, PCR	India, Taiwan[1, 23]
	P. polyphagus	X	X	Histo	India[15]
	P. versicolor	X	X	ISH, PCR	Taiwan[1, 23]
	Scyllarus arctus		X	TEM, ISH, PCR, Dot-blot	France[2]
Crayfish	*Astacus astacus*		X	PCR	Sweden[7]
	A. leptodactylus		X	TEM, ISH, PCR, Dot-blot	France[2]
	Cherax destructor		X	Histo, Dot-blot	Australia[3]
	Pacifastacus leniusculus		X	Histo, ISH	Sweden[6]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
	Procambarus clarkii		X	ISH, PCR	China, Taiwan[1, 5, 23]
	Orconectes limosus		X	TEM, ISH, PCR, Dot-blot	France[2]
Crab	*Atergatis integerrimus*		X	PCR	India[19]
	Calappa philarigus	X	X	Histo, ISH, PCR	India, Taiwan[9, 19]
	Callinectes lophos		X	ISH, PCR	Taiwan[23]
	Cancer pagurus		X	TEM, ISH, PCR, Dot-blot	France[2]
	Carcinus maenas		X	TEM, ISH, PCR, Dot-blot	France[2]
	Charybdis annulata	X	X	Histo, PCR	India[4, 19]
	C. cruciata		X	PCR	India[4]
	C.feriatus	X	X	Histo, ISH, PCR	India, Taiwan[9, 11, 23]
	C. granulata		X	ISH	Taiwan[1, 23]
	C. lucifera	X	X	Histo, PCR	India[12, 19]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
	C. natatus	X	X	Histo, ISH PCR	India, Taiwan, Thailand[9, 19]
	Demania splendida		X	PCR	India[19]
	Doclea hybrida		X	Histo, PCR	India[19]
	Gelasimus marionis nitidus	X		PCR	India[4]
	Grapsus albolineatus		X	Histo, PCR	India[19]
	Halimede ochtodes		X	Histo, PCR	India[19]
	Helice tridens	X		PCR	Taiwan, Thailand[9, 11]
	Liagore rubronaculata		X	Histo, PCR	India[19]
	Liocarcinus depurator		X	TEM, ISH, PCR, Dot-blot	France,India[8, 15]
	L. puber		X	TEM, ISH, PCR, Dot-blot	France, India[8, 15]
	Lithodes maja		X	Histo, PCR	India[19]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
Crab	*Macrophthalmus sulcatus*	X		PCR	India[4]
	Matuta miersi		X	Histo, PCR	India[19]
	M. planipes	X		PCR	India[12]
	Menippe rumphii		X	PCR	India[19]
	Metapograpsus sp.		X	Histo	India, Taiwan[15]
	Metapograpsus messor	X		PCR	India[4]
	Paradorippe granulata		X	Histo, PCR	India[19]
	Paratelphusa hydrodomous		X	Histo, PCR,	India[18]
	P. pulvinata		X	Histo, PCR,	India[18]
	Parthenope prensor		X	Histo, PCR	India[19]
	Phylira syndactyla		X	Histo, PCR	India[19]
	Podophthalmus vigil		X	Histo, PCR	India[19]
	Portunus pelagicus	X	X	Histo, ISH, TEM	Taiwan, Thailand[9, 11, 21]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
	P. sanguinolentus	X	X	Histo, ISH, PCR	India, Taiwan[1, 9, 11, 19, 24]
	Sesarma sp.		X	Histo, ISH, PCR	India, Thailand[8, 15]
	S. oceanica	X		PCR	India[12]
	Scylla serrata	X	X	Histo, ISH, PCR	India, Taiwan, Thailand[8, 9, 11, 15, 19, 21]
	S. tranquebaricca		X	Histo	India[15]
	Thalamite danae		X	Histo, PCR	India[19]
	Uca pugilator		X	Histo, ISH	Thailand[8]
Other	*Sergestoidea, Acetes sp.*	X	X	Histo, ISH, PCR	Thailand[21]
	Cirripedia Balanus sp.	X	X	PCR	Mexico, Thailand[11, 16]
	Branchiopoda Cladocera	X		PCR	Mexico[16]
	Branchiopoda Artemia sp.	X		PCR	India[12]
	Stomatopoda, Squilla mantis	X		PCR	India[4]
	Copepoda	X		PCR	Mexico, Thailand[11, 16]

Appendix Table A1 (Continued)

Animal	Species	Type of infection		Detection method	Country (references)
		Natural	Experimental		
	Chaetognata	X		PCR	Mexico[16]
	Rotifera	X		PCR	China[25]
	Polychaeta, Marphysa sp.	X		PCR	India[22]
	Coleoptera Ephydridae	X		PCR	Taiwan[11]

References: [1]Chang et al. 1998; [2]Corbel et al. 2001; [3]Edgerton 2004; [4]Hossain et al. 2001; [5]Huang et al. 2001; [6]Jiravanichpaisal et al. 2001, 2004; [8]Kanchanaphum et al. 1998; [9]Kou et al. 1998; [10]Lightner et al. 1998; [11]Lo et al. 1996a; [12]Lo et al. 1999; [13]Lu et al. 1997; [14]Rajan et al. 2000; [15]Rajendran et al. 1999; [16]Ramírez-Douriet et al. 2005; [17]Sahul-Hameed et al. 2000; [18]Sahul-Hameed et al. 2001; [19]Sahul-Hameed et al. 2003; [20]Shi et al. 2000; [21]Supamattaya et al. 1998; [22]Vijayan et al. 2005; [23]Wang et al. 1998a; [24]Wang et al. 1998b; [25]Yan et al. 2004; [26]Zhan et al. 1998;.

Note: Histo, Histopathology; PCR, polymerase chain reaction; ISH, in situ hybridization; TEM, transmission electron microscopy; IIF, indirect immunofluorescence

Appendix B

Chemical reagents and substances

1. Protocol for PCR with Phusion™ Hot Start High-Fidelity DNA Polymerase (FINNZYMES)

Component	Volume / 20 µl reaction	Final concentration
Water, nuclease-free	add to 20 µl	-
5x Phusion HF Buffer*	4 µl	1x
10 mM dNTPs	0.4 µl	200 µM each
primer A**	x µl	0.5 µM
primer B**	x µl	0.5 µM
template DNA	x µl	10pg-1µg
Phusion Hot Start DNA Polymerase (2 U/µl)	0.2 µl	0.02 U/µl

* Optionally 5x Phusion GC Buffer can be used, see section 4.2. for details.
** The recommendation for final primer concentration is 0.5 µM, but it can be varied in a range of 0.2-1.0 µM if needed.

2 Reagent for DNA Extraction

2.1 Lysis solution

2.1.1 100 mM NaCl

2.1.2 10 mM Tris/HCl, pH8

2.1.3 25 mM EDTA (ethylene diamine tetra-acetic acid)

2.1.4 0.5% SLS (sodium N-Laurylsarcosinate) or 2% SDS (sodium dodecyl sulfate)

2.1.5 0.5 mg/ml proteinase K (added just before use).

2.2 5 M NaCl

2.3 1/10volume of N-cetyl N,N,N-trimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7M NaCl)

2.4 chloroform/isoamyl alcohol (24/1)

2.5 95% or absolute ethanol

3. Buffer for agarose gel electrophoresis

3.1 20X TAE buffer pH 8.3 stock solution

3.1.1 0.8 M Tris HCl

3.1.2 0.4 M sodium acetate

3.1.3 0.04 M Na_2EDTA

3.1.4 Adjust pH with glacial acetic acid to pH 8.3 and bring to 1 litre with distilled water.

3.2 10X loading buffer / dye

3.2.1 20% glycerol

3.2 2..0.01% bromphenol blue

3.2.3 add TE to final volume

3.3 5 mg/ml ethidium bromide (EtBr)

3.3.1 500 mg EtBr

3.3.2 add distilled water to 100 ml

Appendix C

The standard protocols

1. Agarose gel electrophoresis (Sambrook *et al.*, 1989)

1.1 Prepare an agarose gel, according to recipes list e.g. 1% gel in 1XTAE buffer, by combining the agarose with buffer in the Erlenmeyer flask and wrapped with clear pored plastic (low gel temperature agarose may also be used) and melt the agarose for 3 minutes by microwave oven. Check the gel temperature before pouring the gel onto plate, the desired temperature is about 55-60 °C.

1.2 Pour the gel onto a taped plate with casting combs in place. Allow 20 - 30 mins for solidification.

1.3 Carefully remove the tape and the gel casting combs and place the gel in a horizontal electrophoresis apparatus. Add 1x TAE electrophoresis buffer to the reservoirs until the buffer just covers the agarose gel.

1.4 Add at least one- tenth volume of 5x agarose gel loading dye to each DNA sample, mix, and load into the wells. Electrophoresis the gel at 50 - 100 V/cm until the required separation has been achieved.

1.5 Incubate the agarose gel in EtBr tank for 15-20 minutes.

1.6 Visualize the DNA fragments on a long wave UV light box.

Appendix D

Manufacturer’s instruction of commercial kit

1. Protocol for DNA extraction from agarose gels (NucleoSpin ® Extract II)

1.1 Excise DNA fragment / Solubilize gel slice

1.1.1 Take a clean scalpel to excise the DNA fragment from an agarose gel.

1.1.2 Excise gel slice containing the fragment carefully to minimize the gel volume.

1.1.3 Determine the weight of the gel slice and transfer it to a clean tube. For each 100 mg of agarose gel add 200 µl Buffer NT.

1.1.4 Incubate sample at 50°C until the gel slices are dissolved (5-10 min).

1.1.5 Vortex the sample briefly every 2-3 min until the gel slices are completely dissolved.

1.2 Bind DNA

1.2.1 Place a NucleoSpin® Extract II Column into a CollectionTube (2 ml) and load the sample.

1.2.2 Centrifuge for 1 min at 11,000 x g. Discard flow-through and place the NucleoSpin® Extract II Column back into the Collection Tube.

1.3 Wash silica membrane

Add 600 µl Buffer NT3. Centrifuge for 1 min at 11,000 x g. Discard flow-through and place the NucleoSpin® Extract II Column back into the Collection Tube.

1.4 Dry silica membrane

Centrifuge for 2 min at 11,000 x *g* to remove Buffer NT3 quantitatively. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the Collection Tube.

1.5 Elute DNA

1.5.1 Place the NucleoSpin® Extract II Column into a clean 1.5 ml microcentrifuge tube (not provided).

1.5.2 Add 15-50 µl Elution Buffer NE and incubate at room temperature for 1 min to increase the yield of eluted DNA. Centrifuge for 1 min at 11,000 x g.

Appendix E

IUPAC Nucleotide ambiguity codes

Appendix Table E1 IUPAC Nucleotide ambiguity codes

Nucleotide ambiguity symbol	Nucleotide
A	A
T	T
C	C
G	G
M	A/C
R	A/G
W	A/T
S	C/G
Y	C/T
K	G/T
V	A/C/G
H	A/C/T
D	A/G/T
B	C/G/T
X/N	A/T/C/G

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