โรคตัวแดงดวงขาวในกุ้งขาวแวนนาไม (*Litopenaeus vannamei*) ในประเทศไทย:
ระบาดวิทยา ปัจจัยเสี่ยงของโรคและการจำแนกทางอณูชีววิทยา

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WHITE SPOT DISEASE (WSD) IN PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) IN THAILAND: EPIDEMIOLOGY, DISEASE ASSOCIATED RISK FACTORS AND MOLECULAR TYPING

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ภัทรพล เปี่ยมสมบูรณ์ : โรคตัวแดงดวงขาวในกุ้งขาวแวนนาไม (*Litopenaeus vannamei*) ในประเทศไทย: ระบาดวิทยา ปัจจัยเสี่ยงของโรคและการจำแนกทางอณูชีววิทยา (WHITE SPOT DISEASE (WSD) IN PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) IN THAILAND: EPIDEMIOLOGY, DISEASE ASSOCIATED RISK FACTORS AND MOLECULAR TYPING) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. เจนนุช ว่องธวัชชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. น.สพ. ดร. ชัยเดช อินทร์ชัยศรี, 146 หน้า.

การศึกษานี้สำรวจความชุกของโรคตัวแดงดวงขาวในเขตอุตสาหกรรมการเลี้ยงกุ้งในจังหวัดจันทบุรี ระหว่างปี พ.ศ. 2552 ถึง พ.ศ. 2557 และหาความสัมพันธ์ระหว่างสภาพภูมิอากาศกับการเกิดโรคตัวแดงดวงขาว ทั้งหมด 1,952 ครั้งในช่วงระยะเวลาการศึกษา โดยการใช้โมเดลทางสถิติคือ negative binomial regression (NBR) models โดยจำนวนครั้งของการเกิดโรคนั้นพบมากระหว่างเดือนตุลาคมถึงกุมภาพันธ์ โดยจะเริ่มลดลงตั้งแต่เดือนมีนาคมถึงมิถุนายน และน้อยที่สุดในเดือนพฤษภาคม จากผลของ multivariate NBR model พบว่า จำนวนครั้งการเกิดโรคตัวแดงดวงขาวที่เพิ่มขึ้นสอดคล้องกับอุณหภูมิอากาศที่ลดลง และการเพิ่มของระดับการเปลี่ยนแปลงของอุณหภูมิอากาศระหว่างวัน นอกจากนี้ได้มีการหาปัจจัยเสี่ยงของการเกิดโรคตัวแดงดวงขาวในระดับฟาร์มในเขตดังกล่าวด้วยวิธีการศึกษาเชิงวิเคราะห์แบบย้อนหลังจากผลไปหาเหตุ (case-control study) โดยใช้แบบสอบถามและการสัมภาษณ์เกษตรกรทั้งหมด 157 ฟาร์ม และนำข้อมูลที่ได้มาวิเคราะห์ด้วยโมเดลทางสถิติคือ logistic regression model การศึกษาพบว่าการใช้น้ำร่วมกันจากคลองส่งน้ำ การเลี้ยงกุ้งตลอดปีโดยไม่เว้นช่วง และในกรณีที่เกษตรกรเป็นเจ้าของฟาร์มมากกว่า 1 ฟาร์มนั้นเป็นปัจจัยเสี่ยงของการเกิดโรคตัวแดงดวงขาวในจันทบุรี ในทางกลับกันพบว่าการใช้ปูนขาวสาดพื้นบ่อเพื่อฆ่าเชื้อโรค และการผสมโพรไบโอติกในอาหารนั้นลดโอกาสการเกิดโรค การศึกษานี้ได้เก็บตัวอย่างเชื้อไวรัสตัวแดงดวงขาวจากกุ้งที่เกิดโรคทั้งหมด 137 ตัวอย่างจากภาคตะวันออกและภาคใต้ของประเทศไทยในช่วงปี พ.ศ. 2550 ถึง พ.ศ. 2557 และได้นำมาหาความแตกต่างในระดับพันธุกรรมโดยใช้วิธีการทำปฏิกิริยาลูกโซ่พอลิเมอเรส (polymerase chain reaction; PCR) ในการเพิ่มจำนวนสายพันธุกรรมที่ตำแหน่งที่ใช้จำแนกความหลากหลายทางพันธุกรรม ได้แก่ Indel-I, Indel-II และ Variable number tandem repeats (VNTRs) ที่อยู่ใน ORF ที่ 75, 125 และ 94 ซึ่งผลการวิเคราะห์ Indel-I และ Indel-II พบว่ามีเชื้อไวรัสตัวแดงดวงขาวอย่างน้อย 3 จีโนไทป์ในประเทศไทย ซึ่งจีโนไทป์ดังกล่าวมีความใกล้เคียงกับเชื้อไวรัสตัวแดงดวงขาวจากประเทศ เวียดนาม อินเดีย บราซิล และ ซาอุดิอาระเบีย สำหรับการวิเคราะห์ VNTRs นั้นพบความหลากหลายทางพันธุกรรมมากกว่า โดยพบชนิดของ เชื้อไวรัสตัวแดงดวงขาวอย่างน้อย 33 จีโนไทป์ และจากการวิเคราะห์ในระดับพันธุกรรมทั้งหมดพบว่า พันธุกรรมของเชื้อไวรัสตัวแดงดวงขาวนั้นมีความคงที่ในระหว่างปี พ.ศ. 2550 ถึง พ.ศ. 2557 ซึ่งผลการศึกษานี้อาจนำมาใช้เป็นแหล่งข้อมูลอ้างอิงสำหรับการศึกษาทางระบาดวิทยาของโรคตัวแดงดวงขาวต่อไปในอนาคต

ภาควิชา อายุรศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา อายุรศาสตร์สัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: WHITE SPOT SYNDROME VIRUS / RISK FACTORS / PACIFIC WHITE SHRIMP / EPIDEMIOLOGY / VIRUS GENOTYPING

PATHARAPOL PIAMSOMBOON: WHITE SPOT DISEASE (WSD) IN PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI*) IN THAILAND: EPIDEMIOLOGY, DISEASE ASSOCIATED RISK FACTORS AND MOLECULAR TYPING. ADVISOR: ASSOC. PROF. JANENUJ WONGTAVATCHAI, D.V.M., M.Sc., Ph.D., CO-ADVISOR: ASST. PROF. CHAIDATE INCHAISRI, D.V.M., M.Sc., Ph.D., 146 pp.

Prevalence of white spot disease (WSD) in an intensive shrimp culture area located in Chanthaburi province, Thailand during 2009-2014 was observed. Retrospective data of 1,952 WSD cases were analyzed for the association between WSD occurrence and climate factors negative binomial regression (NBR) models. A high number of WSD cases were found between October to February, while a less number of cases were reported during March to June, and the lowest numbers were reported in May. The multivariate NBR model indicated significant associations between an increased number of WSD cases with decreased atmospheric temperature and more variation of daily atmospheric temperature. Case-control study using logistic regression model was also used to identify the risk of WSD occurrence at farm-level in this area. Results of an interview survey of 157 intensive shrimp farms showed that farms sharing inlet water, culturing shrimp year round and with a single owner operating more than one farm were identified as WSD risk factors. The analysis also showed WSD risks to be reduced at farms that applied lime to disinfect pond bottoms and use of probiotics feeding supplementation. A total of 137 white spot syndrome virus (WSSV) samples causing disease in pond during 2007-2014 were collected from eastern and southern Thailand. The variations in their genome were analyzed using Polymerase chain reaction (PCR) targeting the 5 variable loci, including Indel-I, Indel-II and Variable number tandem repeats (VNTRs) located in ORF75, 125 and 94. Analysis of Indel-I and Indel-II showed the newly 3 WSSV genotypes identified in Thailand. These genotypes were related to WSSV from Vietnam, India, Brazil and Saudi Arabia. Analysis of the VNTRs showed high degree of variation, which at least 33 genotypes were detected. The similarity of WSSV genome in several WSSV isolated collected during 2007-2014 suggested that WSSV genome is now stable. The results from this study may be used as database for further epidemiological study of WSSV.

Department:	Veterinary Medicine	Student's Signature	.....
Field of Study:	Veterinary Medicine	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

AHPND	Acute hepatopancreatic necrosis disease
β	Estimated coefficient
CI	Confidence interval
CFRD	Chanthaburi Coastal Fisheries Research and Development Center
DNA	Deoxyribonucleic acid
EMS	Early mortality syndrome
GPS	Global positioning system
HHNBV	Hypodermal and hematopoietic necrosis beculovirus
Indels	Insertion/deletion sites
IRR	Incident rate ratio
MBV	Monodon baculo virus
NBR	Negative binomial regression
OIE	World Organisation for Animal Health
OR	Odd ratios
ORF	Open reading frame
PAV	Penaeid acute viremia
PCR	Polymerase chain reaction
PL	Postlarvae
PmNOBII	The second non-occluded baculovirus of *P. monodon*
PmNOBIII	The third non-occluded baculovirus of *P. monodon*
PRDV	Penaeid rod-shaped DNA virus
QIC	Quasi Likelihood Independence Model Criterion
RUs	Repeat units
RV-PJ	Rod-shape nuclear virus of *Peneaus japonicas*

SEMBV	Systemic ectodermal and mesodermal baculovirus
SNP	Single nucleotide polymorphism
SPF	Specific pathogens free
TCBS	Thiosulfate-citrate-bile salts-sucrose
VNTR	Variable number tandem repeat
WSD	White spot disease
WSSV	White spot syndrome virus
YHV	Yellow head virus

UNITS

bp	base pair
kb	kilobase pair
℃	degree Celsius
g	gram (s)
g	gravity
km	kilometer (s)
min	minute (s)
sec	second (s)
V	Volt (s)
Ml	milliliter (s)
µl	microliter (s)

CHAPTER I
IMPORTANCE AND RATIONALE

Over the last 40 years, industrialization of penaeid shrimp culture has emerged all over the world, supplying world's food sources and creating hundred thousands of jobs for both skilled and unskilled labor. Thailand is considered one of the world's leading shrimp exporter, with the industry taking part in socioeconomic development of the country by gaining substantial foreign revenue (Lebel et al., 2002; FAO 2014). Shrimp culture has shifted from extensive to intensive system in order to support the rapidly growing industry. Accompanying the growth and poor management of shrimp culture, severe infectious diseases have emerged (Lightner and Redman 1998; Flegel et al., 2008). Economic loss due to diseases was up to approximately US$15 billion in the past 15 years which viral diseases were responsible for 60% of the losses (Flegel 2012). Among viral diseases, white spot syndrome virus (WSSV), an etiological agent of white spot disease (WSD), is one the most devastating agent of all culture shrimp (Flegel 2012). It was considered a disease of Black tiger shrimp (*Penaeus monodon*) at first, but WSSV also affects many other species of penaeid shrimp (Nunan et al., 2001), which the infection can lead to 100% mortality within 3-10 days under culture condition (Nunan et al., 2001). The first WSD outbreak occurred in East Asia in 1991 and subsequently spread throughout the shrimp farming regions across the globe, including South America, Europe and

Australia (Dieu et al., 2010; Muller et al., 2010; Vijayan and Sanil 2012). WSSV pandemic has raised global alert on disease prevention strategies, resulted in increasing number of WSSV researches on many aspects, including epidemiology (Flegel et al., 2008).

The eastern and southern parts of Thailand are two major sites of shrimp farming where WSSV has been introduced since 1994. High prevalence of WSSV detected in wild *P. monodon* broodstock was reported in the cool-monsoon season in Thailand due to the congestion of shrimp in shallow water which induced stress and suitable condition for horizontal transmission of WSD (Withyachumnarnkul et al., 2003). In order to reduce risks of transmitting WSD from wild population, shrimp industry in Thailand has shifted from wild-capture broodstock *P. monodon* to domesticated *L. vannamei* since the beginning of 21st century. However, WSD still causes significant losses in *L. vannamei* cultured (Flegel 2012) and prevalence of WSD in cultured shrimp in Thailand has not been officially reported.

Since there is no effective treatment or vaccine against WSSV, management for pathogens exclusion and stress reduction are needed (Hoa et al., 2005b). In addition, development of preventive measures, such as controlling and monitoring the spread of WSSV using epidemiological methods are necessary. Risk factors analysis may support the construction of intervention strategies (Mohan et al., 2008). Several risk factors associated with WSD in term of pond culture (Tendencia et al.,

2011), transmission (Supamattaya et al., 1998), effects of water physio-chemical parameters (Tendencia et al., 2010b) and carrier organisms (Corsin et al., 2001) have been reported. However, disease risk factors analysis has to be tailor made to suit each particular region and farming system. Study of WSD risk factor in Thailand has never been conducted.

Molecular epidemiology is one of the potential tool for investigating WSSV spread and movement in many spatiotemporal scales, which may support our understanding on virus distribution and evolution (Dieu et al., 2010). Genetic markers have been widely used in both human and veterinary epidemiological studies (Mazars et al., 2001; Knowles and Samuel 2003; Martella et al., 2007). Polymerase chain reaction (PCR) has been used to determine the polymorphic loci in WSSV genome (Dieu et al., 2010). To date, complete WSSV genome sequences of four origins have been carried out, including Taiwan (WSSV-TW; GenBank accession no. AF440570) (Tsai et al., 2000), China (WSSV-CN; GenBank accession no. AF332093) (Yang et al., 2001), Thailand (WSSV-TH; GenBank accession no. AF369029) (van Hulten et al., 2001) and recently from Korea (WSSV-KR; GenBank accession no. JX515788) (Chai et al., 2013). The isolates from Taiwan, China and Thailand show 99.32% sequences similarity (Pradeep et al., 2008b), which indicated that WSSV isolates are closely related and likely to evolve from a common ancestor. The high similarities between the conserved genes were found among WSSV isolates, such as DNA polymerase gene (Chen et al., 2002; Marks et al., 2004) and the major structural

protein genes (Chang et al., 2001; Marks et al., 2004). Therefore, phylogenetic analysis of conserved genes is not suitable for studying WSSV evolution. Molecular markers which have been used to study WSSV epidemiology were major insertion/deletion (Indels) sites between open reading frame (ORF) 14/15 (Indel-I) and 23/24 (Indel-II) (Hoa et al., 2012) and the variable number tandem repeat units (VNTRs) located in ORF 75, 94 and 125 (Wongteerasupaya et al., 2003). Indel-I and Indel-II have been suggested as suitable molecular markers for the study of WSSV distribution and evolution in large and intermediate scales whilst VNTRs were proposed for smaller scale (Dieu et al., 2010). Genomic Indels have been used in many studies as a tool for tracing the occurrences of WSSV and investigating virus evolution in Vietnam (Dieu et al., 2004; Hoa et al., 2012), India (Pradeep et al., 2008b), China (Tan et al., 2009; Tan and Shi 2011), Mexico (Ramos-Paredes et al., 2012) and Brazil (Muller et al., 2010). Pradeep et al., (2008a) used these variable regions to investigate the WSSV outbreaks in India and reported that the Indian isolates were closely related to Thailand's and, Thai isolates may have been introduced to India. In addition, WSSV isolates from Mexico collected during 2000-2005 showed similarity in Indel-I (ORF14/15) with isolates from India (Ramos-Paredes et al., 2012). The epidemiological study of WSSV from Vietnam indicated that WSSV entered Vietnam by multiple introductions (Dieu et al., 2004) and has been moved from central to southern and northern region by trading of shrimp postlarvae (PL)

(Hoa et al., 2012). VNTRs have been used to study WSSV epidemiology at farm or pond-level and to trace movement between infected populations because the high variation of these regions (Pradeep et al., 2008a; Hoa et al., 2011; González-Galaviz et al., 2013). The current status of WSSV genotypes and theirs distribution in Thailand have not been recently investigated, especially in Pacific white shrimp (*Litopenaeus vannamei*), which is a major cultured species in the present time. Constructing genotypic database and study of molecular epidemiology of WSSV may develop the understanding of disease distribution pattern and may be another component to assist in development of preventive measures in Thailand.

Objectives of Study

(1) To investigate the prevalence of white spot disease in cultured shrimp in the intensive shrimp culture area of Thailand.

(2) To identify risk factors associated with WSD occurrence in the intensive shrimp cultured of Thailand.

(3) To use molecular makers to characterize genome of white spot syndrome virus causing outbreaks in cultured shrimp in eastern and southern parts of Thailand.

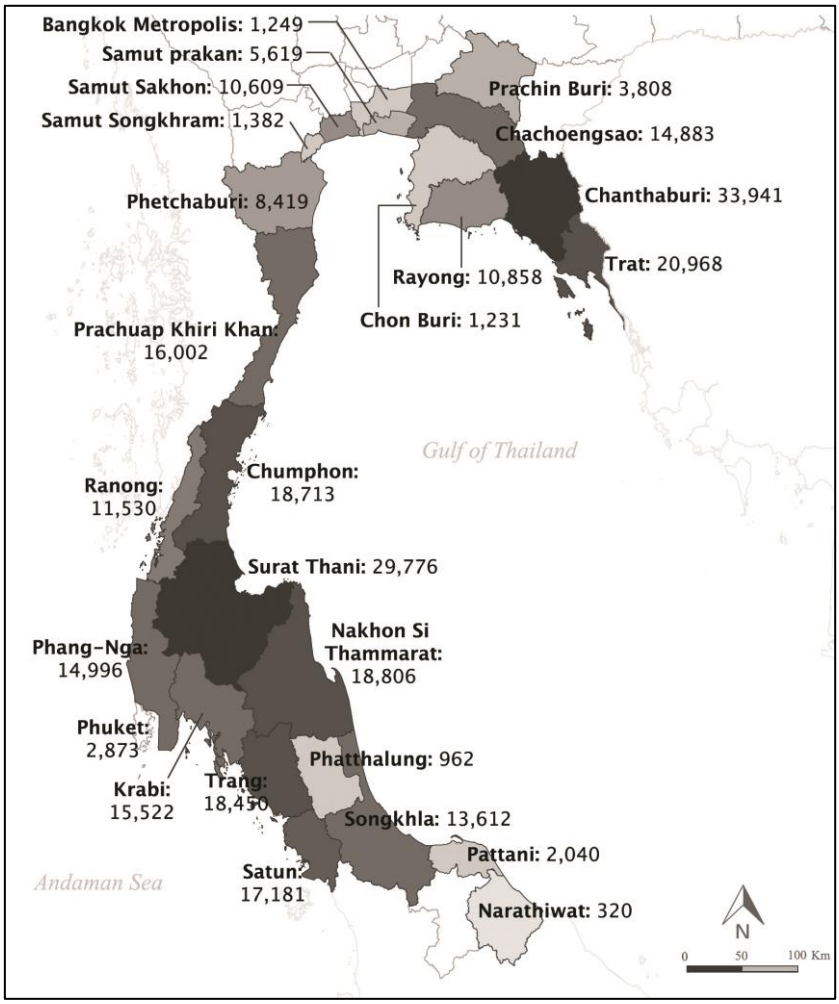
CHAPTER II
LITERATURE REVIEW

2.1 Shrimp culture in Thailand

Shrimp is the largest single seafood commodity by value. Approximately 75% of global shrimp production is from aquaculture. Black tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*) are the most important invertebrate food animals, which entirely dominated the shrimp culture industry (Walker and Winton 2010). Shrimp culture in Thailand has gradually increased since the period of 1970. *P. monodon* was the first major cultured species because of high export value and its ability to grow quickly under farming condition (Szuster 2006). The industry had expanded remarkably after breakthrough of the technique of inducing maturation of captured female broodstock by removing eyestalk. Hatchery produced postlarvae had become a major source for large-scale farming since then (Flegel et al., 2008). Due to the unavailable of technology for disease screening in captured broodstock, improper broodstock sanitary management, and lack of concern regarding diseases and impacts of shrimp farm to the environment; consequently, serious disease outbreaks and environmental degradation has occurred which led to the first collapse of shrimp farming in the Upper Gulf of Thailand region. The industry has turned to the eastern and southern part where good quality of soil and water were still available. In spite of the improved

management and shrimp disease researches during the 1987-1992, viral diseases were still a serious threat to shrimp farmers, for instance, monodon baculovirus (MBV) and yellow head virus (YHV) were the most devastating viruses in *P. monodon* in Asia during that time. Moreover, the emergence of White spot disease (WSD) since 1994 (Marks et al., 2004) had worsened the entire industry which led to significant production losses in Thailand and other Asian countries (Flegel et al., 2008). During this period, production of *P. monodon* which depended on shrimp PL produced from wild capture broodstock were difficult to obtain, especially and disease-free broodstock; therefore, the domesticated lines of specific pathogen-free (SPF) *L. vannamei* was introduced and Thai's shrimp farming industry was resurged since 2001 (Flegel 2012). Nowadays, productions of shrimp are mainly localized in the coastal areas of eastern and southern part of Thailand (Figure 1). Despite the improve biosecurity measures and new shrimp farming technologies (Lightner et al., 2012), losses caused by viral diseases are still occurring and are considered as a major constrain in shrimp farming, especially the White spot syndrome virus which causes high and rapid mortality for all cultivated species (Flegel 2012).

Figure 1. Map of shrimp area of Thailand and total production yield (tons) in 2013. Source: Department of Fisheries, Minister of Agricultural and Cooperative, 2013.



2.2 White spot disease

White spot disease caused by White spot syndrome virus (WSSV), a sole member of the genus *Whispovirus*, within a new virus family *Nimarviridae* (OIE 2015). WSSV is a large double strand DNA virus, ovoid to bacilliform in shape with tail-like appendage at one end (van Hulten et al., 2001). WSSV virions are regular symmetry,

with approximate size of 80-120 nm in diameter and 250-380 nm in length (OIE 2015). Replication of WSSV virions occurs in hypertrophied nuclei of infected cells with the absence of occlusion bodies. WSSV was initially classified as a non-occluded baculovirus based on its appearance (Lightner 2003). Various names had been used to describe this virus. The disease was first called penaeid acute viremia (PAV), and the virus was named after its as penaeid rod-shaped DNA virus (PRDV) or rod-shaped nuclear virus of *P. japonicus* (RV-PJ) (Nakano et al., 1994). In the People's Republic of China, the virus was named Chinese baculo virus or hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Huang et al., 1995). In Thailand, the names systemic ectodermal and mesodermal baculovirus (SEMBV) or the second non-occluded baculovirus of *P. monodon* (PmNOBII) was used (Wongteerasupaya et al., 1995) and in Taipei was called white spot baculovirus (WSBV) or the third non-occluded baculovirus of *P. monodon* PmNOBIII (Lo et al., 1996). However, the virus genome was later characterized and found that it has a unique characteristic different from baculoviruses (Yang et al., 2001).

2.2.1 Susceptible species

Wide range of marine, brackish and fresh aquatic crustaceans can be infected with WSSV, including crabs, crayfish, lobsters and all species of cultured shrimp, such as *P. monodon*, *L. vannamei*, Kuruma prawn *Penaeus japonicas*, Chinese white shrimp *Fenneropenaeus chinensis*, and Indian prawn *Fenneropenaeus indicus*. The

virus can resulted in 100% mortality within 3-10 days after infection under cultured condition (Nunan et al., 2001). All life stages of cultured shrimp from eggs to broodstock are susceptible to WSSV. Postlarvae, juveniles and adults are the most suitable stages for virus detection (OIE 2015). Vertical and Horizontal transmission are possible for WSSV infection, transmission via trans-ovum, consumption of infected tissue (cannibalism) and expose to contaminate water have been reported in previous studies (Corsin et al., 2001; Wu et al., 2001). Infected shrimp may be visible around the edge of the pond, presenting clinical sign of lethargy, reduce feed intake, and reddish to pinkish discoloration of the outer exoskeleton (Escobedo-Bonilla et al., 2008). White spots or patches may be found under the carapace of moribund shrimp which may consequence from virus-induced integument dysfunction (Wang et al., 1998). While WSSV infections in wild aquatic crustacean carriers are presented with variable degree of severity and sometimes clinical disease is absent. These carriers which can be infected with WSSV and express the disease under suitable environmental conditions include, *Acetes* sp., *Alpheus* sp., *Helice* sp., *Hemigrapsus* sp., *Exopalaemon* sp., *Callianassa* sp., *Macrophthalmus* sp., *Macrophthel* sp., *Metaplax* sp., *Mysis* sp., *Orithyia* sp., *Palaemonoidea* sp., *Scylla* sp,. *Sesarma* sp. and *Stomatopoda* sp. Latent infection without disease was reported in several non-decapodal crustaceans, such as *Artemia salina, Balanus* sp., copepods, rotifers and *Tachypleidue* sp. Sea slaters (Isopoda), Euphydradae insect larvae and polychaete worms have also been proven as mechanical vectors of WSSV without any sign of

infection (OIE 2015).

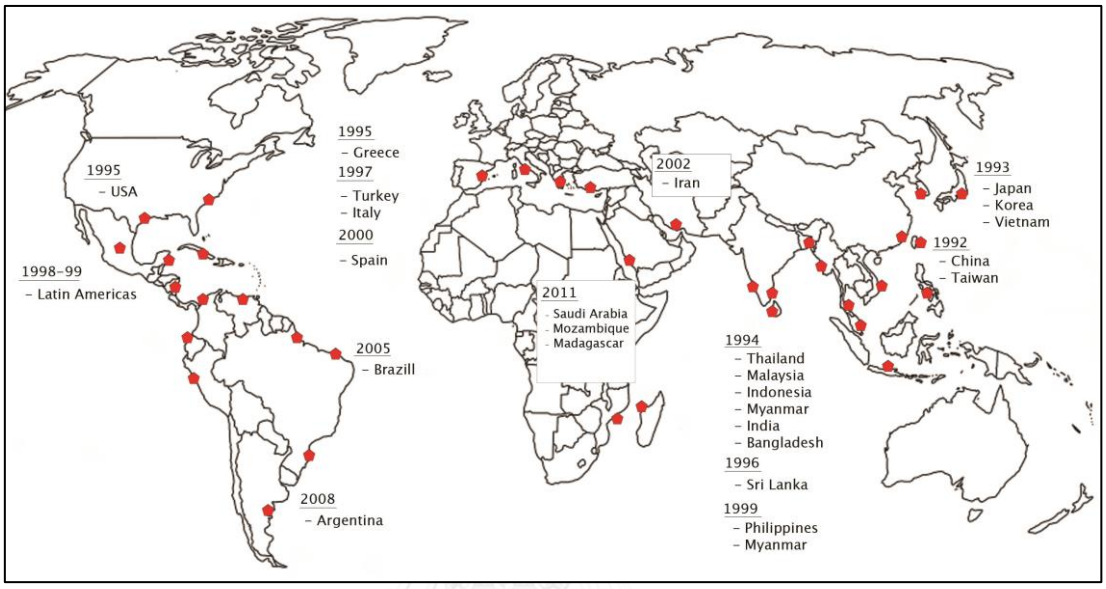
2.2.2 Emerging of WSD and its global distribution

The first occurrence of WSSV was reported in cultures *P. japonicas* in 1992 in Taiwan and Fujian province, China (Chiang and Lo 1995). In 1993, the disease spread to Japan *via* imported juvenile Kuruma shrimps from China (Inouye et al., 1994). In the same year, mass mortality cases of cultured shrimp caused by this virus were also reported in Korea (Park et al., 1998) and Vietnam (Zwart et al., 2010). By 1994, the virus has caused devastating losses in cultured shrimp across Asia, including, India (Karunasagar et al., 1998), Bangladesh (Hossain et al., 2001), Indonesia (Sunarto 2001), Malaysia (Oseko 2006) Thailand (Wongteerasupaya et al., 1995) and followed by Sri Lanka in 1996 (Munasinghe et al., 2010). Philippines and Myanmar was the last two countries in South-East Asia affected by WSD in 1999 (Magbanua et al., 2000; Saw 2004). Transboundary movement of unscreened, grossly normal broodstock and shrimp PL infected with WSSV both legal and illegally was purposed as a major cause of WSSV spread in Asia (Magbanua et al., 2000; Flegel et al., 2008). In 1995, WSSV moved from eastern to western hemisphere, into the United States of America, *via* the infected frozen shrimp and from contaminated waste discharged by processing plants located on the US coastal area, which re-processing the infected frozen shrimp products for value-added (Nunan et al., 1998; Lightner 2003). In addition, Hasson et al., (2006) reported that shrimp used as bait for sport fishing imported from

China were positive with WSSV and could be a potential cause of WSSV contaminant in coastal freshwater and marine crustacean and shrimp farming in the US. Although, WSD has been eradicated from farmed shrimp in the US, WSSV was occasionally detected in wild population of crab and shrimp in southeastern part, which indicated that WSSV may establish in this coastal water (Powell et al., 2015). WSD first appeared in central and South American during 1998-99. Ecuador, which is one of the top shrimp producing country was impacted by the emerging of WSD in mid to late-1999, which resulted in 70% decrease in shrimp export in the following years of 2000-01 (Lightner 2003). WSD was officially confirmed in Brazil in late 2004 to early 2005 (Cavalli et al., 2008) and in Argentina in 2008 (Martorelli et al., 2010). WSD outbreaks were also reported in Europe during 1995-2003. Greece was the first to report WSD cases in 1995, due to the imported shrimp PL from Taiwan, followed by WSD outbreaks in Turkey and Italy (from imported shrimp PL from Turkey) in 1997. WSD form Asian origin caused outbreaks in Spain during 2000-03, by the practice of feeding imported *P. monodon* carcasses to wild harvested *P. japonicas* (Stentiford and Lightner 2011). The first occurrences of WSD in the Middle East were in 2002 in Iran (Simrouni et al., 2014), followed by Saudi Arabia in 2005 from farms that used *P. monodon* broodstock from Southeast Asia (Tang et al., 2012b). Africa was the last continent where WSD occurred in Mozambique and Madagascar in 2011. The virus was believed to reach Africa by oceanic current, ballast water from commercial vessels or imported infected shrimp from Saudi Arabia to the local processing plants

(RAF 2013). Figure 2 illustrates the distribution of WSSV globally.

Figure 2. Global distribution of WSSV, years indicates the first report in each country



2.2.3 Prevention of WSD and factors associated with WSD occurrence

Farm management and effective biosecurity measures that keep WSSV out of the farming system are the most suitable for WSD prevention. Biosecurity in aquaculture aims to prevent pathogens from contracting and spreading within the farm, as well as to eliminate disease carrier and un-desirable health condition (Pruder 2004). Shrimp Infected with WSSV alone do not usually result in disease outbreak. WSD outbreak in pond usually requires multiple factors. Figure 3 summarized the important causes participating in WSD outbreaks in shrimp pond. Standard control measures for reducing the incidence of WSD currently rely on stocking ponds with WSSV-free shrimp PL, using closed zero-water-exchange culture system and/or

biosecure ponds incorporating with crab-proof fencing and bird-proof netting and personal (hands and feet) disinfectant bath (Corsin et al., 2005). However, biosecurity breaches and/or inappropriate farm management may lead to the introduction of disease into a farm. In order to acquire proper biosecurity measures, an epidemiological approach, such as risk factors analysis is one of the tools for establishing preventive strategies for disease control in shrimp culture (Lightner 2005).

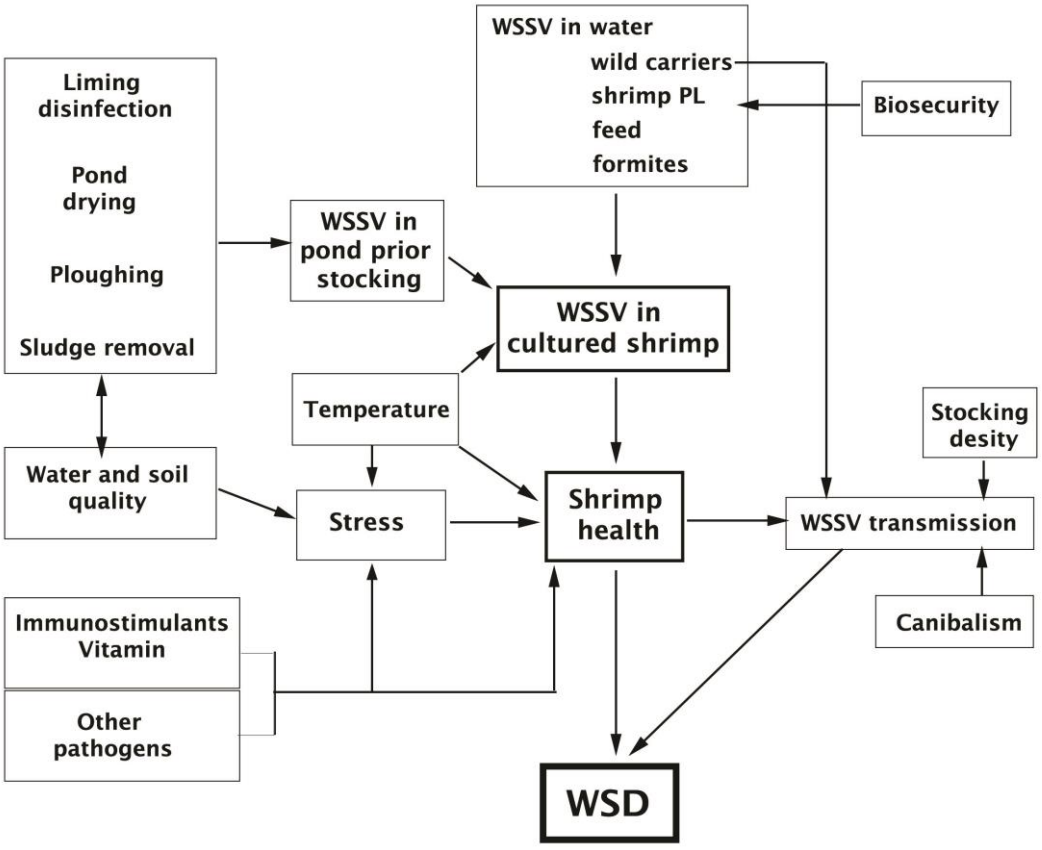
Several studies have applied statistical models to identify risk factor of WSD occurrence in different farming systems. A study in semi-intensive *P. monodon* farm in Philippines found that high stocking densities, feeding of live mollusks, farm sharing common water sources, increasing pond size, and pond water contained high amount of green colonies *Vibrio* on TCBS, were associated with higher risk of WSD occurrence (Tendencia et al., 2011; Tendencia and Verreth 2011). Extensive Vietnamese *P. monodon* farms, located closer to the sea were reported to have higher risk of shrimp to be positive from WSSV at harvest. Authors suggested that these farms had higher chance of receiving WSSV-infected decapods and WSSV-contaminated zooplankton (Corsin et al., 2001). The same study also suggested that increased mangrove to pond area ratios and the use of vitamin dietary supplements, water filtration through 300 µm mesh screens as well as pond bottom dry-out and decontamination, including plowing and sludge removal between crops, reduced the

risk of WSD occurrence (Corsin et al., 2001; Tendencia et al., 2011). In addition, application of aquaculture lime to disinfect the fallowed pond bottoms was reported to be useful in preventing WSD in *P. monodon* farms in India (MPEDA/NACA 2003) and Bangladesh (Islam et al., 2014).

Environmental factors, including temperature and rainfall also play an important role for WSD occurrence. Previous studies suggested that the occurrence of WSD was associated with relatively low and/or fluctuated temperature. High prevalence of WSD outbreaks in Ecuadorian shrimp farm was observed during cool season from June to November (Rodríguez et al., 2003). In Mexico, abrupt water temperature and salinity changes due to 3 days of heavy rain resulted in WSD outbreaks and viral load increased in cultured *L. vannamei* (Peinado-Guevara and López-Meyer 2006). In the Philippines, WSD incident in *P. monodon* farm increased in a month with >14 continuous rain days and relatively low atmospheric temperature (Tendencia et al., 2010a). Environmental factors not only affect shrimp immune system by acting as shrimp stressor, but also influence on biology of the virus. Stress reduces immune responses; consequently, WSSV are able to replicate more efficiently and cause mortality in cultured shrimp (Doan et al., 2009). A crucial shrimp defense mechanism against WSSV infection such as virus-induced apoptosis was also found decreasing at water temperature below 32°C (Granja et al., 2003). Higher mortality rates were observed in *P. monodon* orally-challenged with WSSV at

water temperature ranging from 16°C to 30°C than those of 32°C to 36°C challenges (Raj et al., 2012).

Figure 3. Diagram of Factors effecting WSD outbreak in pond-cultured shrimp, modified from Corsin et al., (2005)



2.2.4 Molecular epidemiology of WSSV

WSSV genome is a large circular double stranded DNA of approximately 300 kb. The virus genome contains 184 putative ORFs which only 11 ORFs are recognized in public databases as genes encoding DNA replication, enzyme for nucleotide metabolism and protein modification (van Hulten et al., 2001). Epidemiological study of WSSV have been carried out using complete WSSV genome sequences of three origins as reference databases, including Taiwan (WSSV-TW; GenBank accession no. AF440570; Tsai et al., 2001), China (WSSV-CN; GenBank accession. no. AF332093; Yang et al., 2001) and Thailand (WSSV-TH; GenBank accession no. AF369029; van Hulten et al., 2001). PCR technique has been used to differentiate a little difference (~0.8%) among the genome of these isolates. The significant differences in WSSV genome were composed of variable regions located between ORF14 and 15, ORF23 and 24, in ORF75, 94, and 125, a transposase sequence that present only in WSSV-TW, and single-nucleotide polymorphisms (SNPs) (Marks et al., 2004).

Genomic regions between ORF14/15 and ORF23/24 are known as major insertions/deletions (indels) sites. Indel I (ORF14/15) contains a variable region prone to recombination whereas Indel-II (ORF23/24) contains a large genomic deletion. WSSV adapted in specific novel environment where it was introduced by gradually removed its redundant sequences (Dieu et al., 2010). By investigating the pattern of these two regions, epidemiologists were able to trace movement and evolution of

WSSV (Pradeep et al., 2008b). Dieu et al., (2010) applied statistical analysis to identify which variable regions are suitable for each particular geographical scale, based on their degree of variation. The authors suggested that Indel-I has lesser degree of genomic variation and is suitable for the continental and global scale (10,000 km), while indel-II has higher degree of variation and therefore appropriate for regional or within the country scale (1,000 km). Each WSSV-TW, WSSV-CN and WSSV-TH contains a unique sequence in the indel-I region, whereas WSSV-CN and WSSV-TH has ~2 kb and ~13.2 kb deletion in Indel-II compared to WSSV-TW, respectively (Figure 4 and 5). This indicated that WSSV were derived from common ancestor and evolved separately by deleting its redundant sequences during the spread from either side of Taiwan Strait to China and Southeast Asia (Dieu et al., 2004). However, after the analysis of WSSV isolate obtained from Thailand in 1996, Mark et al. (2005) found this isolate (WSSV-96-II) contains the combination of genomic sequence in Indel-I region from WSSV-TW, WSSV-CN and WSSV-TH and also contains a full length of 13.2 kb in Indel-II, which similar to WSSV-TW. This created another hypothesis that WSSV-96-II might be as common ancestor of all WSSV which initially moved from Thailand, causing the first outbreak in Taiwan and China and then spread back to Thailand before distributed to other countries (Pradeep et al., 2008b). Introduction of WSSV into several countries were explained using the analysis of these Indels. WSSV entered Vietnam from multiple introductions, from both China and Taiwan to central Vietnam and then spread north and south. WSSV was also directly introduced to

southern Vietnam from Thailand (Dieu et al., 2010). Genotypes of WSSV occurred in India were closely related to Thai isolate (Pradeep et al., 2008b). WSD occurrences in China were caused by the particular genotypes circulated in the area (Tan et al., 2009; Tan and Shi 2011). WSSV samples obtained from Philippines, Indonesia, Cambodia and Iran showed some degree of similarity of WSSV from Thailand (Zwart et al., 2010). In addition, WSSV Mexican isolates was found to have similar pattern of Indel-I with Indian isolate (Ramos-Paredes et al., 2012). Pattern of Indel-I and Indel-II of WSSV collected from different countries are summarized in Figure 4 and 5.

Variable-number tandem repeats (VNTRs) are located in ORF75, 94, 125. These ORFs contain repeat units (RUs) in the middle and are flanked with non-repeated sequence at 5' and 3' ends. ORF75 includes 2 types of RUs, 45 bp and 102 bp. Repeat unit of 54 bp with SNPs at the position 36 or 48 are presented in ORF94 and ORF125 contains 69 bp RU (Muller et al., 2010). These polymorphic loci contain high degree of variation compare to the Indels. Hence, VNTRs have been used to investigate movement of WSSV in smaller scales, for example, among cluster of shrimp culture communities, between pond to pond or farm to farm (Dieu et al., 2010). In addition, VNTRs can be used to trace the source of WSSV causing outbreak in pond from potential carriers such as crab (Hoa et al., 2011). Several studies were conducted to determine pattern of VNTRs of WSSV found in WSD endemic countries and the patterns of VNTRs reported by these countries are shown in Table 1.

Figure 4. Schematic representation of WSSV variable region, Indel-I (ORF14/15), reported from different countries

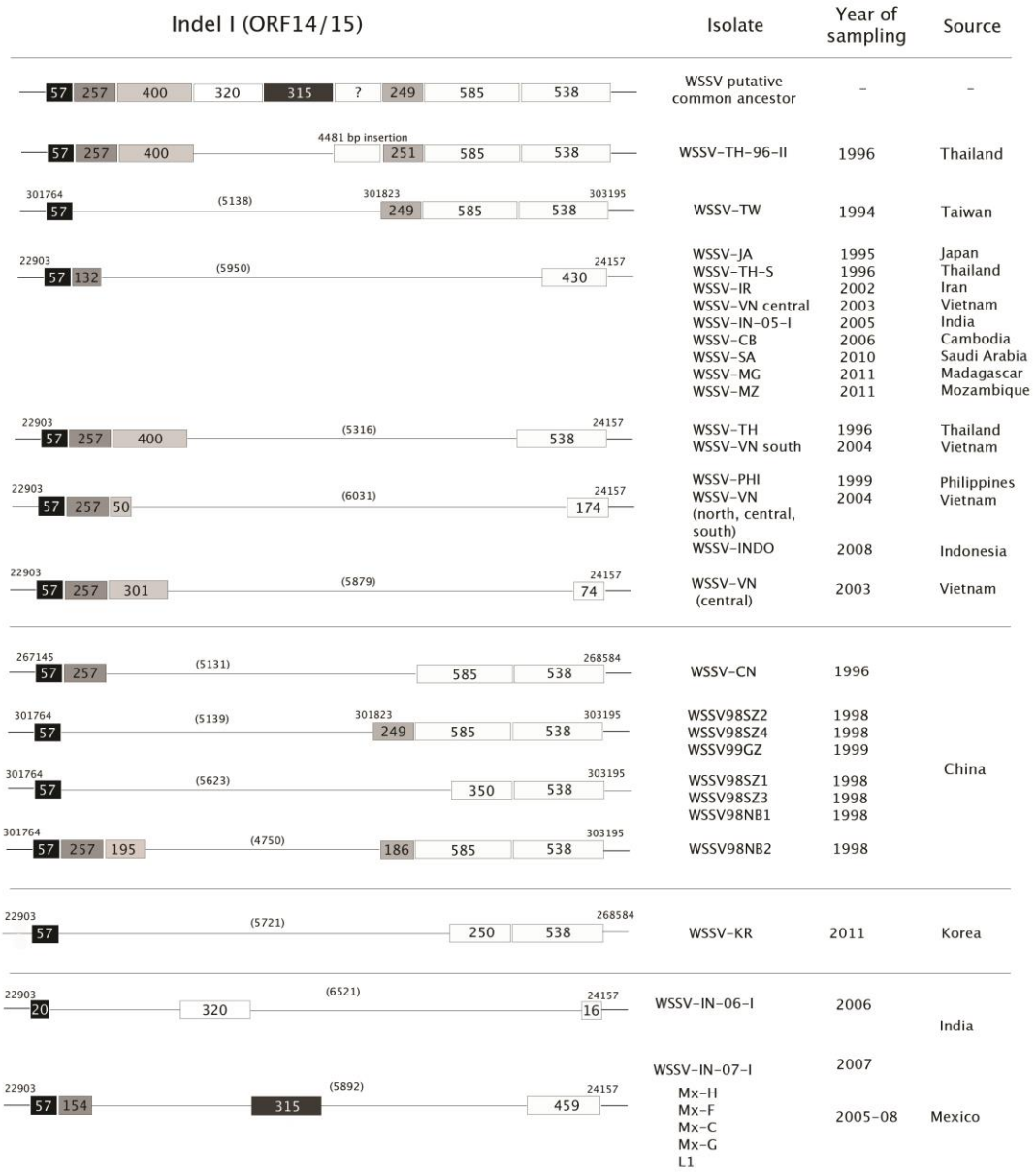


Figure 5. Schematic representation of WSSV variable region, Indel-II (ORF23/124), reported from different countries

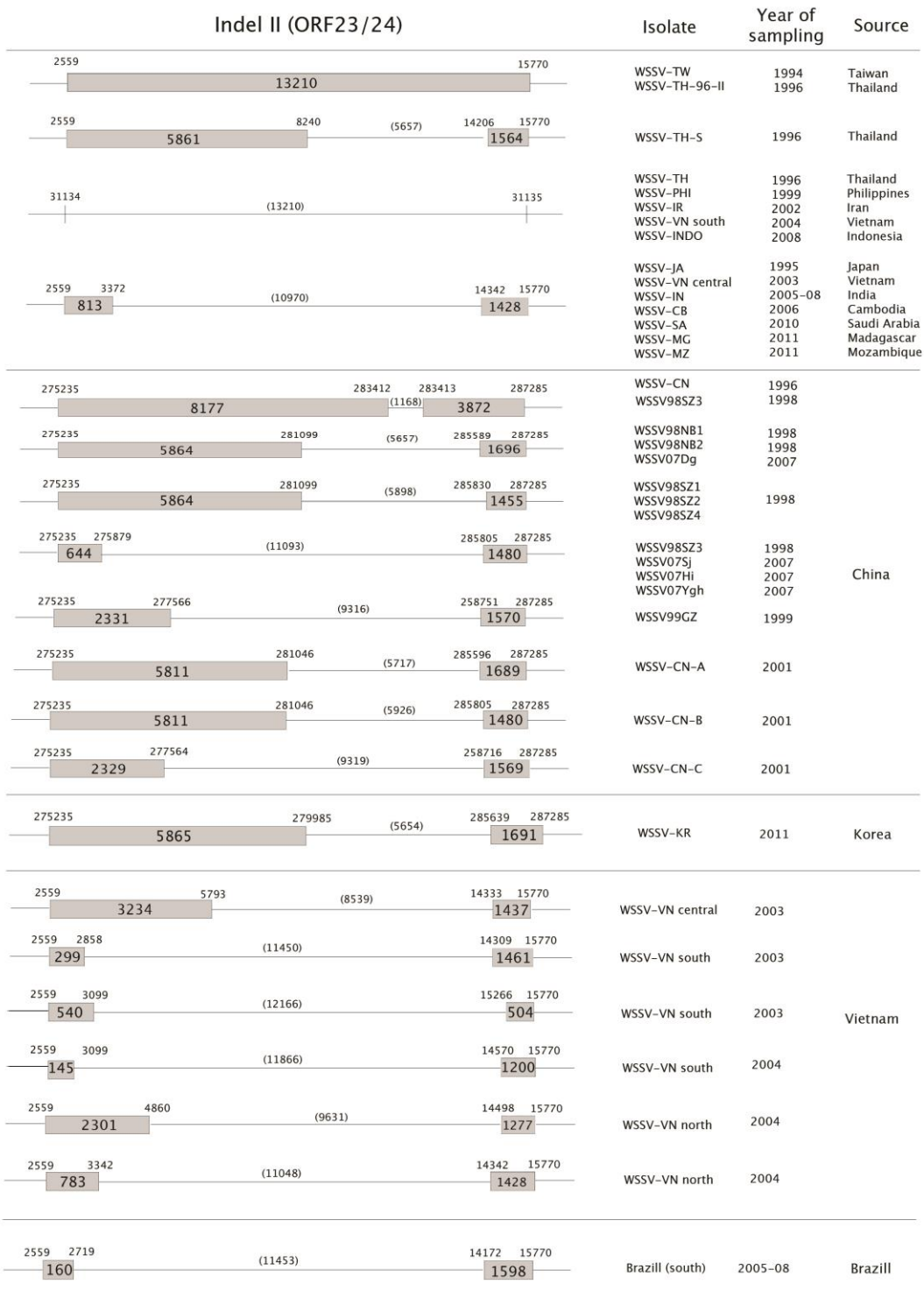


Table 1. Summary of WSSV genotypes, characterized by VNTRs at ORF75, 94 and 125. No. of RUs= number of repeats unit, SNPs=single nucleotide polymorphisms found at the position 48 of the repeated sequence.

Isolate (year)	ORF75; (45, 102) bp No. of RUs	ORF125; 69 bp No. of RUs	ORF94; 54 bp	
			No. of RUs	SNPs
Reference isolate				
WSSV-TW	21 (16, 5)	8	6	TTTTGTT
WSSV-CN	15 (11, 4)	8	12	TTGGGGGGTTTT
WSSV-TH	12 (9, 3)	6	6	TGGGTT
China (1998-1999) (Tan and Shi 2011)				
WSSV98NB1	NA	NA	8	GTGTGTTT
	NA	NA	14	GTGGTTTTTTTT
WSSV98NB2	NA	NA	9	GTGGTTTTT
	NA	NA	6	GTGTTT
WSSVSZ1-3,	NA	NA	6	GTGTTT
WSSVZ4	NA	NA	9	TTTGTGTTT
Thailand (2000-2002) (Wongteerasupaya et al., 2003)				
			6-12, 14-15, 17, 19-20	
Sur1	NA	NA	9	TTTTGTTGT
Sur2, Chu3	NA	NA	8	TTGTTGGT
Chu1	NA	NA	7	TTTGTGT
Chu2	NA	NA	6	TTTGGG

Table 1 (cont). Summary of WSSV genotypes, characterized by VNTRs at ORF75, 94 and 125; No. of RUs= number of repeats unit, SNPs=single nucleotide polymorphisms found at the position 48 of the repeated sequence.

Isolate (year)	ORF75; (45, 102) bp No. of RUs	ORF125; 69 bp No. of RUs	ORF94; 54 bp	
			No. of RUs	SNPs
Vietnam (2003-2004) (Dieu et al., 2004; Dieu et al., 2010)				
North	12 (9, 3)	10	9	NA
	7 (6, 1)	NA	4	NA
Central	5 (3, 2)	6	10	GGGTTTGGTT
	5 (3, 2)	5	17	GTTTTGTTTGTGGGGTT
	5 (3, 2)	6	10	GGGGGGGGGG
	5 (3, 2)	7	7	TTTTGTT
	14 (10, 4)	7	7	TTTTGTT
	6 (4, 2)	6	10	GGGTGGTTTT
South	5 (3, 2)	7	8	NA
	5 (3, 2)	5	4	NA
	5 (3, 2)	7	15	NA
	5 (4, 1)	6	11	NA
	6 (4, 2)	9	10	NA
	NA	4	9	NA
India (2002-2004) (Musthaq et al., 2006)				
			6-13	
	NA	NA	7	GTTTGGT
	NA	NA	9	GTGGGGGTT
	NA	NA	11	GGGGGGTTTTT
	NA	NA	13	GTTTGGGTTTTTT
India (2005-2006) (Pradeep et al. 2008a)				
	2-7	2-5, 7-12, 15	2-10, 12-14, 16	NA

Table 1 (cont). Summary of WSSV genotypes, characterized by VNTRs at ORF75, 94 and 125; No. of RUs= number of repeats unit, SNPs=single nucleotide polymorphisms found at the position 48 of the repeated sequence.

Isolate (year)	ORF75; (45, 102) bp No. of RUs	ORF125; 69 bp No. of RUs	ORF94; 54 bp	
			No. of RUs	SNPs
The Americas (Muller et al. 2010)				
USA				
Texas (1997)	14 (11, 3)	10	5	NA
South	NA	10	5	NA
Hawaii (2004)	6	11	8	NA
Panama (1999)	15 (11, 4)	11	12	NA
Honduras	15 (11, 4)	11	13	NA
Brazil				NA
South (2005)	10 (7, 3)	8	16	TGTTGTGGGGTGGG
South (2007)	10 (7, 3)	8	16	TGTGTTTTTTTGG
South (2008)	10 (7, 3)	8	16	TGTGTTTTTTTTTT
Northeast	11 (8, 3)	9	4	TGGG
Nicaragua	8 (6, 2)	8	14	NA
Mexico (2008)	14 (11, 3)	7	19	NA
Mexico (González-Galaviz et al., 2013)				
S. Babara	-	10	7	NA
Atanasia	4	-	3	NA
Siari (2010)	4	10	7	NA
Riito1 (2010)	-	10	7	NA
Ritto2 (2010)	3	9	11	NA
Ritto3 (2010)	3	-	-	NA
Ritto4 (2008)	15	11	6	NA
Tobari1 (2010)	3	-	3	NA
Tobari2 (2010)	-	-	4	NA
Tastiota	-	-	1	NA

Table 1 (cont). Summary of WSSV genotypes, characterized by VNTRs at ORF75, 94 and 125; No. of RUs= number of repeats unit, SNPs=single nucleotide polymorphisms found at the position 48 of the repeated sequence.

Isolate (year)	ORF75; (45, 102) bp No. of RUs	ORF125; 69 bp No. of RUs	ORF94; 54 bp	
			No. of RUs	SNPs
Iran (Simrouni et al., 2014)				
IRWSSVBU1 (2012)	NA	NA	3	GGG
IRWSSVBU2 (2012)	NA	NA	6	GGGGGG
IRWSSVKH1 (2010)	NA	NA	6	TTTTTT
IRWSSVKH2 (2011)	NA	NA	3	GGG
IRWSSVKH3-6	NA	NA	3	TTT
Saudi Arabia (Tang et al., 2012a)				
10-143 (2010)	NA	6	7	NA
11-065 (2011)	NA	8	13	NA
11-041 (2011)	NA	7	Deletion	NA
Mozambique (Tang et al., 2012a)				
11-312 (2011)	NA	6	Deletion	NA
Korea				
WSSV-KR	4 (3, 1)	5	NA	NA

CHAPTER III

MATERIALS AND METHODS

The present study was composed of 3 phases as follows;

Phase 1 Investigation of WSD prevalence and the impacts of climate factors to the occurrence WSD in cultured penaeid shrimp in Chanthaburi province, Thailand;

Phase 2 WSD risk factors associated with shrimp farming practices and geographical location in Chanthaburi province, Thailand;

Phase 3 Molecular characterization of WSSV isolates from 2007-2014, obtained from WSD outbreaks in the eastern and southern shrimp culture area of Thailand.

Phase 1 Investigation of WSD prevalence and the impacts of climate factors to the occurrence WSD in cultured penaeid shrimp in Chanthaburi province, Thailand.

1.1 Study area

The studied area was located in Chanthaburi province in the eastern part of Thailand (Figure 6). Chanthaburi is one of the largest shrimp grow-out areas in Thailand, expanding from 3,900 hectares in 2009 to 6,800 hectares culture area in 2012. Its annual production yield is more than 60,000 tons, and ninety eight percent of the cultured shrimp is *L vannamei* (Department of Fisheries 2014). Three seasons are described in this area: rainy season, from mid-May to mid-October; cool season, from mid-October to mid-February; and warm season, from mid-February to mid-May.

1.2 Laboratory data and WSD prevalence

Laboratory data of WSD diagnosis during 2009-2014 were retrieved from government research centers (Department of Fisheries, Ministry of Agriculture and Cooperative). The centers provide shrimp disease diagnostic service to local shrimp farmers in the region. A WSD case was diagnosed when a sample was positive with WSSV by a single-step PCR (OIE 2015). Data included in the study were pond-cultured shrimp, from 10-day shrimp PL onward. Because the mass mortality in a pond may occur within 3-10 days after WSSV infection (Nunan et al., 2001), positive samples obtained from an individual client within a 10-day interval were counted as

a single occurrence. Prevalence of WSD was calculated by dividing the number of positive samples by the total number of submitted samples and reported on a monthly and yearly basis.

1.3 Climate data

Meteorological data in the study area were provided by the Meteorological Department of Thailand, Ministry of Information and Communication Technology. Data were recoded every 3 hours and averaged for daily atmospheric temperature, relative humidity, wind speed and total amount of rainfall. Daily atmospheric temperature variation was computed from the differences between minimum and maximum atmospheric temperature. Descriptive statistic was used to aggregate the data to a monthly basis.

1.4 Statistical analysis

All statistical procedures were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL). The negative binomial regression (NBR) model was used to estimate the association between monthly WSD occurrence (count, dependent variable) and climate factors from January 2009 to December 2014 (independent variables). Continuous-scale variables were transformed to a categorical-scale using quartiles when the distribution did not show normality or linearity. Univariate analysis was performed, and variables that were statistically significant at 85% confidence interval were included in the multivariate model. Collinearity between significant variables was examined using Spearman's rank correlations, and correlation coefficients (*r*)

greater than 0.40 were interpreted as an indication of collinearity. Selection of collinear variables included in the final multivariate model was based on significant *P*-value (*P* <0.05) of the final model and acceptability of potential biological cause-effect relationships. Incident rate ratios (IRR) and their 95% confidence intervals (CI 95%) were calculated for statistically significant variables. Interactions among variables in the constructed final model were examined. If no interaction was found, factors that would change the regression coefficient estimates of at least one other factor by more than 25% were considered to be confounders and subsequently included in the final model to adjust the confounding effect of those variables. The equations for negative binomial regression are as follow (Hilbe 2011):

$$Y_i|E_i \sim Poisson(E_i)$$

given Y_i = the number of WSD occurrence; E_i = the number of expected WSD occurrence, which Y_i follows a Poisson probability distribution.

$$E_i = \mu_i$$

μ_i = mean of Y_i;

$$\ln(\mu_i) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + .. \beta_m X_m$$

β_0 = constant; X = independent variable; β_m = estimate coefficient of X_m;

$$E_i \sim Gamma(\lambda_i, k_i)$$

E_i follows a Gamma distribution with parameters (λ_i, k_i).

Phase 2 White spot disease risk factors associated with shrimp farming practices and geographical location in Chanthaburi province, Thailand

2.1 Study population and size

The study focused on *L. vannamei* farms located in the coastal area of Chanthaburi province, Thailand (Figure. 6) and was conducted between October 2011 and December 2013 in collaboration with the Chanthaburi Coastal Fisheries Research and Development Centre (CFRD), Department of Fisheries. Chanthaburi province is one of the largest shrimp aquaculture regions in Thailand, producing >60,000 tons/year (Department of Fisheries 2014). Farmers generally purchase shrimp PL (PL10 - PL12) from local or nearby (< 300 km) hatcheries for grow-out in earthen ponds for 90-120 days.

The case-control study used a questionnaire to interview farmers (APPENDIX E). Farms were selected arbitrarily from 886 located in the CFRD study area. Farms were divided into WSD case and control groups based on their disease status in the CFRD records. WSSV was detected using the nested PCR method endorsed by OIE (2014). Farms were assigned to the WSD case group when WSSV was detected in association with disease in at least one crop over the study period. Farms were assigned to the control group when monthly samples were WSSV-negative over the study period. Based on assuming an odds ratio of 4 with a 95% confidence interval

and 80% statistical power with an expected proportion exposed amongst controls of 8%, at least 66 farms were required per group. The farm sample size of 100 farms for both the case and control groups was thus selected for interview.

2.2 Information collection

Information on farming practices was collected using a structured farm owner questionnaire combined with interviewer observations. The questions were approved by local government officers and farm personnel to ensure they were easily understood. For consistency, all farm owners and/or managers were interviewed by Dr. P. Piamsomboon on the basis that their responses would be anonymous. Questions on potential risk factor variables covered farm characteristics and management practices as well as various other factors described in studies in other Asian countries (Tendencia et al., 2011, Table 4).

Farm global positioning system (GPS) x, y coordinates were obtained using a Garmin eTrex® 10 handheld GPS device. For each farm, distances to the nearest point on the coastline, public canal, national highway and mangrove forest were determined using the Euclidean distance calculation function in the Spatial Analyst Tools, ArcToolbox, ArcGIS 10.0 software (ESRI). The relevant spatial data of road networks, coastline, canals and mangrove forests were provided by the Royal Thai Survey Department, Ministry of Defense.

2.3 Statistical analysis

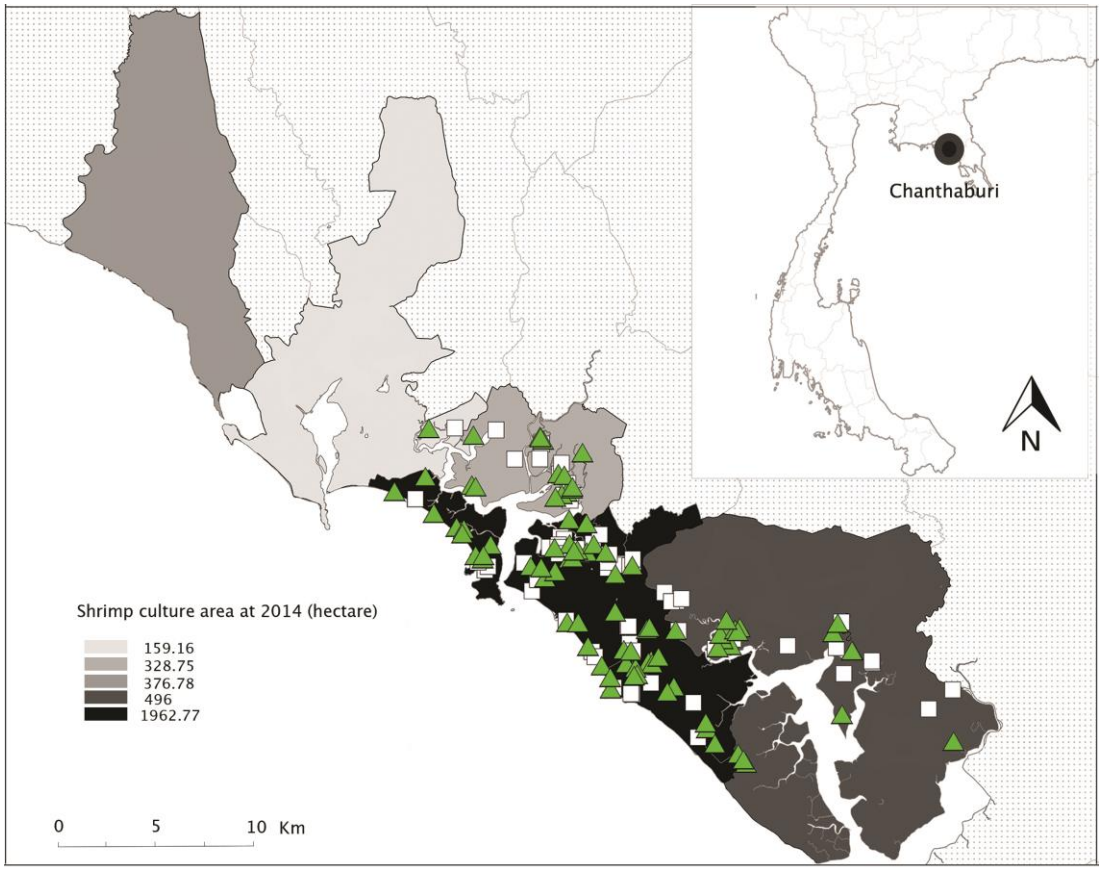
Statistical analyses utilized the SPSS software (version 22, SPSS Inc.). Continuous-scale risk factor variables were tested for normality and linearity of effect. If the distribution was not normal or linear, the variable was transformed to a categorical-scale using quartiles. The WSD status of the farm was used as the dependent variable in each analysis. Univariate logistic regression was initially performed in order to identify a subset of statistically significant risk factor variables using P <0.1. Spearman rank correlations were used to examine for collinearity between the significant risk factor variables. Correlation coefficients >0.40 were interpreted to indicate collinearity. Amongst pairs of collinear variables, a choice was made based on acceptability of potential biological cause-effect relationships to select the variable to include in the multivariable analysis. Subsequently, variables that were not collinear were included in a multivariable logistic regression analysis using a backward stepwise variable selection approach based on P >0.05. Variables that changed the regression coefficient estimates of at least one other variable in the model by more than 20% were considered to be confounders and therefore included in the final model to adjust for the confounding effect of that risk factor. Odds ratios (OR) and their 95% confidence intervals were calculated for statistically significant risk factor variables.

The equation for binary logistic regression (Liang and Zeger 1986) is as follows:

$$\ln \frac{p}{1-p} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + .. \beta_i X_i + e$$

given p = chance of WSD occurrence, then $1-p$ = chance of no WSD occurrence; β_0= constant; X = independent variable; βi = estimate coefficient of Xi; e = error

Figure 6. The study area of phase 1 and 2, the five coastal shrimp culture districts in Chanthaburi province, Thailand. Shading represents shrimp culture area density of each district at year 2014. For phase 2 of the study, locations of case (▲) and control (□) farms are shown.



Phase 3 Molecular characterization of WSSV isolates from 2007-2014, obtained from WSD outbreaks in the eastern and southern shrimp culture area of Thailand.

3.1 WSSV isolates

A total of 137 WSSV isolates from year 2007 to 2014 were used in the study. WSD positive shrimp samples of processed *L. vannamei* were provided from the National Institute of Animal Health, Department of livestock development, Ministry of Agriculture and Cooperative. These samples were collected at the processing plants located in the southern part of Thailand. WSD positive sample of grown-out *L. vannamei* were provided by the Coastal Fisheries Research and Development Centers (Department of Fisheries, Ministry of Agriculture and Cooperative) located in the eastern and southern parts of Thailand. WSSV isolates used in the present study are shown in Table 2.

3.2 DNA extraction

Approximately 25-50 g of gill and/or pleopod tissues was collected. Commercial DNA extraction kit (DNAzol®, Life Technologies, USA) was used for extraction of WSSV genome. Briefly, the tissues were added with 1 ml of DNAzol® reagent and were homogenized with hand held glass homogenizer. Then, the homogenized samples were centrifuged at 12,000 *g*, 4°C for 10 min. Following centrifugation, supernatant was transferred to a new sterile tube and absolute ethanol was added to capture precipitate DNA. The tube was stored vertically for 30

sec to 1 min to allow the DNA to settle to the bottom of the tube. Supernatant was disposed and DNA precipitate was washed twice with 0.8-1.0 ml of 75% ethanol. After ethanol was removed, DNA precipitate was air dried in an open tube for 5 to 15 sec. DNA precipitate was completely dissolved using sterile distill water. Extracted DNA samples were stored in absolute ethanol at -20°C until used.

3.3 PCR amplification of WSSV variable loci

The extracted DNA samples were re-confirmed for WSSV positive by nested PCR method as described by OIE (2015). Primers used for reconfirmation of WSSV and analysis of WSSV variable loci were from previous reported and from newly design primers using Primer3 software version 0.40 (http://bioinfo.ut.ee/primer3-0.4.0) (Table 3). PCR reactions were performed in a final volume of 20 µl, containing 10 µl of Taq Ready Mix DNA polymerase (KAPA Biosystem, USA), 0.8 µl of forward and reverse primers (Sigma-genosys, Singapore), 6.4 µl distilled water and 2 µL of DNA template. For the large WSSV fragments (1.5- 2 kb) PCR reactions were carried out using KAPA2G Robust HotStart ReadyMIx PCR kit (KAPA Biosystem, USA), which is the second generation DNA polymerase suitable for the amplification of large DNA fragment of <5 kb. The 25 reactions consisted of 12.5 µl of Robust Taq DNA polymerase, 1.25 µl of forward and reverse primers, 8 µl distilled water and 2 µL of DNA template. PCR amplifications were conducted using a PCR Thermalcycler (Tpersonal combi mode, Biometra®, Germany). The PCR thermal cycling protocol included initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation

at 95°C for 30 sec. Annealing temperature and time applied to the reactions were depended on primers (Table 3), followed by extension at 72°C for 1 min/kb. After 35 cycles, the final extension step was set at 72°C for 2 min. The PCR thermal cycling protocol for KAPA2G Robust HotStart ReadyMIx included initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension at 72°C for 30 sec/kb. The final extension step was set at 72°C for 1 min/kb. The PCR products was analyzed using 1.5% agarose gel electrophoresis at 100 V for 20 min (Sigma-Aldrish®, USA), in 1X Tris-acetate/EDTA buffer. The products were purified using NucleoSpin Gel Extraction kit (Nucleospin®, Germany).

3.4 Analysis of variable loci

A total of 10 WSSV isolates were used as the representative for each variable region. The purified PCR products were sequenced by Dideoxy Chain Termination method (1st BASE DNA sequencing Service, Singapore). Results of DNA sequencing were assembled and validated with Seqman II software version 5.03 (DNASTAR, Madison, WI, USA). For the variable regions “Indel-I” and “Indel-II”, the DNA fragments were aligned with MegAlign software version 5.03 (DNASTAR, Madison, WI, USA) and compared with reference strains of WSSV from Taiwan (WSSV-TW; GenBank accession no. AF440570) or China (WSSV-CN; GenBank accession no. AF332093), or Thailand (WSSV-TH; GenBank accession no. AF369029 and WSSV-TH-96-II GenBank accession no AY864668). Reference data were downloaded from the BLAST

database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The recombination and deletion patterns of "Indel-I" and "Indel-II" were compared with WSSV isolates from previous studies in Southeast Asia, the Indian subcontinent and South America (Dieu et al., 2004; Pradeep et al., 2008b; Tan et al., 2009; Dieu et al., 2010; Muller et al., 2010; Zwart et al., 2010; Hoa et al., 2012). The number of tandem repeats in the variable regions ORF75, 94 and 125 were determined using the Tandem Repeat Finder software (Benson 1999).

Table 2 WSSV isolates used in the present study

Province	Year	No. of isolate	Isolate	Types of sample
1. Chanthaburi	2007	5	Ch1-5/07	Pond cultured
	2008	4	Ch1-4/08	Pond cultured
	2009	4	Ch1-4/09	Pond cultured
	2011	15	Ch1-15/11	Pond cultured
	2012	12	Ch1-12/12	Pond cultured
	2013	5	Ch1-5/13	Pond cultured
	2014	4	Ch1-4/14	Pond cultured
2. Rayong	2012	18	R1-18/12	Pond cultured
3. Trat	2012	7	T1-7/12	Pond cultured
4. Surat Thani	2009	9	Su1-9/09	Processed
	2010	2	Su1-2/10	Processed
5. Songkhla	2009	5	Sk1-5/09	Processed
	2012	15	Sk1-15/12	Pond cultured
6. Phuket	2012	13	Phu1-13/12	Pond cultured
7. Krabi	2012	11	K1-11/12	Pond cultured
8. Prachuap Khiri Khan	2008	2	Pac1-2/08	Processed shrimp
9. Chumporn	2008	2	Chu1-2/08	Processed
10. Ranong	2008	2	Ran1-2/08	Processed
11. Pattani	2008	2	Pat1-2/08	Processed
Total		**137**		

Table 3 Primers used in the present study

Primers	Sequence (5'-3')	Annealing Temperature (°C)/ Elongation time(sec)	WSSV sequence coordinates	Size of PCR product (bp)
WSSV confirmation				
146F1	ACTACTAACTTCAGCCTATCTAG	55/120	-	1447
146R1	TAATGCGGGTGTAATGTTCTTACGA		-	
146F2	GTAACTGCCCCTTCCATCTCCA	55/120	-	941
146R2	TACGGCAGCTGCTGCACCTTGT		-	
WSSV variable loci				
Indel-I (ORF14/15)				
TJW14/15-F	TCAACAACCCAAATCCCATT	60/90†	21834-21854#	3,515#
TJW14/15-R	CTCTCAATCTTCCCCCAACA		25176-25156#	
VR14/15-screen-F[b]	GAGATGCGAACCACTAAAAG	49/120	22904–22923#	1,254#
VR14/15 screen-R[b]	ATGGAGGCGAGACTTGC		24157–24141#	
Indel-II (ORF23/24)				
VR23/24-1 F[b]	ATGGGCTCTGCTAACTTG	60/120†	4359–4376*	10,833*
VR23/24-1 R[b]	ATGATTGTATTCGTCGAAGG		15191–15172*	
VR23/24-south-F[b]	CTACAACGGCCAAGTCAT	60/120†	30701–30718#	1555#
VR23/24-south-R[b]	CGCAATTCTCCTCGCAGTT		32255–32237#	
VNTR loci				
TJW75-F	TCTGAAGCTGGG GGAACTAA	50/60	107680-	1452#
TJW75-R	GAGCAACTCTGCACAGCATC		109131-	
ORF94-flank-F[b]	GTGCCGCAGGTCTACTC	51/80	142656–	682#
ORF94-flank-R[b]	CATACGACTCTGCTTCTTG		143337–	
ORF125-flank-F[b]	CGAAATCTTGATATGTTGTGC	52/100	187791–	652#
ORF125-flank-R[b]	CCATATCCATTGCCCTTCTC		188442–	

[a] Primers recommended by OIE (2014); [b] Primers reported by Dieu et al. (2004)

*Sequence according to WSSV-TW; # Sequence according to WSSV-TW

† KAPA2G Robust HotStart ReadyMIx PCR kit was used.

CHAPTER IV
RESULTS

Phase 1 Investigation of WSD prevalence and the impacts of climate factors to the occurrence WSD in cultured penaeid shrimp in Chanthaburi province, Thailand.

1.1 WSD occurrence

The WSD cases were reported in many stages of shrimp PL, from 9 to 115 days of stocking. A total of 1,952 WSD cases were reported during the study period (January 2009 to December 2014). An annual WSD prevalence and the numbers of WSD case ranged from 61.79%, 482 cases (year 2011) to 12.09%, 210 cases (year 2014). Pattern of WSD prevalence and numbers of case in 2009 - 2014 were variable between seasons and high prevalence and case were notified from October to February. The decreased WSD prevalence and cases were observed in March to June with the lowest of WSD in May (Table 4).

Table 4. WSD Prevalence (%) and numbers of WSD case reported from 2009 to 2014 in Chanthaburi province, Thailand.

Month/year	Prevalence (positive cases/ total submitted cases)					
	2009	2010	2011	2012	2013	2014
January	63.16 (24/38)	66.67 (32/48)	89.47 (34/38)	66.67 (52/78)	22.64 (24/106)	16.10 (18/112)
February	58.62 (34/58)	58.70 (54/92)	69.44 (50/72)	54.76 (46/84)	25.00 (26/104)	25.50 (28/110)
March	39.29 (22/56)	32.56 (28/86)	68.42 (52/76)	44.00 (44/100)	14.29 (18/126)	5.80 (8/138)
April	32.14 (18/56)	29.17 (14/48)	51.85 (28/54)	40.00 (16/40)	8.54 (14/164)	5.90 (10/170)
May	41.67 (10/24)	6.67 (2/30)	28.57 (12/42)	17.86 (10/56)	4.55 (6/132)	11.10 (16/144)
June	35.29 (24/68)	33.33 (10/30)	40.00 (20/50)	21.88 (14/64)	5.15 (10/194)	4.00 (8/200)
July	31.25 (20/64)	17.65 (6/34)	55.56 (30/54)	23.21 (26/112)	7.92 (16/202)	7.40 (16/216)
August	50.00 (36/72)	50.00 (30/60)	51.85 (56/108)	20.41 (20/98)	17.65 (24/136)	8.00 (12/150)
September	30.77 (8/26)	26.09 (12/46)	72.73 (48/66)	30.43 (28/92)	6.82 (6/88)	10.20 (10/98)
October	67.92 (72/106)	68.97 (40/58)	68.89 (62/910)	20.18 (44/218)	28.57 (32/112)	11.30 (14/124)
November	42.86 (24/56)	80.65 (50/62)	65.85 (54/82)	27.78 (40/144)	26.67 (32/120)	16.70 (22/132)
December	78.95 (30/38)	95.24 (40/42)	75.00 (36/48)	46.67 (28/60)	34.38 (44/128)	33.80 (48/142)
Annual prevalence	48.64 (322/662)	50.00 (318/636	61.79 (482/780	32.11 (368/1146	15.63 (252/1612	12.09 (210/173

1.2 Climate factors associated with WSD occurrence

Observed data of all climate factors except daily temperature variation were not normally distributed; therefore, they were equally divided into 4 groups using quartile rank. The forth quartile of each factor was used as reference. Univariate NBR analysis showed significant association between WSD occurrences with all climate factors (Table 5). Spearman's correlation showed positive correlations among the atmospheric temperature factors and rainfall factors. Average wind speed correlated negatively with atmospheric temperature and rainfall. In addition, significant negative correlation (r = -0.81) was found between daily atmospheric temperature variation and total amount of rainfall (Table 6). Because of their negative correlation, both factors cannot be concurrently introduced into the multivariate model. The final multivariate NBR model was constructed based on the smallest Quasi Likelihood Independence Model Criterion (QIC) value, of which the lesser value indicates the better fit of a model. Consequently, two factors were selected for the final model, average atmospheric temperature (IRR: 2.53, CI 95%: 1.72 – 3.75) and daily atmospheric temperature variation (IRR: 1.08, CI 95%: 0.97 – 1.20) (Table 7). Interaction and confounding among the factors in the final model were not observed. The multivariate NBR model indicated that WSD occurrence increased with a decreasing atmospheric temperature, and the highest WSD occurrence was found at the atmospheric temperature between 24.5 – 27.2°C. The variation of daily atmospheric temperature >10°C also substantially raised the

disease incidence. Figure 7 illustrates the relationship between WSD occurrences and the significant climate factors.

Table 5. Univariate NBR model for the association between monthly WSD occurrence and climate factors; β: estimated coefficient, IRR (CI: 95%): incident rate ratios and its 95% confidence interval, QIC: Quasi Likelihood Independence Model Criterion.

Factors	β	IRR (CI: 95%)	*P*-value	QIC
Atmospheric temperature (°C)				
Average			<0.0001	31.27
quartile 1 (24.5 – 27.2)	0.99	2.71 (1.86 – 3.95)		
quartile 2 (27.3 – 27.9)	0.62	1.86 (1.21 – 2.86)		
quartile 3 (28.0 – 28.5)	0.55	1.74 (2.16 – 2.58)		
quartile 4 (28.6 – 29.8)		-*		
Minimal average			<0.0001	31.99
quartile 1 (19.7 – 23.8)	0.88	2.41 (1.68 – 3.46)		
quartile 2 (23.9 – 24.7)	0.55	1.73 (1.18 – 2.56)		
quartile 3 (24.8 – 25.8)	0.09	1.10 (0.68 – 1.77)		
quartile 4 (25.9 – 26.2)		-*		
Maximal average			0.07	38.45
quartile 1 (29.9 – 31.7)	0.45	1.57 (1.01 – 2.41)		
quartile 2 (31.8 – 32.5)	0.30	1.35 (0.83 – 2.18)		
quartile 3 (32.6 – 33.3)	0.55	1.73 (1.13 – 2.65)		
quartile 4 (33.4 – 34.8)		-*		
Daily variation			0.03	35.57
8.2 + 1.4 (Mean ± SD)	0.11	1.12 (1.01 – 1.25)		
Rainfall				
Total amount (mm)			0.01	36.23
quartile 1 (0 – 54.5)	0.64	1.90 (1.28 – 2.81)		
quartile 2 (54.6 – 190.1)	0.07	1.08 (0.69 – 1.68)		
quartile 3 (190.2 – 411.5)	0.16	1.18 (0.76 – 1.81)		
quartile 4 (411.6 – 1035.4)		-*		
Number of rain days			0.05	38.10
quartile 1 (0 - 6)	0.52	1.67 (1.12 – 2.51)		
quartile 2 (6 - 16)	0.30	1.35 (0.92 – 2.00)		
quartile 3 (17 - 24)	0.81	1.08 (0.70 – 1.68)		
quartile 4 (>28)		-*		

Table 5 (cont). Univariate NBR model for the association between monthly WSD occurrence and climate factors; β: estimated coefficient, IRR (CI: 95%): incident rate ratios and its 95% confidence interval, QIC: Quasi Likelihood Independence Model Criterion.

Factors	β	IRR (CI: 95%)	*P*-value	QIC
Average relative humidity (%)			<0.0001	37.57
quartile 1 (57.0 – 74.3)	0.69	2.00 (1.32 – 3.04)		
quartile 2 (74.4 – 80.5)	0.16	1.17 (0.74 – 1.85)		
quartile 3 (80.6 – 84.0)	0.16	1.17 (0.75 – 1.83)		
quartile 4 (84.1 – 88.0)		-*		
Average wind speed (knot)			0.02	36.13
quartile 1 (0.90 – 1.30)	-0.63	0.53 (0.37 – 0.78)		
quartile 2 (1.40 – 1.60)	-0.59	0.55 (0.38 – 0.81)		
quartile 3 (1.70 – 2.10)	-0.27	0.76 (0.53 – 1.11)		
quartile 4 (2.11 – 4.50)		-*		

Factor	Atmospheric temperature			Rain fall		Average relative humidity	Average wind speed
	Minimal average	Maximal average	Daily variation	Total amount	Rain days		
Atmospheric temperature							
Average	0.72 (<0.0001)	0.58 (<0.0001)	-0.19 (0.10)	0.25 (0.03)	0.18 (0.12)	0.12 (0.47)	-0.45 (0.02)
Minimal average	-	0.12 (0.03)	-0.64 (<0.0001)	0.65 (<0.0001	0.59 (<0.0001)	0.56 (<0.0001)	-0.43 (<0.0001)
Maximal average	-	-	0.50 (<0.0001)	-0.33 (0.004)	-0.35 (0.003)	-0.40 (0.002)	-0.12 (0.28)
Daily variation	-	-	-	-0.81 (<0.0001	-0.79 (<0.0001)	-0.82 (<0.0001)	0.36 (0.02)
Rain fall							
Total amount					0.88 (<0.0001)	0.80 (<0.0001)	-0.43 (<0.0001)
Rain days					-	0.80 (<0.0001)	-0.46 (<0.0001)
Average relative humidity					-		-0.65 (<0.0001)

Table 6 Spearman's correlation among significant factors; correlation coefficients are presented with the *P*-value in the parenthesis.

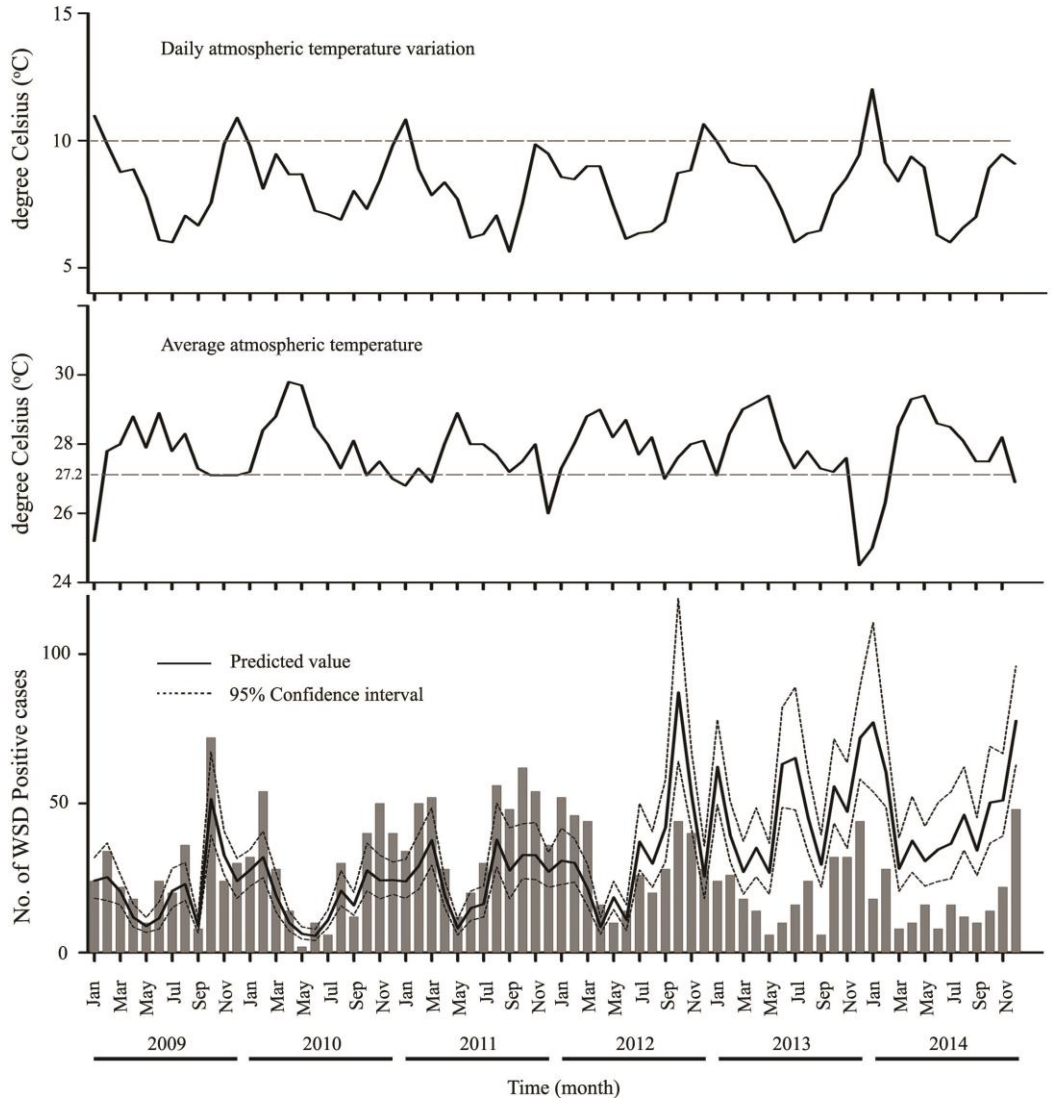
Table 7 Multivariate NBR model for the association between monthly WSD occurrence and climate factors in Chanthaburi province, Thailand; **β**: estimated coefficient, IRR (CI: 95%): incident rate ratios and its 95% confidence interval

Factor	β	IRR (CI: 95%)	*P*-value
Constant	-1.56	0.21 (0.08-0.51)	0.001
Average atmospheric temperature (°C)			<0.0001
quartile 1 (24.5 – 27.2)	0.93	2.53 (1.72 – 3.75)	
quartile 2 (27.3 – 27.9)	0.64	1.91 (1.25 – 2.92)	
quartile 3 (28.0 – 28.5)	0.55	1.74 (1.12 – 2.59)	
quartile 4 (28.6 – 29.8)		Reference	
Daily atmospheric temperature variation			0.09
8.2 + 1.4 (Mean + SD)	0.08	1.08 (0.97 – 1.20)	

The final multivariate NBR model indicates that the incident rate of WSD occurrence for the shrimp farming community in Chanthaburi province, Thailand, can be estimated using the following equation:

$$\ln(\mu_i) = -1.56 + 0.93(q1_{\text{AvgTemp}}) + 0.64(q2_{\text{AvgTemp}}) + 0.55(q3_{\text{AvgTemp}}) + (0.08)(\text{Difference from the minimal value})$$

Figure 7. WSD occurrences in Chanthaburi province, Thailand and predicted values obtained from the multivariate NBR model. Climate factors significantly associated with WSD occurrence are presented.



Phase 2 WSD risk factors associated with shrimp farming practices and geographical location in Chanthaburi province, Thailand.

2.1 Descriptive analysis

Of the 200 farms that had been in operation for at least 5 years and were selected for study, 157 comprising 88 case-and 69 control farms, agreed to be interviewed. These included 87 (55.4%) small farms, 46 (29.3%) intermediate-sized farms and 24 (15.3%) large farms (Table 8). The total culture area covered by the farms represented 25.1% of total area of Chanthaburi province.

In terms of farm biosecurity, 26.2% were fenced and 6.4% had vehicle tire baths and personnel disinfection systems at their entrance. At most (77%) farms, water was released directly into the environment at harvest rather than being recycled. Very few (<1%) farms practiced water decontamination prior to release. Many (42%) farms had crab-proof fencing and 27.4% had bird-proof netting.

2.2 Univariate and multivariate analyses

The univariate analysis identified 15 risk factors associated with WSD occurrence in Chanthaburi province, Thailand (Table 8). Out of 15 variables, 6 variables were selected for inclusion in the final multivariable analysis, based on the given significant *P*-value. The final model initially included variable “water source”, “continuous culture”, “owner of multiple farms”, “lime application”, “use of probiotics and “caretaker”. Of these variables, significant collinearity were found

between “caretaker” and “water source” variable (*r* = 0.54) and between “caretaker” with “continuous culture” variable (*r* = 0.46). The “water source” and “continuous culture” variables were thus selected for the final model based on them being identified previously as potential WSD risk factors (Tendencia et al. 2011). The reintroduction of the ‘distance to national highways’ variable resulted in a >20% change in one category coefficient estimate for the water source variable, and it was thus included as a confounder in the model. The 95% confidence interval, odds ratio, and level of statistical significance for each variable are detailed in Table 9.

Table 8. Univariate logistic regression analysis of the association between white spot disease (WSD) occurrences and farm characteristics and farm management factors. (*) and values in bold indicate significant risk factors ($P < 0.1$); N: number of farms; OR (95% CI): odds ratio and its 95% confident interval.

Variable	Explanation	N	*P*-value	OR (95% CI)
Farm area (Hectares)				
Farm size	Total farm area			
	< 2.4	87	0.15	0.48 (0.19-1.26)
	> 2.4 - 8	46	0.76	0.85 (0.30-2.41)
	> 8	24		Reference
Culture area*	Total area used for shrimp culture			
	< 0.88	39	**0.01**	0.31 (0.12-0.81)
	> 0.88 - 4.32	80	**0.09**	0.49 (0.22-.14)
	> 4.32	38		Reference
Water reserve area*	Total area of reservoir pond			
	0	52	0.86	0.93 (0.40-2.13)
	> 0 - 0.32	44	0.45	0.71 (0.30-1.70)
	> 0.32 – 1.04	22	**0.01**	8.57 (1.76-41.78)
	> 1.04	39		Reference
Sludge pond area*	Total area of sludge pond			
	0	47	0.88	0.93 (0.38-2.28)
	> 0 - 0.16	42	**0.04**	0.39 (0.16-0.97)
	> 0.16 - 0.48	31	0.57	1.33 (0.49-3.66)
	> 0.48	37		Reference
Farm features				
Number of ponds/farm	Total number of ponds used for shrimp culture per farm			
	≤ 10	127	0.20	0.57 (0.25-1.33)
	> 10	30		Reference
Cul/Res ratio*	Ratio between shrimp culture and reservoir pond		**0.03**	1.04 (1.003-1.07)

Table 8 (cont). Univariate logistic regression analysis of the association between white spot disease (WSD) occurrences and farm characteristics and farm management factors. (*) and values in bold indicate significant risk factors ($P < 0.1$); N: number of farms; OR (95% CI): odds ratio and its 95% confident interval.

Variable	Explanation	N	*P*-value	OR (95% CI)
Caretaker*	Person responsible for farm operation:			
	Owner	80	**0.04**	0.42 (0.19-0.98)
	Owner and worker(s)	39	**0.08**	0.42 (0.17-1.10)
	Farm manager and	38		Reference
Water source*	Source of water used in farm for shrimp culture:			
	Sea	23	0.37	1.85 (0.49-6.98)
	Public canal(s)	117	**0.01**	3.98 (1.31-12.1)
	Underground water	17		Reference
Owner of multiple farms	Farmer operate more than one farm, each located in different areas			
	Yes	48	**0.08**	1.89 (0.93-3.84)
	No	109		Reference
Water recycling	Reuse of water from the previous shrimp crop to culture the next			
	Yes	36	0.23	0.63 (0.30-1.33)
	No	121		Reference
Adjacent farms	Presence of other shrimp farms next to the observed farm			
	Yes	138	0.20	1.90 (0.72-5.01)
	No	19		Reference
Limited access	No access of unauthorized personnel to farm			
	Yes	59	0.98	0.99 (0.52-1.90)
	No	98		Reference
Fence	Presence of barrier or other upright structure surrounding the farm			
	Yes	41	0.27	1.51 (0.73-3.14)
	No	116		Reference
Pet in farm	Presence of other animals roaming freely in the farm, for example, dogs and chickens			
	Yes	69	0.45	1.28 (0.67-2.42)
	No	88		Reference

Table 8 (cont). Univariate logistic regression analysis of the association between white spot disease (WSD) occurrences and farm characteristics and farm management factors. (*) and values in bold indicate significant risk factors ($P < 0.1$); N: number of farms; OR (95% CI): odds ratio and its 95% confident interval.

Variable	Explanation	N	*P*-value	OR (95% CI)
Vehicle disinfection	Disinfection processes used for vehicles entering the farm, for example, tire baths and vehicle sprays			
	Yes	10	0.36	1.90 (0.47-7.64)
	No	147		Reference
Separate workers	Different worker allocated to each pond			
	Yes	39	0.96	1.02 (0.49-2.11)
	No	118		Reference
Continuous culture*	Farm that stock shrimp continuously year round, producing more than 2 crops per year			
	Yes	86	**0.09**	1.73 (0.91-3.29)
	No	71		Reference
Pond preparation				
Sludge removal	Disposal of soil at the bottom of the pond after each crop is			
	Yes	82	0.53	0.82 (0.43-1.53)
	No	75		Reference
Lime application*	Application of lime to dried pond bottom for disinfection			
	Yes	57	**0.01**	0.41 (0.21-0.80)
	No	100		Reference
Water preparation				
Water filter	Water in culture ponds is filtered using a trawling net			
	Yes	105	0.53	0.80 (0.41-1.58)
	No	52		Reference
Animal waste	Chicken/pig manure or cow dung used as pond fertilizer			
	Yes	53	0.92	1.03 (0.53-2.01)
	No	104		Reference

Table 8 (cont). Univariate logistic regression analysis of the association between white spot disease (WSD) occurrences and farm characteristics and farm management factors. (*) and values in bold indicate significant risk factors ($P < 0.1$); N: number of farms; OR (95% CI): odds ratio and its 95% confident interval.

Variable	Explanation	N	*P*-value	OR (95% CI)
Inorganic fertilizer	Use of inorganic fertilizer to adjust water color before stocking the			
	Yes	99	0.87	0.95 (0.49-1.82)
	No	58		Reference
Insecticide	Use of insecticide to eliminate aquatic decapods during water			
	Yes	108	0.79	1.10 (0.55-2.17)
	No	49		Reference
Copper	Use of copper to eliminate shellfish before stocking the shrimp			
	Yes	101	0.55	0.81 (0.42-1.60)
	No	56		Reference
Tea seed*	Use of tea seed cake or powder to kill small fish before stocking			
	Yes	138	**0.07**	0.35 (0.11-1.11)
	No	19		Reference
Pond features				
PE-lined pond	Use of polyethylene to cover the pond slope			
	Yes	74	0.43	1.30 (0.68-2.47)
	No	83		Reference
Bird-proof netting	String or net installed above the pond to prevent access by birds			
	Yes	43	0.69	0.87 (0.43-1.75)
	No	114		Reference
Crab-proof fencing	Nylon/plastic screen installed on dike surrounding the pond to prevent crabs from entering			
	Yes	66	0.75	0.90 (0.47-1.70)
	No	91		Reference
Hand- and foot-baths	Containers that contain chemicals for hand and foot disinfection, placed at pond entrance			
	Yes	24	0.12	2.12 (0.82-5.45)
	No	133		Reference

Table 8 (cont). Univariate logistic regression analysis of the association between white spot disease (WSD) occurrences and farm characteristics and farm management factors. (*) and values in bold indicate significant risk factors ($P < 0.1$); N: number of farms; OR (95% CI): odds ratio and its 95% confident interval.

Variable	Explanation	N	*P*-value	OR (95% CI)
Feed additive				
Vitamin C	Use of commercial vitamin C mixed into feed			
	Yes	22	0.76	1.16 (0.46-2.89)
	No	135		Reference
Probiotics mix in feed*	Use of commercial probiotics mixed into feed			
	Yes	136	**0.05**	0.35 (0.12-1.01)
	No	21		Reference
Postlarvae				
Source of shrimp PL*	Provinces from which farmers obtain postlarvae			
	Province 1	63	**0.03**	0.41 (0.18-.93)
	Province 2	47	0.50	0.74 (0.30-.80)
	Province 3	43		Reference
Virus detection of PL	Test for abnormalities and the presence of important viruses in post			
	Yes	88	0.92	0.97 (0.51-1.82)
	No	69		Reference
Stocking density	Number of postlarvae released to culture pond (PL/ m^2)			
	< 62.5	80	0.33	0.63 (0.25-1.60)
	> 62.5-81.25	40	0.92	1.06 (0.35-3.21)
	> 81.25	37		Reference

Table 8 (cont). Univariate logistic regression analysis of the association between white spot disease (WSD) occurrences and farm characteristics and farm management factors. (*) and values in bold indicate significant risk factors (*P*< 0.1); N: number of farms; OR (95% CI): odds ratio and its 95% confident interval.

Variable	Explanation	N	*P*-value	OR (95% CI)
Distance variables[a]	Nearest distance categorized into quartiles[b] from farms to the particular features (Km)			
To coastline*	quartile 1 (0.03 - 0.58)	39	0.17	1.87 (0.76-4.60)
	quartile 2 (0.60 - 1.26)	39	**0.04**	4.07 (1.57-10.53)
	quartile 3 (1.27 - 2.34)	39	**0.07**	2.30 (0.93-5.70)
	quartile 4 (2.35 - 6.44)	39		Reference
To nearest national highway*	quartile 1 (0.01 - 1.00)	39	**0.01**	0.30 (0.11-0.77)
	quartile 2 (1.09 - 2.09)	39	0.10	0.45 (0.17-1.16)
	quartile 3 (2.13 - 4.16)	39	**0.01**	0.30 (0.11-0.77)
	quartile 4 (4.17 - 9.51)	39		Reference
To nearest public canal	quartile 1 (0.06 - 0.38)	39	0.50	0.73 (0.30-1.79)
	quartile 2 (0.39 - 0.85)	39	0.24	1.74 (0.69-4.40)
	quartile 3 (0.86 - 1.85)	39	0.50	0.73 (0.30-1.79)
	quartile 4 (1.86 - 5.68)	39		Reference
To nearest mangrove forest*	quartile 1 (0.01 - 1.00)	39	0.29	0.61 (0.25-1.53)
	quartile 2 (1.09 - 2.09)	39	0.74	1.17 (0.46-2.98)
	quartile 3 (2.13 - 4.16)	39	**0.08**	0.45 (0.18-1.12)
	quartile 4 (4.17 - 9.51)	39		Reference

[a] One of the farm locations was missing, resulting in a total of 156 farms for the distance

Table 9. Multivariate logistic regression model of white spot disease (WSD) risk factors in intensive Pacific white shrimp *Litopenaeus vannamei* culture systems in Chanthaburi province, Thailand; N: number of farms; β: estimated coefficient; OR (95% CI): odds ratio and its 95% confident interval.

Variable		N	β	OR (95% CI)	*P*-value
	Constant		0.80	1.26 (0.2 - 7.83)	0.9
Water source	Sea	23	0.10	0.5 (0.08 - 3.09)	0.46
	Canal	117	1.17	3.22 (1.05 - 10.57)	0.05
	Underground	17		Reference	
Lime application	Yes	57	-0.93	0.39 (0.18 - 0.86)	0.02
	No	100		Reference	
Probiotic used in feed	Yes	136	-1.10	0.33 (0.11 - 0.98)	0.05
	No	21		Reference	
Owner of multiple farms	Yes	48	0.91	2.50 (1.07 - 5.08)	0.03
	No	109		Reference	
Continuous culture	Yes	86	0.82	2.27 (1.03 - 5.02)	0.04
	No	71		Reference	
Distance to the nearest national highway	quartile 1	39	-1.07	0.34 (0.12 - 1.01)	0.05
	quartile 2	39	-0.81	0.44 (0.16 - 1.26)	0.12
	quartile 3	39	-1.20	0.30 (0.11 - 0.87)	0.03
	quartile 4	39		Reference	

The final multivariate logistic regression model indicates that the probability of WSD occurrence associated with farming practices in Chanthaburi province, Thailand, can be estimated using the following equation:

$$\ln\frac{p}{1-p} = 0.8 + 1.17CanalWater + (-0.93)Lime + (-1.1)Probiotic + 0.91Owner + 0.82ContinuousCulture + (-1.07)Quartile1 + (-1.2)Quartile3$$

Phase 3 Molecular characterization of WSSV isolates from 2007-2014, obtained from WSD outbreaks in the eastern and southern shrimp culture area of Thailand.

A total of 120 samples out of 137 samples were WSSV-positive by nested PCR methods as described by OIE (2015). Positive samples included 43 samples from Chanthaburi, 17 samples from Rayong, 3 samples from Trat, 10 samples from Surat Thani, 17 samples from Songkhla, 12 samples from Phuket, 7 samples from Krabi, and 2 samples from Prachuap Khiri Khan, Chumporn, Ranong and Pattani province. Positive samples were analyzed for variable regions indel-I, Indel-II and VNTR loci at ORF75, 94 and 125.

3.1 Indel-I (ORF14/15)

PCR primers set VR14/15-screen was able to amplify the variable region ORF14/15 in 102 from 120 samples. Twelve out of fifteen samples that were negative from a single-step PCR were successfully amplified by nested PCR, using primer set TJW14/15 with primer set VR14/15-screen. From 117 ORF14/15-amplified samples, the present study found 2 amplicon types of ~600 bp (112/114) and ~500 bp (2/114) (Figure 8A). The ~600 bp amplicons were detected in all studied province, whereas the ~500 bp amplicons were only detected from 2 samples from Krabi province. Sequence analysis showed 620 bp amplicons and 507 bp amplicons which have 5,950 bp and 6,031 bp deletions, respectively, comparing to the representative genotype of the WSSV common ancestor in Southeast Asia (WSSV-TH-

96-II ORF14/15; GenBank accession no. AY753327.1). The 620 bp isolates contained 132 bp out of the 257 bp in the 5′ region and 430 bp out of the 538 bp in the 3′ region found in WSSV TH-96-II. In addition, the 620 bp isolates presented 99% nucleotide identity with isolate WSSV-IN-05-I from India (GenBank accession. no. EU 327501). Sequence of the 507 bp showed the full 257 bp and 50 bp out of the 400 bp in the 5′ region, and 174 bp out of the 538 bp in the 3′ region found in WSSV TH-96-II. Multiple alignments among 5 isolates of the 620 bp isolates and 5 isolates of the 506 bp isolates presented with 98.5% and 99.3% nucleotide identity, respectively. Figure 9 shows schematic diagram of variable region Indel-I (ORF14/15) of WSSV found in the present study with reference isolates from previous reports.

3.2 Indel-II (ORF23/24)

Amplification of 55 out of the 120 samples were accomplished using primer set VR23/24-south which resulted in 2 types of amplicons, including ~3500 bp (42/55) and ~3000 bp (13/55). The VR23/24-south forward primer and VR23/24-1 reverse primer were then applied to obtain small amplicons suitable for accurate DNA sequencing. PCR product of ~3500 bp and ~3000 bp were reduced to ~2000 bp and ~1500 bp respectively (Figure 8B). The ~2000 bp isolates were identified in Chanthaburi, Rayong, Trat, Phuket, Krabi, Ranong and Pattani province, while the ~1500 bp were found specifically in the eastern part of Thailand. The ~2000 bp isolates were aligned with WSSV-TW and they showed 99% nucleotide identity from position 2144 to 3380 followed by a deletion of 10,970 and then complete

nucleotide identity from positions 14351– 15171 bp. Aligning the sequence of ~1500 bp isolates also revealed the 99% nucleotide identity from position 2144 to 2791 with 11632 bp deletion and followed by 99% nucleotide similarity from position 14351– 15171 bp. Multiple alignments of Indel-II variable region among 5 isolates of ~2000 bp and 5 isolates of ~1500 bp showed 96.3% and 97.9% nucleotide identity, respectively. Figure 10 shows schematic representation of variable region Indel-II (ORF23/24) of WSSV found in the present study with reference isolates from previous reports.

Figure 8. PCR products of WSSV variable regions; Indel-I (A), 2 amplicon sizes were detected, ~600 bp (Lane 1, 3, 4, 5) and ~500 bp (Lane 2), primer set: VR14/15 screen; Indel-II (B), 2 amplicon sizes were detected, ~2000 bp (Lane 1-5) and ~1500 bp (Lane 6-8), primer: VR23/24-south-F and VR23/24-1-R. M: marker.

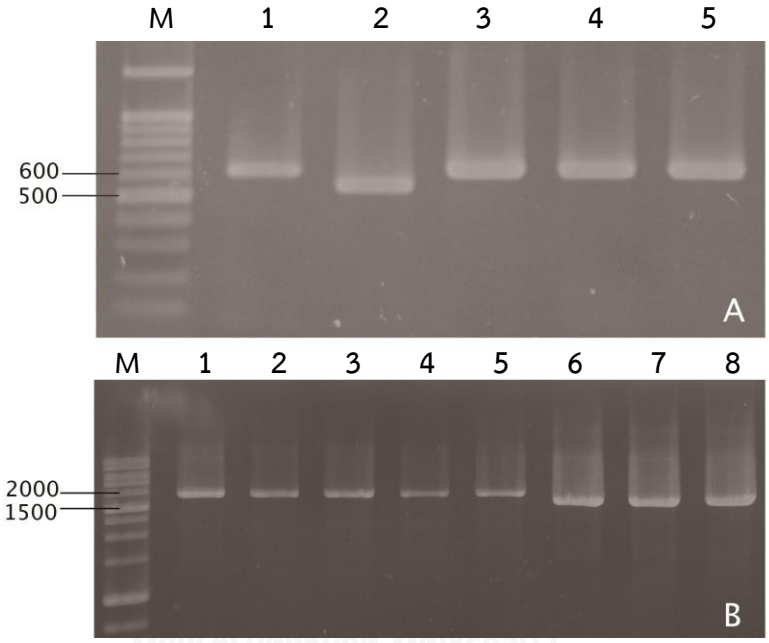


Figure 9. Schematic diagram of the variable region Indel-I (ORF14/15) of WSSV putative common ancestor, WSSV-TH-96-II, WSSV-TW, WSSV-CN, WSSV-TH, WSSV-VN and other WSSV isolates related to WSSV isolates found in in cultured *L. vannamei* in the present study (WSSV-TH-14* and WSSV-TH-12*). Genomic sequence number according to GenBank sequence are indicated above each isolate. Line (——) indicates deletion in the sequence. Fragment lengths are adhered to NCBI database and described in boxes. Arrows represent primer binding sites. Information regarding sources, year of sample collection and host species of those isolates were provided.

Indel-I (ORF14/15)

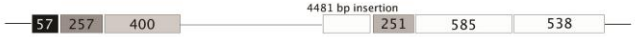
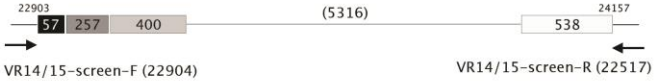
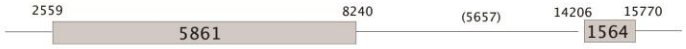
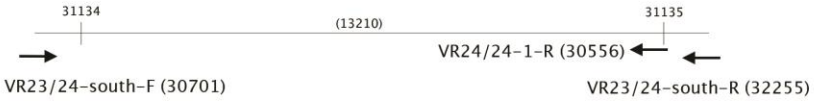
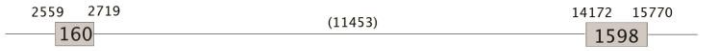
Schematic representation	Isolate	Source: Country	Source: Place	Year of sampling	Host species
	WSSV putative common ancestor				
	WSSV-TH-96-II	Thailand	Surat Thani	1996	*P. monodon*
	WSSV-TW	Taiwan	South	1994	*P. monodon*
	WSSV-CN	China	Xiamen	1996	*P. japonicus*
	WSSV-TH WSSV-VN south	Thailand Vietnam	Surat Thani Ba Ria	1996 2004	*P. monodon*
	WSSV-VN central	Vietnam	Nihn Thuan	2003	*P. monodon*
	WSSV-JA WSSV-TH-S WSSV-IR WSSV-VN central WSSV-IN-05-I WSSV-CB WSSV-SA WSSV-MG WSSV-MZ	Japan Thailand Iran Vietnam India Cambodia Saudi Arabia Madagascar Mozambique	No data No data Abadan Phu Yen Andhra Pradesh ShihanukVille No data Nodata Nodata	1995 1996 2002 2003 2005 2006 2010 2011 2011	*P. japonicus* *P. monodon* *P. indicus* *P. monodon* *P. monodon* *P. monodon* *P. indicus* *P. monodon* *P. monodon*
	WSSV-TH-14*	**Thailand**	**East and South[a]**	**2007-2014**	***L. Vannamei***
	WSSV-PHI	Philippines	Iloilo	1999	*P. monodon*
	WSSV -VN north, central, south,	Vietnam		2004	*P. monodon*
	WSSV-INDO	Indonesia	Jambak, Java	2008	Polychaete
	WSSV-TH-12*	**Thailand**	**Krabi**	**2012**	***L. Vannamei***

Figure 10. Schematic diagram of the variable region Indel-II (ORF23/24) of WSSV-TW, WSSV-CN, WSSV-TH, WSSV-VN and other WSSV isolates related to WSSV isolates found in cultured *L. vannamei* the present study (WSSV-TH-14* and WSSV-TH-14-E*). Genomic sequence number according to GenBank sequence are indicated above each isolate. Line (—) indicates deletion in the sequence. Arrows represent primer binding sites. Information regarding sources, year of sample collection and host species of those isolates were provided.

Indel-II (ORF23/24)

Schematic diagram	Isolate	Source: Country	Source: Place	Year of sampling	Host species
	WSSV-TW WSSV-TH-96-II	Taiwan Thailand	South Surat Thani	1994 1996	*P. monodon*
	WSSV-CN	China	Xiamen	1996	*P. japonicus*
	WSSV-TH-S	Thailand	No data	1996	*P. monodon*
	WSSV-TH WSSV-PHI WSSV-IR WSSV-VN south WSSV-INDO	Thailand Philippines Iran Vietnam Indonesia	Surat Thani Iloilo Abadan South Jambak, Java	1996 1999 2002 2004 2008	*P. monodon* *P. monodon* *P. indicus* *P. monodon* Polychaete
	WSSV-JA WSSV-VN central WSSV-IN WSSV-CB WSSV-SA WSSV-MG WSSV-MZ	Japan Vietnam India Cambodia Saudi Arabia Madagascar Mozambiqu	No data Phu Yen Andhra Pradesh ShihanukVille No data No data No data	1995 2003 2005-08 2006 2010 2011 2011	*P. japonicus* *P. monodon* *P. monodon* *P. monodon* *P. indicus* *P. monodon* *P. monodon*
	WSSV-TH-14*	**Thailand**	**East and South[a]**	**2007-2014**	***L. Vannamei***
	Brazill (south)	Brazill	Bahia Santa Catarina	2005-08	*L. vanamei*
	**WSSV-TH-14-E***	**Thailand**	**East[b]**	**2007-2014**	***L. Vannamei***

3.3 Variable number tandem repeat

3.3.1 ORF75

Primer set ORF75-flank (Dieu et al., 2004) was not able to produce PCR amplicon from all samples. Conseqently, primer set TJW75 was designed to extend the coverage of the previous flanking primers. Blast results of PCR products derived from TJW75 primer set showed 99% nucleotide sequence with the WSSV-TH ORF75 (GenBank accession no. AF369029). Sequence analysis of PCR products found in the present study showed five amplicon sizes ranging from 644 to 1556 bp (Figure 11A). the numbers of RUs were between 0 to 22 RUs and 2 types of repeated sequence were detected, including 45 bp, and 102 bp. The pattern of RUs are showed in parenthesis (45 RU, 102 RU) in Table 10. The 0 and 5 RUs in ORF75 were the most commonly found throughout study area. The 22 repeats isolates were the dominent and local RUs type of Songkhla province. The least prevalence RUs were 11 and 3 RUs, which 11 RUs were found only in Rayong province, while the 2 RUs were found in Rayong, Surat Thani, Krabi and Songkhla province (Figure 12).

3.3.2 ORF125

Eight types of 69 bp RUs in the ORF125 variable region were found in the present study. Sequencing of PCR products ranged between 421 to 978 bp (Figure 11B), and the 3 to 11 RUs were detected (Table 10). Blast results showed 99% nucleotide sequence similarity with the WSSV-TH ORF125 (GenBank accession no. AF369029). The most commonly RUs found throughout eastern and southern part of

Thailand included 5, 6 and 4 RUs (Figure 12), whereas 1 RUs was the least frequent and found only in one WSSV isolate from Chanthaburi province.

3.3.3 ORF94

PCR targeting ORF94 from 79 WSSV samples, 10 amplicon sizes of 501 to 986 bp were detected (Figure 11C) and a total of 10 patterns of RUs from 3 to 14 were found. The SNPs at the position 48 of the RUs were found in the isolates with 3, 8, 10 and 14 RUs (Table 10). Sequence analysis of each PCR products showed 99% similarity with the nucleotide sequence of WSSV-TH ORF94 (GenBank accession no. AF369029). The 7, 8 and 4 RUs were the most frequent RUs types found in both eastern and southern part of Thailand. The least frequent RUs detected were 12 RUs in a single WSSV isolate from Rayong, 14RUs and 3 RUs from Chanthaburi isolates (Figure 12).

3.3.4 WSSV genotyping and its distribution in Thailand

Genotyping of WSSV were based on the genomic pattern of Inde-I and Indel-II with the VNTRs associated with the DNA minisatellites in the WSSV genome (Tang et al., 2013). Based on Indel-I and Indel-II, at least 3 genotyps were found. The genotype "WSSV-TH-14" has 5950 bp deletion in the Indel-I region and 10970 bp deletion in Indel-II region. This genotype was found in our studied provinces and was a majority (74%) in Thailand. The second prevalent genotype found (23%) was "WSSV-TH-14-E" which has 5950 bp deletion in the Indel-I region and 11632 bp deletion in Indel-II region. This genotype was detected only in the eastern part of

Thailand. The genotype “WSSV-TH-12” was detected only for 2%. It contained 6031 bp deletion in the Indel-I region; however, the amplification of the Indel-II for this genotype was unsuccessful.

Analysis of the 3 VNTR loci indicated that at least 33 WSSV genotypes were presented in shrimp cultured area of Thailand. Genotyping of WSSV in the present study was based on different numbers of RU found within each VNTR locust. The RUs were then arranged from the least variation to the highest variation (ORF75, ORF125, ORF94). Table 11 summarizes WSSV genotypes found in the present study. In Chanthaburi province, a total of 17 genotypes were found which the 11 genotypes were found only in Chanthaburi. Seven WSSV genotypes were detected in Rayong province which 5 genotypes were localized in this province. Three samples from Trat province were genotypically different and two genotypes were only presented in Trat. Five genotypes were recorded from Krabi province, which 3 genotypes were only detected in the province. Phuket province was presented with 6 genotypes which only 2 genotypes were locally found in the Phuket’s sample. One genotype (22, 5, 7) was dominated and only detected in Songkhla province, whereas 4 other genotypes found in the area were minority and were detected in other provinces. Two WSSV genotyps found in Surat Thani province and one WSSV genotype found in Pattani province were similar to one of the Shongkhla and Phuket isolates. Figure 12 summarizes spatial distribution of WSSV in the present survey.

Table 9. Numbers of RUs found in ORF75, 125, and 94 from WSSV in the present study. ORF75 possess 45 and 102 bp RUs, respectively, which are given in parentheses. SNP = Single nucleotide polymorphism presented at the position 48 of the RUs

ORF75 (45 and 102 bp RUs)			ORF125 (69 bp RUs)			ORF94 (54 bp RUs)			
RUs	Amplicon size (bp)	N (%)	RUs	Amplicon size (bp)	N (%)	RUs	Amplicon size (bp)		SNP*
0	644	24 (30.4%)	3	421	4 (5.1%)	3	501	2 (2.5%)	GGT
3 (1,2)	702	5 (6.3%)	4	489	11 (13.8%)	4	548	13 (16.5%)	
5 (3, 2)	810	35 (44.3%)	5	561	27 (34.2%)	5	603	7 (8.9%)	
11 (8, 3)	897	3 (3.8%)	6	634	21 (26.6%)	6	659	6 (7.6%)	
22 (18, 4)	1556	12 (15.2%)	7	701	6 (7.6%)	7	712	25 (31.6%)	
			8	764	4 (5.1%)	8	766	14 (17.7%)	GTGTGTTT
			9	838	5 (6.3%)	10	818	5 (6.3%)	GGGGTTTGGG
			11	978	1 (1.3%)	11	929	4 (5.1%)	
						12	986	1 (1.3%)	
						14	1047	2 (2.5%)	GGGGGGGTGGG TTTT

Figure 11. PCR products of WSSV VNTR variable regions; ORF75 (A), 8 ORF125 (B) and ORF 94 (C); M: marker.

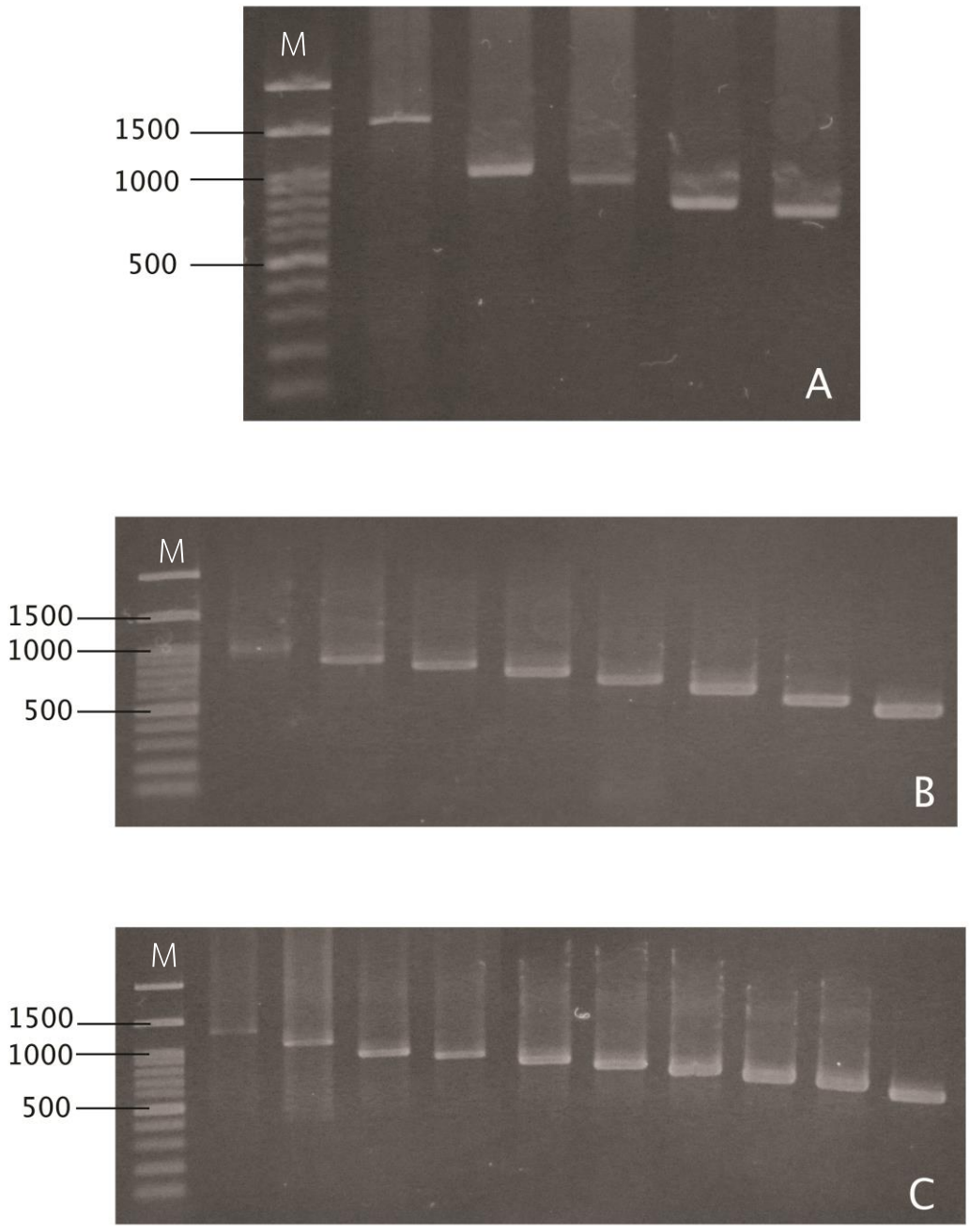


Table 10. Thirty-three WSSV genotypes found in cultured *L. vannamei* during 2007-2014 in Thailand. Numbers of repeat units (RUs) are used to differentiate each WSSV isolate. WSSV genotypes are classified as: "RUs of ORF75, RUs of ORF125, RUs of ORF94".

Genotype	Isolate	Province	Year
0, 3, 7	K1/12, K11/12	Krabi	2012
0, 5, 4	Ch8/12	Chanthaburi	2012
	R7/12, R8/12, R10/12, R15/12	Rayong	2012
	Sk2/09	Songkhla	2009
	K4/12	Krabi	2012
0, 5, 5	T2/12	Trat	2012
	Sk5/09	Songkhla	2009
	Phu1/12, Phu2/12, Phu3/12	Phuket	2012
0, 5, 7	Phu9/12, Phu10/12, Phu11/12	Phuket	2012
0, 5, 8	R5/12, R9/12, R13/12, R14/12	Rayong	2012
0, 5, 12	R17/12	Rayong	2012
0, 5, 14	Ch1/11	Chanthaburi	2011
0, 6, 10	R1/12	Rayong	2012
3, 4, 4	R6/12	Rayong	2012
3, 6, 4	K2/12	Krabi	2012
3, 9, 11	Su3/09, Su5/09	Surat Thani	2009
	Sk4/09	Songkhla	2009
5, 3, 8	Ch12/12	Chanthaburi	2011
5, 3, 11	K5/12	Krabi	2012
5, 4, 6	Ch11/12	Chanthaburi	2011
5, 4, 7	Phu12/12, Phu13/12	Phuket	2012
	Pat1/08, Pat2/08	Pattani	2008

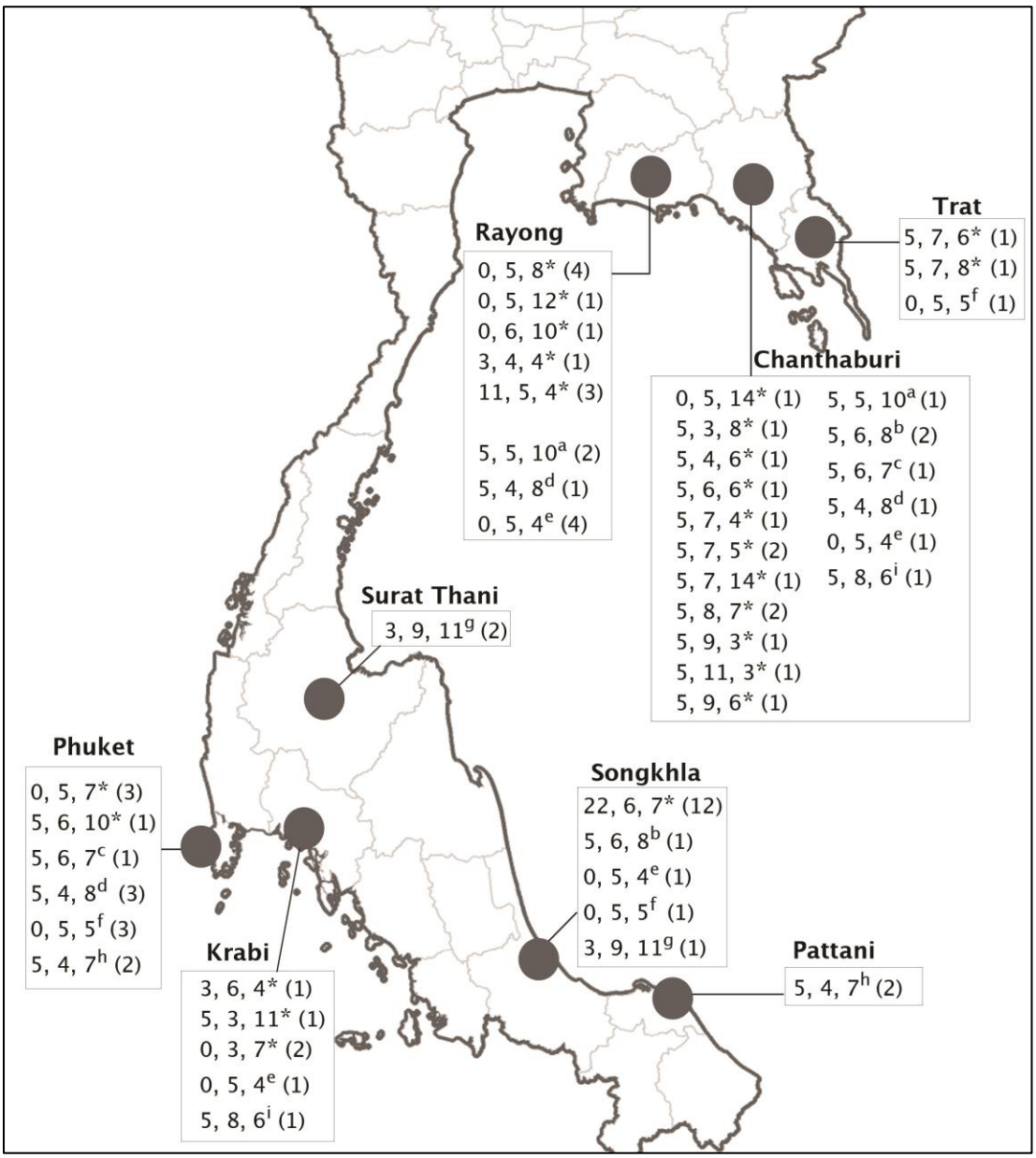
Table 11 (cont). Thirty-three WSSV genotypes found in cultured *L. vannamei* during 2007-2014 in Thailand. Numbers of repeat units (RUs) are used to differentiate each WSSV isolate. WSSV genotypes are classified as: "RUs of ORF75, RUs of ORF125, RUs of ORF94".

Genotype	Isolate	Province	Year
5, 4, 8	Ch2/11	Chanthaburi	2011
	R2/12	Rayong	2012
	Phu5/12, Phu6/12, Phu7/12	Phuket	2012
5, 5, 10	Ch1/09	Chanthaburi	2009
	R16/12, R18/12	Rayong	2012
5, 6, 6	Ch4/07	Chanthaburi	2007
5, 6, 7	Ch7/11	Chanthaburi	2007
	Phu8/12	Phuket	2012
5, 6, 8	Ch9/12, Ch10/12	Chanthaburi	2012
	Sk10/12	Songkhla	2012
5, 6, 10	Phu4/12	Phuket	2012
5, 7, 4	Ch1/13	Chanthaburi	2013
5, 7, 5	Ch3/07	Chanthaburi	2007
	Ch2/14	Chanthaburi	2014
5, 7, 6	T1/12	Trat	2012
5, 7, 8	T6/12	Trat	2012
5, 7, 14	Ch3/09	Chanthaburi	2009
5, 8, 6	Ch2/09	Chanthaburi	2009
	K3/12	Krabi	2012
5, 8, 7	Ch8/11	Chanthaburi	2011
	Ch1/14	Chanthaburi	2014
5, 9, 3	Ch5/07	Chanthaburi	2007
5, 9, 6	Ch5/11	Chanthaburi	2011
5, 11, 3	Ch1/07	Chanthaburi	2007

Table 11 (cont). Thirty-three WSSV genotypes found in cultured *L. vannamei* during 2007-2014 in Thailand. Numbers of repeat units (RUs) are used to differentiate each WSSV isolate. WSSV genotypes are classified as: "RUs of ORF75, RUs of ORF125, RUs of ORF94".

Genotype	Isolate	Province	Year
11, 5, 4	R3/12, R4/12, R11/12	Rayong	2012
22, 6, 7	Sk3/09	Songkhla	2009
	Sk1/12, Sk2/12, Sk3/12, Sk5/12, Sk6/12, Sk8/12, Sk11/12, Sk12/12, Sk13/12, Sk14/12, Sk15/12	Songkhla	2012

Figure 12. Thirty-three WSSV genotypes detected in cultured *L. vannamei* during 2007-2014 in Chanthaburi, Rayong, Trat, Surat Thani, Songkhla, Phuket, Krabi and Pattani province.; *Genotype presented only within the province; [a-i] indicates provinces where the particular genotypes were found; [a]Chanthaburi and Rayong; [b]Chanthaburi and Songkhla; [c]Chanthaburi and Phuket; [d]Chanthaburi, Rayong and Phuket; [e]Chanthaburi, Rayong, Krabi and Songkhla; [f]Trat, Songkhla and Phuket; [g]Surat Thani and Songkhla; [h]Pattani and Phuket; [i]Chanthaburi and Krabi. (N) = numbers of isolate.



CHAPTER V
DISCUSSION

WSSV has been a cause of serious disease in shrimp farmed in Thailand and has impacted both *P. monodon* and *L. vannamei* in hatcheries as well as grow-out ponds (Withyachumnarnkul et al., 2003; Flegel 2012). WSD occurrence during 2009-2014 in Chanthaburi province, Thailand was significantly influenced by atmospheric temperature. A high number of WSD cases in this study were observed during months with relatively low atmospheric temperature and a high degree of daily atmospheric temperature variation. Multivariate NBR analysis indicated that incident rate of WSD increased gradually when atmospheric temperature decreased. It was evident that WSD cases occurred 2.53 times more in months with an average atmospheric temperature of 24.5 – 27.2℃ then those in months with an average air temperature of 28.6 – 29.8℃. Atmospheric temperature is positively correlated to pond-water temperature (Nargis and Pramanik 2008), and decreased water temperature was reported as an important factor that supports viral replication but adversely affects shrimp immune response. Experiment infection of WSSV in *L. vannamei* at water temperature 27℃ to 30℃ resulted in the development of WSD gross signs, presence of WSSV-infected cells and 100% mortality rate, while none of these outcomes were found in a similar experiment at 33℃ (Rahman et al., 2006). WSSV proliferation in *F. chinensis* evaluated using real time PCR indicated that the

optimum temperature for WSSV proliferation was at 25°C (Gao et al., 2011). A crucial shrimp defense mechanism such as the WSSV-induced apoptosis was found to decrease at water temperature below 32°C (Granja et al., 2003). In addition, higher mortality rates were observed in *P. monodon* orally-challenged with WSSV at water temperature ranging from 16°C to 30°C than those of 32°C to 36°C challenges (Raj et al., 2012).

The statistical model of the observed data revealed that an increase of daily atmospheric temperature variation by 1 degree raised the rate of WSD occurrence by 8% (IRR: 1.08, Table 8). The temperature change within a day usually affects fluctuation of water temperature, and this condition was shown to induce WSD outbreaks with a low-level of WSSV infection in cultured shrimp (Hsu et al., 2000; Kautsky et al., 2000). The fluctuation of 3-4°C in water temperature during a day activated WSD outbreaks in cultured *P. monodon* in the Philippines (Tendencia and Verreth 2011). The impairment of immune function, decrease in total haemocyte count, phenoloxidase and nitric oxide synthase activity (NOS), as well as high rate of lipid peroxidation activity which was indicated by the increased malondialdehyde (MDA) were observed in *L. vannamei* during a sudden drop (22°C to 16 °C) of water temperature (Jia et al., 2014). An earlier study suggested that high amount of rain induced WSD occurrence due to the change in water temperature and salinity (Peinado-Guevara and López-Meyer 2006); however, our study found lower WSD occurrence during months with a high amount of rain. Strong negative correlation

(r = -0.81) between total amount of rainfall and daily atmospheric temperature variation observed in this study indicated that there was less atmospheric temperature fluctuation in months with a high amount of rain. The moderate minimum water temperature in rainy months may contribute lower WSD occurrence observed in our study. It was also reported that cloud accumulation during a rainy period in the Philippines slowed the cooling process of pond water during the night, and subsequently resulted in higher average minimum water temperature in shrimp pond (Tendencia et al., 2010a).

The highest number of WSD occurrence (482 cases) was observed in year 2011. It should be noted that the mid-2010 to early 2011 weather in the Asia-Pacific region was strongly impacted by the La Niña event. This phenomenon also caused decreasing atmospheric temperature during the cool season in Thailand in 2011 (Ueangsawat and Jintrawet 2013). La Niña was also believed to be a cause of high WSD prevalence in Ecuador in 1999 due to a lower atmospheric temperature than normal (Rodríguez et al., 2003). The present survey provided another example of the effect of climate change on an infectious disease in a marine organism.

A dramatic decline of WSD prevalence from 2012 to 2014 was observed in this study. The decrease of WSD prevalence could be associated with the emergence of the early mortality syndrome (EMS), now known as acute hepatopancreatic necrosis disease (AHPND), in this area since late-2011. In addition to the sudden prevalence of EMS/AHPND that caused mass mortality in early

stocking PL, shrimp farmers in this area decreased their operations. Shrimp cultured areas and production yield of Chanthaburi province decreased over 40% in 2014 compared to 2011 (Department of Fisheries 2014). This also explains the marked deviation of the predicted values in the multivariate NBR model, while the predicted values during 2009 to 2011 corresponded to observed values. Despite the decrease in WSD prevalence, the number of WSD cases remained the same, and the influence of climate factors was still apparent in those cases. The emergence of a new disease may affect the prevalence of endemic WSD; however, this study's findings suggest that WSD is still a major constraint in this shrimp farming community, especially when temperatures are low and fluctuate.

Control of WSD in Thailand's shrimp farming has been challenging due to small to medium-scale farms making up a large part of the industry. These farms are usually clustered, share common water sources and disregard recommended disease management practices. Despite strict biosecurity measures being used actively at the large-scale study farms, WSD remained an issue due to biosecurity being neglected at neighboring small-scale farms. Better management practices (BMPs) have been introduced to small-scale aquaculture farms in many developing countries (Mohan et al., 2008; Phan et al., 2009; Umesh et al., 2010). These BMPs aim to provide small-scale farmers with knowledge of cheap and practical farm management and feeding procedures to assist them improve yields and minimize losses caused by infectious diseases (Padiyar et al., 2003). In India, BMPs have been implemented in shrimp

culture communities affected seriously by WSD, and risk factor analyses have been pivotal to identifying and quantifying the value of various BMPs (Padiyar et al., 2003; Mohan and De Silva 2010).

In the Chanthaburi province study area, WSD occurrence was associated profoundly with the sourcing of pond water from communal canals conveying water to a cluster of farms from either the sea or a river. This has also been identified to be an important WSD risk factor in the Philippines, particularly when the canal is used for both farm inlet and outlet water, and more so when used as a water outlet during emergency harvests (Tendencia et al., 2011). At all study farms, however, canal inlet water was typically chlorinated before being used to fill ponds, and all employed zero water exchange grow-out systems. However, not all farms applied pond biosecurity systems to prevent entry of WSSV carriers likely to reside in the communal canals, and thus such carriers might represent a source of disease (OIE 2015). WSD outbreaks in farms in Vietnam have been associated with the introduction of WSSV-infected decapods and WSSV-contaminated zooplankton (Corsin et al., 2001). The use of communal canals also increases the likelihood of ponds receiving poor-quality hypertrophic or eutrophic water with potential to cause stress that could in turn induce disease (Lyle-Fritch et al., 2006; Huang et al., 2011).

In the study area, shrimp farms most distant from highways tended to be those either using or located nearest to communal canals. Therefore, this variable was also correlated with higher farm densities and WSD occurrence. The higher risk

of WSD at farms where the owner operated several farms might be due to increased movements of staff and vehicles between the farms together with inadequate biosecurity precautions.

Experienced shrimp farmers in Thailand and the Philippines generally avoid stocking ponds during colder weather due to higher risks of WSD (Withyachumnarnkul et al., 2003; Tendencia et al., 2010a). Higher WSD risks do occur at farms growing 3 or more crops each year compared to farms growing less than 2 crops during the warmer months. WSD becomes less problematic in grow-out water temperatures >30°C, and WSSV has been identified to replicate more effectively in Pacific white shrimp at water temperatures of ~26°C compared to ~32°C (Vidal et al., 2001). These findings are supported by WSSV gene expression in subcuticular epithelial cells being higher among Pacific white shrimp in ~26°C water compared with 33°C water (Reyes et al., 2007). Cell apoptosis caused by WSSV infection is also lower at water temperatures below 32°C (Granja et al., 2003). While growing shrimp at colder water temperatures poses higher risks of WSD, several farmers in the study region disregarded this due to attractive shrimp prices during cooler periods. In addition, farmers who produce >2 crops a year would have less time for pond drying. WSSV has been shown to remain infectious for up to 19 days in the sediment of pond being sun-dried and up to 35 days in undrained pond (Kumar et al., 2013). Considering that each production cycle of Pacific white shrimp in the study area usually takes 145-165 days inclusive of 30 days pre-stocking for pond preparation,

100-120 days for shrimp grow-out and 15 days for post-harvest pond drying, only those farmers growing 1 or 2 crops a year can set aside adequate time for pond drying.

The multivariate regression analysis showed the application of lime to disinfect the bottoms of fallow ponds to be useful in preventing WSD, as also reported from findings in India (MPEDA/NACA 2003) and Bangladesh (Islam et al., 2014). After each harvest, farmers usually removed the sludge pile and dried the pond bottom before applying lime, as is standard practice in shrimp aquaculture (Cruz-Lacierda et al., 2008). Farmers in the study region also applied lime at concentrations sufficient to generate a pond bottom soil pH >10 which has proven to be an effective disinfectant (Boyd and Massaut 1999; Boyd 2003).

The final logistic model indicated that the use of probiotic feed supplements was a preventive factor against WSD. Bacillus spp. probiotics from either commercial or government sources were used commonly in the study region. Many farmers used pineapple or banana as probiotics due to them containing substantial amounts of Vitamin C (Klimczak et al., 2007) that has been shown to improve stress and non-specific defense responses in shrimp (Lee and Shiau 2002; Qiao et al., 2011). Some lactic acid bacteria have been reported to enhance growth of shrimp and fish (Kesarcodi-Watson et al., 2008; Tuan et al., 2013; Aguilera-Rivera et al., 2014). Probiotics have been suggested to enhance the resistance of cultured shrimp to WSSV by mechanisms involving competitive exclusion and/or immune stimulation

(Li et al., 2009). For example, probiotic organisms such as *Staphylococcus hemolyticus* and *Pediococcus pentosaceus* have been found to protect *L. vannamei* against WSSV and Infectious hypodermal and hematopoietic necrosis virus (Leyva-Madrigal et al., 2011). However, it is possible that the use of probiotics at a farm was simply an indicator of the farmer having the financial ability to better manage crop grow-out, which might confound the survey data correlating probiotics use with lowered WSD risks.

With *P. monodon*, stocking ponds with WSSV-infected PL has been reported to be useful in mitigating WSD risks (Limsuwan 1997; Withyachumnarnkul 1999), and PCR screening of PL for WSSV is generally recommended for shrimp cultured in Thailand (Flegel 2012). However, PCR data on shrimp PL screened over the study period identified no correlation between WSSV detection and WSD occurring during grow-out. Other WSSV infection entry routes into ponds such as intake water or carrier species thus appear to have overridden any benefits of PL screening. While this finding supports PL screening for WSSV being non-mandatory in Thailand, our findings are unlikely to dissuade farms with capacity to accommodate screening from continuing with this practice as part of their disease risk management strategy.

The primary findings of the shrimp farmer survey and PL testing undertaken in the Chanthaburi province study region were that the use of a communal water sources by many independent farms was the major WSD risk factor. Thus, mitigating WSD needs to be a shared responsibility of shrimp farming communities using such

water sources and supported by appropriate government incentives. The study also identified a need for farms utilizing communal water canals to exercise care with water and pond management practices, including the use of lime at concentrations adequate to disinfect the pond bottom soil during dry-out. Farms undertaking continuous culture cycles need to consider employing an adequate period for pond drying and avoiding shrimp culture during cooler months. While surveillance for WSD carriers in communal water canals and mandatory PL testing might also be considered to reduce the risks of WSD, key to the success of such measures will be the active support and participation of local shrimp farming communities.

WSSV causing outbreaks in the intensive shrimp cultured areas of Thailand during 2007-2014 were also characterized using PCR targeting the variable regions in its genome. Among the five variable regions in WSSV genome analyzed in this study, Indel-I, and Indel-II variable regions presented with less variation compared to the VNTRs loci of ORF75, 125 and 94. For WSSV samples collected in Thailand during 2007-2014, at least 3 genotypes of WSSV were identified using Indel-I and Indel-II; while at least 33 WSSV genotypes were identified when the VNTR markers were used. This finding supported the suggestion of Dieu et al., (2010) that the less variation of Indel-I and Indel-II were more suitable for differentiating WSSV in continental and regional scales. Analyzing of Indel-I and Indel-II indicated that the most frequent isolate (WSSV-TH-14) contained a deletion of 5950 bp in the Indel-I and a deletion of 10970 bp in the Indel-II. These isolates were similar to WSSS from japan (WSSV-JA,

collected in 1995), Cambodia (WSSV-CB, collected in 2006) (Zwart et al., 2010), India (WSSV-IN-05, collected in 2005) (Pradeep et al., 2008b), Saudi Arabia (WSSV-SA, collected in 2010), Madagascar and Mozambique (WSSV-MA and WSSV-MZ, collected in 2011-2012) (Tang et al., 2013). Another WSSV isolate (WSSV-TH-14-E) was found only in the eastern part of Thailand. Interestingly, this isolate have the same 5950 bp deletion in the Indel-I with WSSV-TH-14, but in the Indel-II, different pattern of 11632 bp deletion was observed. This 11632 bp deletion was closely related to 11453 bp deletion in the Indel-II of WSSV isolates collected in Brazil during 2005-2008 (Muller et al., 2010). These two isolates have the same 160 bp sequence before ~11 kb deletion. Unfortunately, information regarding the Indel-I of the Brazilian isolates was not available; consequently, partial conclusion can only be made for the relationship between WSSV-TH-14-E and Brazilian isolates. Two isolates from Krabi province, collected in 2012 was identified as WSSV-TH-12. This isolate have similar deletion of 6031 bp in Indel-I as WSSV from Philippines (WSSV-PHI, collected in 1999), Indonesia (WSSV-INDO, collected in 2008) and north, central and south Vietnam (WSSV-VN, collected during 2003-2004) (Dieu et al., 2010). However, PCR amplification of Indel-II of the WSSV-TH-12 isolate was unsuccessful, while other isolates from those countries with similar Indel-I pattern showed the ~13.2 kb deletion. The unsuccessful PCR amplifications of Indel-II have been reported in WSSV isolates from Vietnam (Dieu et al., 2004), India (Pradeep et al., 2008b) and Mexico (Ramos-Paredes et al., 2012). These authors suggested that the deletion of

Indel-II is extended beyond the coverage of primers designed from Asian WSSV sequence. Indel-I and Indel-II of WSSV isolates collected during 2007-2014 in Thailand were completely different with the 1996 reference WSSV isolates from Thailand, WSSV-TH and WSSV-TH-96-II. The only isolate close to the isolates found in our study was WSSV-TH-S (collected in 1996 in Surat Thani province) which has the similar pattern of Indel-I, but has an additional sequence ~5 kb in Indel-II (Zwart et al., 2010).

The analysis of three DNA minisatellites (ORF75, ORF125, and ORF94) indicated that at least 33 WSSV genotypes were presented in Thailand during 2007-2014, which only 3 genotypes were similar to the previous reports. The genotype found in Trat province (T6/12) have the same RUs in all 3 minisatellites (5, 7, 8) as WSSV isolate from Ba Ria province, located in southern Vietnam. The genotype "5, 6, 10" detected in 1 isolate from Phuket province (Phu4/12) was similar to isolate from Da nang province in central Vietnam. However, these isolates were presented with different deletion pattern in the 2 Indels, which suggested that these isolated may not be related. The samples "Ch7/11" and "Phu8/12" were the only two isolates that have 4 out 5 variable loci identical to the isolate from the previous study in Saudi Arabia (Tang et al., 2013). These isolates contained 5,950 bp deletions in Indel-I, 10970 deletions in Indel-II and 6 and 7 RUs in ORF125 and ORF94, respectively. The only difference was in the RUs of ORF75. This finding supported

the suggestion of (Tang et al., 2012a; Tang et al., 2013) that WSSV entered Saudi Arabia *via* imported shrimp broodstock and shrimp PL from Southeast Asia.

The highest variation of RUs in the present study was found in ORF94, followed by ORF 125 and ORF75. This was in concordance with the previous studies in Vietnam (Dieu et al., 2010), India (Pradeep et al., 2008a), Saudi Arabia, Madagascar, Mozambique (Tang et al., 2013) and American continent (Muller et al., 2010). The numbers of RUs in ORF94 were reported between 2 RUs in India (Pradeep et al., 2008a)) and up to 20 RUs in Thailand (Wongteerasupaya et al., 2003). In this study, 10 variants of ORF94 were found, ranging from 3 to 14 RUs which the 7 RUs was the most common. The high prevalence of ORF94-7 RUs was also reported in Vietnam (Hoa et al., 2005a), India (Pradeep et al., 2008a) and Thailand during 2000-2002 (Wongteerasupaya et al., 2003). Among the 12 of ORF94 variants reported in Thailand (6 to 20 RUs with 13, 16 and 18 RUs absent), we reported the first detection of 3, 4, 5 RUs variants in Thailand. To our beast knowledge, the 8 variants of ORF125 and 5 variants of ORF75 were also reported in Thailand for the first time.

PCR amplifications of ORF75 using primer set ORF75-flank were unsuccessful. With the primer set TJW75 design to extend the flanking site, PCR products were achieved. Primer binding site of the ORF75-flank reverse primer was located in the DNA sequences of the products; on the contrary, forward primer binding site was not presented. The deletion of binding site of ORF75-flank forward was also reported in WSSV isolates from Saudi Arabia, Madagascar and Mozambique (Tang et al., 2013).

The movement of WSSV between Thailand and other counties cannot be concluded due to the fact that WSSV in Thailand had never been characterized for almost 2 decades. Our hypothesis would be there were significant movements of WSSV between Thailand, Vietnam and India. The adaptation in genome size by removing redundant sequence or genomic recombination with local existing isolates might occur. These phenomena have been suggested when the virus is introduced into specific novel environment (Dieu et al., 2010). In addition, WSSV in Brazil including other countries in Southeast Asia and the Middle East were possibly originated from Thailand.

The results of this study also suggested that WSSV Indels and VNTRs appear to stabilize overtime and among different hosts. Isolates from 1995 (Zwart et al., 2010) to 2014 in the present study showed the same pattern of Indels. Shrinkage of WSSV genome was likely to occur during the early spread of WSSV (Dieu et al., 2010). Larger genome which loci are more distance would have a higher probability of homologous recombination and large deletion (Zwart et al., 2010). The pattern of Indels detected in cultured *L. vannamei* in our study were similar to WSSV in different hosts including *P. japanicus, P. indicus, P, monodon* and polychaete, which were from different geographical locations and farming systems (Dieu et al., 2004; Pradeep et al., 2008b; Dieu et al., 2010; Zwart et al., 2010). Stability in VNTR loci was also observed in our study. Several isolates from 2007 showed the same RUs pattern as the isolates from 2012 to 2014. Therefore, results of the present study

can be used as a Thailand's WSSV genomic database for the future epidemiological investigation.

The non-similarity genomic pattern in 5 variable loci of our WSSV isolates compared to the rest of the world, and the distribution of the 33 WSSV genotypes in Thailand that showed only 9 genotypes were presented in more than one province may imply that the major source of WSSV causing disease in farmed shrimp was inhabited and localized in each shrimp farming community. Transboundary movement of WSSV may be limited due to the improvement of diagnostic technique and effective enforcement in trading regulations of the world commodities.

CONCLUSION AND SUGGESTION

The present study investigated the prevalence of WSD and identified risk factors associated with WSD occurrence in cultured shrimp in the intensive shrimp culture area of Thailand. Molecular makers were applied to characterized genome of WSSV causing outbreaks in cultured shrimp in eastern and southern parts of Thailand. The conclusions are as follows:

1. The prevalence and number of WSD cases in Chanthaburi province were high between Octobers to February, while it decreased during March to June were, and the lowest were observed in May.
2. The statistical analysis showed that the increase in WSD cases was associated with decreased atmospheric temperature and more variation of atmospheric temperature during a day.
3. The important risk factors associated with farming practice obtained include farms sharing inlet water and culturing shrimp year round. The managements of lime application to disinfect the pond bottoms and used probiotics mixed with feed were also found to reduce risk of WSD occurrence in farm.
4. The genomic pattern of Indels indicated that WSSV in Thailand were related to WSSV from Vietnam, India, Brazil, Saudi Arabia, Madagascar and Mozambique. At 33 WSSV genotype were characterized using 3 DNA minisatellite. The only one isolate showed 4 out of 5 variable loci

identical to WSSV from Saudi Arabia, which indicated transbounday movement of WSSV.

The information obtained from the study could benefit shrimp farming sectors as follows:

1. The understanding of WSD prevalence and climate factors that effect WSD occurrence in this area may assist in the development of disease control plan to accommodate dissimilarities of shrimp culture conditions.
2. Identification of WSD risk factor associated with sharing common water source might be used to guide government and farm WSD control policies. The study encourages the shared responsibility of shrimp farming communities using such water sources and active support and participation of local shrimp farming communities.
3. WSSV genomic database was constructed and can be used for the future WSSV epidemiological investigation.

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APPENDIX

APPENDIX A

Climate data of Chanthaburi province between January 2009 to December 2014.

Predicted WSD incident and its 95% Confident interval are also presented.

DATE	Avgtemp	AvgMinTemp	AvgMaxTemp	TotalRain	Rday	Humid	AvgWindSpp	DayVar	MeanPredict	Lower	Upper
JAN 2009	25.2	20.2	31.2	0.5	1.0	62.0	4.2	10.97	24.1	18.22	31.88
FEB 2009	27.8	23.4	33.3	0.1	3.0	75.0	1.7	9.83	25.3	17.44	36.71
MAR 2009	28.0	24.4	33.2	243.2	16.0	78.0	1.6	8.76	20.47	16.03	26.15
APR 2009	28.8	25.3	34.2	186.2	18.0	79.0	1.5	8.87	11.87	8.62	16.34
MAY 2009	27.9	25.0	32.7	633.3	26.0	84.0	1.4	7.76	8.9	6.73	11.76
JUN 2009	28.9	26.2	32.3	258.1	22.0	80.0	2.6	6.09	11.58	7.89	17
JUL 2009	27.8	25.4	31.4	543.4	24.0	83.0	2.2	6.00	20.65	15.13	28.18
AUG 2009	28.3	25.8	32.9	236.7	22.0	81.0	1.8	7.05	23	17.53	30.2
SEP 2009	27.3	24.7	31.4	689.4	25.0	86.0	1.1	6.66	8.84	6.64	11.77
OCT 2009	27.1	24.2	31.8	307.7	22.0	85.0	1.1	7.56	51.43	39.33	67.25
NOV 2009	27.1	22.7	32.5	0.5	1.0	70.0	3.9	9.87	32.58	26.06	40.74
DEC 2009	27.1	22.3	33.2	0.0	0.0	69.0	2.1	10.90	23.96	18.2	31.55
JAN 2010	27.2	22.9	32.7	30.2	6.0	73.0	2.0	9.78	27.72	22.23	34.55
FEB 2010	28.4	24.8	32.9	100.2	6.0	79.0	1.8	8.11	31.96	25.12	40.66
MAR 2010	28.8	24.5	33.9	77.0	5.0	74.0	1.8	9.47	19.11	13.64	26.76
APR 2010	29.8	26.0	34.7	159.1	8.0	77.0	1.5	8.67	10.02	7.3	13.74
MAY 2010	29.7	26.1	34.8	281.6	18.0	79.0	1.5	8.68	6.27	4.57	8.6
JUN 2010	28.5	25.6	32.8	492.8	27.0	85.0	1.4	7.24	5.59	4.02	7.78
JUL 2010	28.0	25.2	32.3	567.4	27.0	85.0	1.5	7.10	10.91	8.33	14.28
AUG 2010	27.3	24.6	31.5	512.7	30.0	87.0	1.3	6.89	20.76	15.67	27.51
SEP 2010	28.1	24.8	32.8	225.9	22.0	83.0	1.3	8.02	15.87	12.47	20.21
OCT 2010	27.1	24.3	31.6	375.3	22.0	83.0	2.4	7.31	27.59	20.75	36.68
NOV 2010	27.5	24.0	32.4	5.7	5.0	68.0	4.4	8.43	24.23	18.02	32.58
DEC 2010	27.0	22.7	32.6	28.2	3.0	68.0	2.9	9.81	24.31	19.49	30.34
JAN 2011	26.8	22.0	32.9	0.0	1.0	57.0	3.9	10.83	23.84	18.18	31.26
FEB 2011	27.3	23.5	32.3	32.9	8.0	76.0	1.6	8.87	29.13	21.25	39.92
MAR 2011	26.9	23.5	31.4	100.3	12.0	74.0	2.7	7.85	37.7	29.31	48.48
APR 2011	28.0	24.7	33.1	193.9	12.0	81.0	1.4	8.36	19.13	15.05	24.32
MAY 2011	28.9	25.6	33.3	290.9	23.0	81.0	1.3	7.70	8.12	5.9	11.16
JUN 2011	28.0	25.5	31.7	534.4	28.0	85.0	1.6	6.18	14.93	10.81	20.62
JUL 2011	28.0	25.3	31.7	422.2	27.0	83.0	1.7	6.32	16.29	11.9	22.3
AUG 2011	27.7	24.8	31.8	563.0	26.0	84.0	1.5	7.06	37.9	28.69	50.07
SEP 2011	27.2	25.0	30.6	860.7	24.0	87.0	1.2	5.63	27.5	18.07	41.84
OCT 2011	27.5	24.5	32.1	264.2	23.0	82.0	1.7	7.52	32.73	24.81	43.16
NOV 2011	28.0	23.7	33.6	54.2	5.0	69.0	2.9	9.85	32.66	24.5	43.55
DEC 2011	26.0	21.9	31.4	0.0	2.0	61.0	5.0	9.48	27.08	21.86	33.54
JAN 2012	27.3	23.7	32.3	61.2	13.0	74.0	2.0	8.57	30.83	22.8	41.69
FEB 2012	28.0	24.3	32.8	101.8	8.0	78.0	1.3	8.48	30.05	23.62	38.24
MAR 2012	28.8	24.8	33.8	91.4	7.0	76.0	1.0	8.99	21.39	15.5	29.53
APR 2012	29.0	25.2	34.2	60.9	11.0	78.0	1.0	8.99	8.56	6.2	11.82
MAY 2012	28.2	25.0	32.5	545.9	28.0	84.0	1.1	7.50	18.55	14.4	23.9
JUN 2012	28.7	26.0	32.1	286.6	26.0	81.0	1.6	6.14	10.94	7.47	16.01
JUL 2012	27.7	25.2	31.5	475.1	27.0	85.0	1.3	6.36	37.18	27.66	49.98
AUG 2012	28.2	25.4	31.8	245.5	21.0	82.0	1.5	6.43	29.82	21.94	40.53
SEP 2012	27.0	24.5	31.3	379.5	29.0	88.0	0.7	6.81	42.06	30.5	58.01
OCT 2012	27.6	24.1	32.9	172.6	19.0	82.0	1.1	8.72	87.17	64.05	118.63
NOV 2012	28.0	24.5	33.3	204.4	15.0	82.0	1.2	8.83	52.93	41.39	67.7
DEC 2012	28.1	23.2	33.8	0.0	0.0	69.0	2.0	10.65	25.45	18.12	35.74
JAN 2013	27.1	22.7	32.7	66.8	7.0	69.0	2.0	9.98	62.21	49.57	78.08
FEB 2013	28.3	24.4	33.5	43.7	4.0	75.0	1.2	9.15	39.22	30.38	50.63
MAR 2013	29.0	25.0	34.1	61.2	5.0	75.0	1.5	9.02	27.02	19.56	37.33
APR 2013	29.2	25.4	34.4	223.8	12.0	79.0	1.0	9.00	35.13	25.44	48.5
MAY 2013	29.4	25.8	34.1	140.8	20.0	81.0	0.9	8.30	26.74	19.55	36.59
JUN 2013	28.1	25.2	32.5	562.3	26.0	86.0	1.0	7.28	63.16	48.63	82.03
JUL 2013	27.3	24.9	30.9	1035.4	26.0	88.0	0.9	6.00	65.21	47.79	88.97
AUG 2013	27.8	25.1	31.5	498.7	24.0	85.0	1.1	6.35	45.11	33.54	60.67
SEP 2013	27.3	24.9	31.8	46.9	1.0	87.0	0.8	6.46	29.45	21.98	39.45
OCT 2013	27.2	24.0	31.9	327.0	19.0	84.0	1.7	7.87	55.68	43.36	71.5
NOV 2013	27.6	24.1	32.6	72.1	8.0	76.0	3.4	8.51	47.19	34.98	63.65
DEC 2013	24.5	20.5	29.9	2.7	2.0	66.0	5.2	9.44	71.97	58.14	89.09
JAN 2014	25.0	19.7	31.7	0.0	0.0	67.0	3.9	12.02	77.15	53.86	110.53
FEB 2014	26.3	22.2	31.4	40.2	8.0	77.0	1.8	9.13	60.36	48.84	74.6
MAR 2014	28.5	24.9	33.3	115.8	4.0	74.0	1.8	8.39	28.17	20.58	38.55
APR 2014	29.3	25.2	34.6	55.3	6.0	80.0	1.6	9.37	37.47	26.85	52.29
MAY 2014	29.4	25.7	34.7	170.6	16.0	79.0	1.2	8.95	30.7	22.27	42.34
JUN 2014	28.6	26.0	32.5	482.1	25.0	85.0	1.9	6.29	34.59	23.83	50.21
JUL 2014	28.5	26.2	32.2	496.0	24.0	88.0	2.1	6.00	36.54	24.76	53.91
AUG 2014	28.1	25.5	32.1	276.0	18.0	85.0	1.4	6.57	46.18	34.29	62.19
SEP 2014	27.5	24.8	31.7	712.0	24.0	86.0	1.4	7.00	34.23	25.89	45.25
OCT 2014	27.5	24.3	33.1	259.5	20.0	82.0	2.1	8.91	50.3	36.64	69.06
NOV 2014	28.2	24.3	33.8	76.7	9.0	76.0	2.5	9.46	51.02	39.02	66.7
DEC 2014	26.9	22.9	32.0	12.3	1.0	65.0	4.7	9.09	77.66	62.83	96

APPENDIX B

Univariate analysis of the association between climate factors and WSD occurrence using binomial logistic regression model.

Model Information

Dependent Variable	Positive2
Probability Distribution	Negative binomial (1)
Link Function	Log
Offset Variable	lnTotalCase2
Subject Effect 1	ID
Working Correlation Matrix Structure	Independent

Case Processing Summary

	N	Percent
Included	72	100.0%
Excluded	0	0.0%
Total	72	100.0%

1. Average temperature

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	29.789	1	.000
Q_AvgTemp	27.910	3	.000

Dependent Variable: Positive2
Model: (Intercept), Q_AvgTemp, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.916	.1604	-1.230	-.601	32.579	1	.000	.400	.292	.548
[Q_AvgTemp=1.00]	.997	.1920	.621	1.374	26.995	1	.000	2.711	1.861	3.950
[Q_AvgTemp=2.00]	.622	.2190	.193	1.052	8.079	1	.004	1.864	1.213	2.863
[Q_AvgTemp=3.00]	.553	.2031	.155	.951	7.411	1	.006	1.738	1.167	2.588
[Q_AvgTemp=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

2. Average minimal temperature

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	28.003	1	.000
Q_AvgMinT	30.533	3	.000

Dependent Variable: Positive2
Model: (Intercept), Q_AvgMinT, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.753	.1561	-1.060	-.447	23.287	1	.000	.471	.347	.639
[Q_AvgMinT=1.00]	.875	.1823	.517	1.232	23.013	1	.000	2.398	1.677	3.428
[Q_AvgMinT=2.00]	.545	.1949	.163	.927	7.820	1	.005	1.725	1.177	2.527
[Q_AvgMinT=3.00]	.090	.2416	-.383	.563	.139	1	.709	1.094	.682	1.757
[Q_AvgMinT=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

3. Average maximal temperature

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	21.336	1	.000
Q_AvgMaxTemp	6.983	3	.072

Dependent Variable: Positive2
Model: (Intercept), Q_AvgMaxTemp, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.676	.1783	-1.025	-.327	14.375	1	.000	.509	.359	.721
[Q_AvgMaxTemp=1.00]	.450	.2203	.018	.882	4.167	1	.041	1.568	1.018	2.415
[Q_AvgMaxTemp=2.00]	.302	.2449	-.178	.782	1.519	1	.218	1.352	.837	2.186
[Q_AvgMaxTemp=3.00]	.552	.2168	.127	.977	6.474	1	.011	1.736	1.135	2.655
[Q_AvgMaxTemp=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

4. Total rainfall

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	22.942	1	.000
Q_TotalRainmm	15.690	3	.001

Dependent Variable: Positive2
Model: (Intercept), Q_TotalRainmm, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.562	.1622	-.880	-.244	11.978	1	.001	.570	.415	.784
[Q_TotalRainmm=1.00]	.636	.1968	.251	1.022	10.450	1	.001	1.890	1.285	2.779
[Q_TotalRainmm=2.00]	.072	.2247	-.369	.512	.102	1	.749	1.074	.692	1.669
[Q_TotalRainmm=3.00]	.157	.2158	-.266	.580	.528	1	.468	1.170	.766	1.786
[Q_TotalRainmm=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

5. Number of raindays

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	21.982	1	.000
Q_Rday	7.769	3	.051

Dependent Variable: Positive2
Model: (Intercept), Q_Rday, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.561	.1495	-.855	-.268	14.102	1	.000	.570	.425	.765
[Q_Rday=1.00]	.515	.2037	.116	.915	6.400	1	.011	1.674	1.123	2.496
[Q_Rday=2.00]	.299	.1948	-.083	.681	2.357	1	.125	1.349	.921	1.976
[Q_Rday=3.00]	.076	.2209	-.357	.509	.119	1	.730	1.079	.700	1.664
[Q_Rday=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

6. Average Relative humidity

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	22.997	1	.000
Q_Humid	23.657	3	.000

Dependent Variable: Positive2
Model: (Intercept), Q_Humid, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.684	.2034	-1.083	-.286	11.318	1	.001	.504	.339	.752
[Q_Humid=1.00]	.822	.2258	.380	1.265	13.263	1	.000	2.275	1.462	3.542
[Q_Humid=2.00]	.216	.2465	-.267	.700	.771	1	.380	1.242	.766	2.013
[Q_Humid=3.00]	.283	.2411	-.190	.755	1.375	1	.241	1.327	.827	2.128
[Q_Humid=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

7. Average wind speed

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	20.952	1	.000
Q_AvgWindSpp	14.997	3	.002

Dependent Variable: Positive2
Model: (Intercept), Q_AvgWindSpp, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.049	.1195	-.185	.283	.168	1	.682	1.050	.831	1.327
[Q_AvgWindSpp=1.00]	-.626	.1891	-.997	-.256	10.967	1	.001	.535	.369	.774
[Q_AvgWindSpp=2.00]	-.595	.1924	-.972	-.218	9.577	1	.002	.551	.378	.804
[Q_AvgWindSpp=3.00]	-.266	.1884	-.636	.103	2.000	1	.157	.766	.530	1.108
[Q_AvgWindSpp=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									
(Negative binomial)	1[b]									

APPENDIX C

Multivariate negative binomial regression model of WSD between climate factors and WSD occurrence

Goodness of Fit[a]

	Value
Quasi Likelihood under Independence Model Criterion (QIC)[b]	32.101
Corrected Quasi Likelihood under Independence Model Criterion (QICC)[b]	38.961

Dependent Variable: Positive2
Model: (Intercept), Q_AvgTemp, DayVar, offset = lnTotalCase2

a. Information criteria are in smaller-is-better form.

b. Computed using the full log quasi-likelihood function.

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	5.489	1	.019
Q_AvgTemp	22.482	3	.000
DayVar	2.279	1	.131

Dependent Variable: Positive2
Model: (Intercept), Q_AvgTemp, DayVar, offset = lnTotalCase2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-1.556	.4592	-2.456	-.656	11.484	1	.001	.211	.086	.519
[Q_AvgTemp=1.00]	.931	.1990	.541	1.321	21.886	1	.000	2.537	1.717	3.746
[Q_AvgTemp=2.00]	.646	.2177	.220	1.073	8.811	1	.003	1.909	1.246	2.924
[Q_AvgTemp=3.00]	.554	.2020	.158	.950	7.524	1	.006	1.740	1.171	2.585
[Q_AvgTemp=4.00]	0[a]	.	.	.	.	.	.	1	.	.
DayVar	.079	.0521	-.023	.181	2.279	1	.131	1.082	.977	1.198
(Scale)	1									
(Negative binomial)	1[b]									

APPENDIX D

Data of farms (A=control farm; B=case farms) used in the survey of WSD risk factors associated with farm management

Data include farm location, responses to statistically significant variables, probability of WSD occurrence and its 95% confident interval.

FarmID	lattitude	longitude	WSD	Lime	WaterSource	ConCulture	ProFeed	OwnerW	MeanPredi	LowerCl	UpperCl
1A	12.40630	102.17906		0	1	2	2	1	2	0.78	0.547	0.913
2A	12.41200	102.14045		0	1	1	1	1	2	0.018	0.002	0.131
3A	12.41242	102.13976		0	1	1	1	1	1	0.265	0.084	0.587
4A	12.54092	102.09637		0	1	2	2	1	2	0.42	0.18	0.705
5A	12.41898	102.15247		0	2	1	2	1	1	0.718	0.34	0.926
6A	12.44087	102.23882		0	1	2	2	1	2	0.206	0.073	0.462
7A	12.56761	102.08536		0	1	3	1	1	2	0.039	0.007	0.181
8A	12.47050	102.16795		0	2	2	1	1	1	0.874	0.631	0.966
9A	12.57664	102.05666		0	2	2	2	1	2	0.413	0.179	0.695
10A	12.49042	102.12608		0	1	3	2	1	2	0.315	0.119	0.608
11A	12.55853	102.06831		0	2	2	1	1	2	0.58	0.343	0.785
12A	12.46932	102.16902		0	2	2	2	1	2	0.797	0.539	0.93
13A	12.43076	102.11545		0	1	1	2	1	2	0.157	0.028	0.548
14A	12.44546	102.20456		0	1	2	1	2	2	0.376	0.133	0.704
15A	12.38491	102.18178		0	2	1	2	1	1	0.861	0.576	0.966
16A	12.47670	102.07822		0	1	3	1	1	2	0.149	0.037	0.443
17A	12.49001	102.04874		0	2	2	1	2	2	0.866	0.582	0.968
18A	12.41635	102.12893		0	1	1	1	1	2	0.081	0.016	0.323
19A	12.41239	102.34248		0	1	3	1	1	2	0.149	0.037	0.443
20A	12.49175	102.13584		0	1	3	2	1	2	0.287	0.107	0.576
21A	12.45107	102.17020		0	1	2	1	1	2	0.094	0.026	0.287
22A	12.43563	102.11734		0	2	1	1	1	1	0.472	0.153	0.816
23A	12.47480	102.16168		0	2	2	1	1	2	0.259	0.1	0.523
24A	12.55841	102.08411		0	2	2	1	1	2	0.22	0.092	0.438
25A	12.49285	102.13302		0	1	2	1	1	1	0.343	0.141	0.626
26A	12.45396	102.13823		0	2	2	1	1	2	0.601	0.389	0.781
27A	12.45424	102.13876		0	2	2	2	1	1	0.74	0.439	0.912
28A	12.42984	102.29220		0	1	2	1	2	1	0.71	0.347	0.919
29A	12.45797	102.09943		0	2	2	1	1	1	0.554	0.294	0.788
30A	12.53260	102.10281		0	1	2	1	1	2	0.608	0.321	0.835
31A	12.43091	102.29184		0	1	1	1	1	1	0.291	0.086	0.642
32A	12.53570	102.09073		0	2	2	1	1	2	0.461	0.232	0.707
33A	12.51004	102.09654		0	2	2	2	1	2	0.662	0.388	0.858
34A	12.50845	102.09886		0	2	2	1	1	2	0.461	0.232	0.707
35A	12.50504	102.09522		0	2	2	1	1	2	0.22	0.092	0.438
36A	12.49215	102.05064		0	1	1	2	1	1	0.134	0.026	0.472
37A	12.49212	102.13584		0	1	3	2	1	2	0.315	0.119	0.608
38A	12.48361	102.08192		0	2	3	2	1	2	0.182	0.061	0.434
39A	12.48368	102.08168		0	2	3	1	1	2	0.088	0.024	0.278
40A	12.57820	102.03085		0	2	2	2	1	2	0.392	0.168	0.674
42A	12.49156	102.14009		0	2	2	2	1	2	0.413	0.179	0.695
43A	12.45817	102.09943		0	2	1	1	1	1	0.166	0.04	0.484
44A	12.49232	102.14204		0	2	2	1	2	2	0.589	0.288	0.835
45A	12.45101	102.20366		0	1	2	1	1	2	0.388	0.183	0.641
46A	12.46910	102.16593		0	1	2	1	1	1	0.343	0.141	0.626
47A	12.47090	102.17235		0	1	2	1	1	2	0.114	0.035	0.318
48A	12.55554	102.09766		0	1	2	1	1	2	0.094	0.026	0.287
49A	12.50560	102.11694		0	2	2	1	1	2	0.58	0.343	0.785
50A	12.49567	102.14194		0	1	3	2	1	2	0.287	0.107	0.576
51A	12.44344	102.13926		0	1	2	2	1	2	0.206	0.073	0.462
52A	12.43978	102.19319		0	2	2	1	1	2	0.58	0.343	0.785
53A	12.50356	102.08945		0	2	3	1	1	2	0.304	0.087	0.667
54A	12.43945	102.26947		0	2	2	1	1	2	0.259	0.1	0.523

APPENDIX D (cont)

FarmID	lattitude	longitude	WSD	Lime	WaterSource	ConCulture	ProFeed	OwnerW	MeanPredi	LowerCl	UpperCl
55A	12.49411	102.07333		0	1	1	1	1	1	0.291	0.086	0.642
56A	12.49402	102.12621		0	1	2	1	2	2	0.327	0.089	0.707
57A	12.45515	102.27305		0	2	2	1	1	2	0.235	0.102	0.453
58A	12.40076	102.32730		0	1	2	2	1	2	0.592	0.341	0.803
59A	12.53458	102.00534		0	2	1	2	1	2	0.605	0.248	0.877
60A	12.43889	102.14138		0	1	2	1	1	2	0.357	0.168	0.605
61A	12.51102	102.12123		0	2	3	2	1	1	0.452	0.153	0.791
62A	12.49506	102.04443		0	1	1	2	1	2	0.362	0.106	0.731
63A	12.54290	102.10159		0	2	2	2	1	2	0.906	0.757	0.967
64A	12.50360	102.09475		0	2	2	1	1	2	0.22	0.092	0.438
65A	12.49914	102.10979		0	2	2	1	1	2	0.22	0.092	0.438
66A	12.49973	102.09530		0	1	2	1	1	2	0.094	0.026	0.287
67A	12.49877	102.12724		0	2	3	2	1	2	0.169	0.048	0.453
68A	12.42321	102.27400		0	2	2	2	1	1	0.928	0.739	0.983
69A	12.50254	102.10654		0	2	2	1	1	2	0.58	0.343	0.785
1B	12.57005	102.08630		1	2	3	2	1	1	0.506	0.186	0.822
2B	12.44164	102.20318		1	2	2	2	1	1	0.723	0.436	0.899
4B	12.54264	102.04107		1	2	2	1	1	2	0.461	0.232	0.707
5B	12.51673	102.03107		1	2	2	1	2	2	0.951	0.815	0.989
6B	12.39033	102.18733		1	2	2	1	1	2	0.807	0.605	0.92
7B	12.44881	102.26777		1	1	2	1	1	1	0.693	0.385	0.89
9B	12.57803	102.01433		1	2	2	2	1	2	0.906	0.757	0.967
10B	12.39810	102.27259		1	2	2	1	1	2	0.807	0.605	0.92
11B	12.50552	102.05246		1	2	2	2	1	2	0.906	0.757	0.967
12B	12.45130	102.16832		1	2	2	1	1	2	0.58	0.343	0.785
13B	12.57229	102.08443		1	1	2	2	1	1	0.855	0.617	0.956
14B	12.52060	102.10212		1	2	2	1	2	2	0.8	0.528	0.935
15B	12.38095	102.19229		1	1	2	2	1	1	0.935	0.763	0.985
16B	12.45033	102.20492		1	2	2	2	1	1	0.941	0.765	0.987
17B	12.45708	102.10018		1	2	1	2	2	2	0.72	0.299	0.94
18B	12.54929	102.09562		1	1	1	1	1	2	0.075	0.011	0.373
19B	12.57366	102.04243		1	1	2	1	2	2	0.748	0.421	0.924
20B	12.43299	102.15271		1	2	2	1	1	2	0.601	0.389	0.781
21B	12.41567	102.12707		1	2	1	2	1	1	0.691	0.35	0.903
22B	12.50043	102.11300		1	2	2	2	1	2	0.76	0.501	0.909
23B	12.44209	102.11312		1	2	1	2	1	2	0.093	0.012	0.46
24B	12.45026	102.20100		1	2	2	2	2	2	0.948	0.788	0.989
25B	12.37434	102.20693		1	1	2	2	1	2	0.78	0.547	0.913
26B	12.54104	102.10297		1	1	2	2	1	2	0.78	0.547	0.913
27B	12.36871	102.21179		1	2	1	2	1	1	0.861	0.576	0.966
28B	12.53776	102.10074		1	1	2	2	1	2	0.78	0.547	0.913
29B	12.51554	102.03564		1	2	2	2	2	2	0.902	0.69	0.974
30B	12.45668	102.10716		1	1	1	2	1	1	0.452	0.191	0.743
31B	12.49914	102.04284		1	2	1	1	1	2	0.12	0.02	0.482
32B	12.45219	102.15030		1	2	2	1	1	1	0.859	0.615	0.959
33B	12.49202	102.07751		1	2	2	1	1	1	0.586	0.298	0.826
34B	12.51271	102.03465		1	2	2	1	1	2	0.807	0.605	0.92
35B	12.45327	102.15185		1	2	2	2	1	2	0.797	0.539	0.93
36B	12.43185	102.15109		1	1	2	2	1	2	0.561	0.32	0.776
37B	12.43754	102.27985		1	2	2	2	1	1	0.941	0.765	0.987
38B	12.43732	102.27937		1	2	2	2	1	1	0.941	0.765	0.987
39B	12.43088	102.15346		1	2	2	1	1	2	0.601	0.389	0.781
40B	12.57164	102.08471		1	2	3	2	2	2	0.542	0.204	0.845

APPENDIX D (cont)

FarmID	lattitude	longitude	WSD	Lime	WaterSource	ConCulture	ProFeed	OwnerW	MeanPredi	LowerCl	UpperCl
41B	12.53631	102.09770		1	1	2	2	1	1	0.746	0.446	0.914
42B	12.48486	102.08663		1	2	3	2	1	2	0.554	0.262	0.813
43B	12.50548	102.10228		1	1	2	2	1	1	0.826	0.545	0.949
44B	12.52565	102.01689		1	2	2	1	1	2	0.807	0.605	0.92
45B	12.54170	102.04335		1	2	2	1	2	1	0.942	0.742	0.989
46B	12.44542	102.19792		1	2	2	1	2	2	0.866	0.582	0.968
47B	12.41635	102.16683		1	1	2	2	1	1	0.491	0.232	0.755
48B	12.43118	102.13708		1	1	2	2	1	1	0.513	0.254	0.766
49B	12.44037	102.19491		1	2	2	1	1	2	0.58	0.343	0.785
50B	12.53464	102.09362		1	1	2	2	1	1	0.935	0.763	0.985
51B	12.43503	102.15739		1	1	2	1	1	1	0.343	0.141	0.626
52B	12.42999	102.12163		1	2	1	2	1	2	0.385	0.099	0.783
53B	12.50324	102.09267		1	2	2	1	1	2	0.58	0.343	0.785
54B	12.49649	102.10204		1	2	2	2	1	2	0.76	0.501	0.909
55B	12.42834	102.14297		1	2	2	2	1	2	0.775	0.536	0.912
56B	12.42433	102.14385		1	2	2	2	1	1	0.74	0.439	0.912
57B	12.42354	102.14181		1	2	2	2	2	1	0.985	0.886	0.998
58B	12.38061	102.34264		1	2	2	1	1	1	0.874	0.631	0.966
59B	12.48658	102.13091		1	2	2	2	1	2	0.797	0.539	0.93
60B	12.46321	102.13045		1	2	2	1	1	1	0.554	0.294	0.788
61B	12.43968	102.13564		1	2	2	1	2	2	0.889	0.67	0.969
62B	12.41325	102.16274		1	1	2	2	1	1	0.826	0.545	0.949
63B	12.39386	102.18675		1	2	2	1	1	2	0.461	0.232	0.707
64B	12.48861	102.09289		1	1	2	2	1	2	0.592	0.341	0.803
65B	12.50174	102.10702		1	2	2	1	1	2	0.58	0.343	0.785
66B	12.49701	102.10402		1	2	2	1	1	1	0.848	0.591	0.956
67B	12.54823	102.01187		1	2	2	2	1	2	0.906	0.757	0.967
68B	12.43887	102.14008		1	2	2	1	1	1	0.554	0.294	0.788
69B	12.54803	102.09943		1	2	2	2	1	2	0.662	0.388	0.858
70B	12.42268	102.12681		1	2	2	1	2	2	0.876	0.629	0.967
71B	12.50127	102.10495		1	2	2	1	2	2	0.568	0.258	0.833
72B	12.51800	102.11296		1	2	2	2	2	1	0.974	0.853	0.996
73B	12.50502	102.11750		1	2	2	1	1	1	0.533	0.286	0.765
74B	12.53933	101.99230		1	1	1	2	1	1	0.697	0.361	0.903
75B	12.49713	102.04734		1	2	2	1	2	2	0.8	0.528	0.935
76B	12.49918	102.04787		1	1	2	1	1	2	0.24	0.078	0.54
77B	12.49117	102.08414		1	2	2	1	1	2	0.259	0.1	0.523
78B	12.53977	102.10422		1	2	2	1	1	2	0.807	0.605	0.92
79B	12.54087	102.10446		1	2	2	1	1	2	0.807	0.605	0.92
80B	12.44874	102.19656		1	1	2	2	1	2	0.592	0.341	0.803
81B	12.45134	102.20590		1	2	2	1	1	2	0.632	0.388	0.823
82B	12.45259	102.20887		1	2	2	1	1	2	0.632	0.388	0.823
83B	12.45015	102.20763		1	2	2	1	2	2	0.62	0.322	0.849
84B	12.50011	102.12479		1	1	3	1	1	2	0.139	0.026	0.491
85B	12.45736	102.20052		1	2	2	1	1	2	0.601	0.389	0.781
86B	12.45455	102.27067		1	1	2	1	1	2	0.357	0.168	0.605
87B	12.49169	102.14162		1	2	3	2	1	2	0.522	0.255	0.777
88B	12.37073	102.21011		1	2	1	2	1	2	0.605	0.248	0.877

APPENDIX E

Questionnaire used in the present study

1. Thai version

แบบสอบถามเรื่อง ปจจัยเสี่ยงที่มีผลตอการเกิดโรคตัวแดงดวงขาว (White spot disease)

การศึกษานี้มีวัตถุประสงคเพื่อวิเคราะหหาปจจัยเสี่ยงของการเกิดโรคตัวแดงดวงขาวและโรคตายดวน ในสภาพการ เลี้ยงกุงของประเทศไทย แบบสอบถามเปนสวนหนึ่งของวิทยานิพนธของนิสิต บัณฑิตศึกษา หลักสูตรวิทยาศาสตรดุษฎีบัณฑิตสาขาอายุรศาสตรสัตวแพทย คณะสัตวแพทยศาสตร จุฬาลงการณมหาวิทยาลัย โดยขอมูลที่ไดรับจากแบบสอบถามนี้จะถูกปดเปนความลับ และใช ประกอบการศึกษาเทานั้น

ขอขอบคุณในความรวมมืออยางยิ่ง

นายสัตวแพทย ภัทรพล เปยมสมบูรณ

1 ขอมูลเบื้องตน

ชื่อ-นามสกุล เจาของฟารม.....ที่อยู่ .....หมู่.....

ตำบล .....อำเภอ .....พิกัด .....

ประวัติการเกิดโรคในฟาร์มภายใน 3 ปี (โรคที่เกิด/ความถี่/บ่อ/อื่นๆ)

.....

.....

2. ผลทางห้องปฏิบัติการณ์

อาการ

☐ อัตราการตาย.....

☐ ตัวออกสีแดง ☐ มีจุดขาวที่หัว ☐ ว่ายน้ำผิดปกติ.....

อายุ.....วัน ปล่อยความหนาแน่น.....

ผลตรวจ Hepatopancrease (HP) fresh smear

☐ ปกติ

☐ ปลายคอดกิ่ว

☐ ปลายคอดกิ่ว เริ่มพบการฝ่อดำ melanization

☐ ฝ่อดำมาก เสียรูปร่าง

- ผลตรวจปรสิตภายนอก ☐ พบ ระบุ.....

☐ ไม่พบ

- นับจำนวนแบคทีเรียใน HP ☐ Green colony .....CFU/g

☐ Yellow colony.....CFU/g

3. ลักษณะฟาร์มและการจัดการ

พื้นที่ฟารม.....(ไร (โดยมีจำนวนบอเลี้ยง.....(บ่อ)

เจ้าของมีฟาร์มอื่นในการดูแลหรือไม่ ☐ มี ☐ ไม่มี

การดูและฟาร์ม ☐ 1 ☐ 2 ☐ 3

ชนิดของสัตวน้ำที่เลี้ยง ☐ กุ้งขาว ☐ กุ้งขาวรวมกับสัตว์น้ำชนิดอื่น ☐ กุ้งดำ

ระบบการเลี้ยง ☐ วนน้ำใช้ 100 % ☐ วนน้ำใช้บางส่วน ☐ ปล่อยน้ำออกทุกครั้งหลังจับ

แหลงน้ำที่นำมาใชในการเลี้ยงกุง ☐ ทะเล

☐ แมน้ำ หรือ คลอง .....

☐ น้ำบาดาล

มีฟาร์มที่อยู่ติดกันหรือไม่ ☐ มี ☐ ไม่มี

ท่านเลี้ยงกุ้งกี่รอบต่อปี ☐ 1 ☐ 2 ☐ 3 รอบ/ปี (ต่อเนื่องหรือไม่ต่อเนื่อง)

ฟารมมีการจำกัดการเขาออกของบุคคลภายนอกหรือไม ☐ มี ☐ ไม่มี

ฟาร์มมีรั้วรอบขอบชิดหรือไม่ ☐ มี ☐ ไม่มี

กอนเขาฟารมมีการฆ่าเชื้อโรคโดยวิธีเหล่านี้หรือไม่

☐ ที่จุมเทา ☐ สเปรยฆาเชื้อและที่จุมยางรถยนต ☐ ไม่มี

มีสัตวเลี้ยงอยู่ในฟาร์มหรือไม ☐ มี ☐ ไม่มี

แยกคนงานแต่ละบ่อ ☐ มี ☐ ไม่มี

มีการแยกอุปกรณ์แต่ละบ่อเลี้ยง ☐ มี ☐ ไม่มี

- บ่อ

☐ บอพักน้ำ จุดประสงค์ของบ่อพักน้ำ ..... พื้นที่.....

☐ บ่อเก็บเลน ☐ บ่อบำบัดน้ำ

ที่บ่อมีการปู PE หรือไม่ ☐ ปูทั้งบ่อ (1) ☐ ปูขอบบ่อ (2) ☐ ไม่มี (3)

แต่ละบ่อมีเชือกกันนกหรือไม ☐ มี ☐ ไม่มี

แต่ละบ่อมีรั้วกันปูหรือไม่ ☐ มี ☐ ไม่มี (หรือมีแต่ชำรุด)

หญ้ารอบบ่อ ☐ มี (หญ้ายาว) ☐ ไม่มี

มีที่จุมเทาฆาเชื้อกอนเขาไปยังบริเวณบอหรือไม ☐ มี ☐ ไม่มี

มีที่ล้างมือฆาเชื้อกอนเขาไปยังบริเวณบอหรือไม ☐ มี ☐ ไม่มี

- การเตรียมน้ำ

☐ Insecticide (Trichlorfon, Dichlorvos) ☐ Copper ☐ กากชา

☐ Probiotics ☐ Iodine ☐ CL ☐ BKC

มีการกรองขณะสูบน้ำเข้าฟาร์มหรือไม่ ☐ มี ☐ ไม่มี

- การเตรียมบอ

มีการกำจัดตะกอนเลนทุกครั้งในขั้นตอนการเตรียมบ่อ ☐ มี ☐ ไม่มี

มีการตากบอหรือไม ☐ มี .....วัน ☐ ไม่มี

มีการใชปูนขาวสาดพื้นบอกอนใสน้ำหรือไม่ ☐ มี ☐ ไม่มี

การทำสีน้ำ (ตอบได้มากกว่า 1 ข้อ)

☐ มีการใชปุยคอกหรือมูลสัตว ☐ สารเคมี (สีเทียม, ปูนชนิดต่างๆ ฯลฯ) ☐ ใช้จุลินทรีย์

- การใชจุลินทรียหรือสารเสริมอื่นๆ

การใชจุลินทรียรสาดบ่อระหวางการเลี้ยงหรือไม ☐ มี ☐ ไม่มี

จุลินทรีย์ที่ใชขึ้นทะเบียนหรือไม ☐ มี ☐ ไม่มี ความถี่ในการใช.....

การใชสารเสริมอื่นๆระหวางการเลี้ยง ☐ มี ☐ ไม่มี

โปรดระบุ .....

สารที่ใชขึ้นทะเบียนหรือไม ☐ มี ☐ ไม่มี

-ลูกพันธุ์

แหลงของลูกพันธุ์ ☐ ชลบุรี (1) ☐ ฉะเชิงเทรา (2) ☐ ตราด (3) ☐

ภาคใต้ (4) มีการตรวจไวรัสในลูกกุงกอนปลอยหรือไม ☐ มี ☐ ไม่มี

ช้อคิดเห็นอื่นๆ

.....

.....

.....

.....

.....

2. English version

Questionnaire: White spot disease risk factors associated with shrimp farming practices and geographical location in Chanthaburi province, Thailand

The questionnaire is a part of a research project of the Faculty of Veterinary Science, Chulalongkorn University and is used for research purposes only. All information regarding farm owners will remain classified.

Thank you, for your cooperation.

Patharapol Piamsomboon

Part I Laboratory data

1. General information

1.1 Owner's name/address .....House No. .....Village No. .....

Sub-district..... District..... Province.....

Tel:.....

1.2 Farm's coordinates: Latitude.....Longitude.....

2. Disease history

2.1 PCR confirmation of WSD ☐ Yes ☐ No (Skip to article 3)

2.2 Date of WSD occurrence.....

2.4 Cultured species ☐ White pacific shrimp ☐ Black tiger shrimp

2.5 Observed clinical sighs:

- Mortality rate.....
- Reddish to pinkish discoloration ☐ Yes ☐ No
- Presence of white inclusion ☐ Yes ☐ No
- Swimming pattern.....

2.6 Laboratory examination

Hepatopancrease (HP) fresh smear ☐ Normal

☐ Shrinkage at the tips of HP lobes

☐ Presence of melanization

☐ Severe deformity and melanization

- External parasite ☐ Found .....

☐ Not found

- Bacterial culture from HP ☐ Green colony .....CFU/g

☐ Yellow colony.....CFU/g

2.7 Age of affected shrimp..... Days of Stocking

2.8 Stocking density..... PL/ m^2

2.9 Source of PL

☐ Province 1 ☐ Province 2 ☐ Province 3

2.10 Are these PL submitted for virus screening before stocking ☐ Yes ☐ No

Part II Questionnaire survey

3. Farm characteristic

3.1 Farm area..... 3.2 Culture area .....

3.3 Number of pond .....

3.4 Reservoir pond ☐ Yes, water reserver area..... ☐ No

3.5 Sludge pond ☐ Yes, sludge area..... ☐ No

3.6 Water recying in farm (use water from previous crop for the next crop)

☐ 100% recycle

☐ Partial reclycle

☐ Release all water at shrimp harvest

3.7 Do you treat water before release?

☐ Yes, method: .....

☐ No

3.8 Where does the water used for shrimp culture come from?

☐ Sea ☐ Public canal ☐ Underground water

3.9 Personnel who operate the farm

☐ Yourself ☐ Yourself and workers

☐ Appointed manager and workers

3.10 Do you have other farms in your care? ☐ Yes, how many?.....

☐ No

3.11 Other shrimp farms located next to the observed farm ☐ Yes ☐ No

3.12 Is the farm fenced? ☐ Yes ☐ No

3.13 Do you allow non-related personnel to enter a farm freely ☐ Yes ☐ No

3.14 Do you have any pets roaming freely in farm

☐ Yes, what kind of pet..... ☐ No

3.15 Do you apply any disinfection practice for vehicals entering a farm?

☐ Vehical spray ☐ Tire bath ☐ None

3.16 Do you have hand- and foot- disinfection bath for personnel entering a farm?

☐ Yes ☐ No

3.17 Do you separate work and equipment for each pond or cluster of ponds?

☐ Yes ☐ No

3.18 How many crop do you produce per year?.....

4 Pond features

4.1 Are the ponds lined with a polyethylene sheet?

☐ Whole pond ☐ Slope ☐ None

4.2 Pond biosecurity

☐ Bird-proof netting ☐ Crab-proof fencing

☐ Hand- and foot- disinfectant baths ☐ None

5. Pond and water preparation

5.1 Is the sludge (soil at the bottom of the pond) removed after each harvest?

☐ Yes ☐ No

5.2 Do you dry the pond before use?

☐ Yes, how long? ..... ☐ No

5.3 Do you apply lime to the pond bottom?

☐ Yes, concentration..... ☐ No

pleas describe the method.....

.....

5.4 Is the water is filtered through a trawling net before entering culture ponds

☐ Yes, size of mesh..... How many layers?..... ☐ No

5.5 Do you use chicken/pig manure or cow dung to fertilize a pond?

☐ Yes ☐ No

5.6 Do you use inorganic fertilizer to adjust water color?

☐ Yes ☐ No

5.7 Water treatment, which are the following substance(s) that you use?

☐ Insecticide (Trichlorfon, Dichlorvos) ☐ Copper ☐ Tea seed ☐ Chlorine

☐ Iodine

☐ Quaternary ammonium compounds (QACs) ☐ Probiotics (license, non-license)

☐ Other .....

5.8 Please describe how you prepare the water for shrimp culture:

.....

.....

6. Feed management

6.1 Feed

☐ Commercial feed ☐ Live feed .....

6.2 Feed supplementation

☐ Probiotics (license, non-license) Type .....

☐ Immunostimulant.....

☐ Other .....

Please describe how you apply feeding supplementation

.....

6.3 Feeding ratio (%) and frequency (per day)

.....

Other note or comment

.....

.....

End of the questionnaire

APPENDIX F

Univariate logistic regression analysis of farm management risk factors of WSD

Model Information

Dependent Variable	concase[a]
Probability Distribution	Binomial
Link Function	Logit
Subject Effect 1	Farm ID
Working Correlation Matrix Structure	Independent

a. The procedure models 1 as the response, treating 0 as the reference category.

Case Processing Summary

	N	Percent
Included	157	100.0%
Excluded	0	0.0%
Total	157	100.0%

1. Farm size

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	4.438	1	.035
Farmsize	3.526	2	.172

Dependent Variable: concase
Model: (Intercept), Farmsize

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.693	.4330	-.156	1.542	2.562	1	.109	2.000	.856	4.673
[Farmsize=1]	-.716	.4832	-1.663	.231	2.197	1	.138	.489	.190	1.260
[Farmsize=2]	-.159	.5299	-1.198	.880	.090	1	.764	.853	.302	2.410
[Farmsize=3]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

2. Culture area

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.500	1	.114
Culatea123	5.786	2	.055

Dependent Variable: concase
Model: (Intercept), Culatea123

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.898	.3577	.197	1.599	6.302	1	.012	2.455	1.218	4.948
[Culatea123=1.00]	-1.156	.4819	-2.100	-.211	5.752	1	.016	.315	.122	.810
[Culatea123=2.00]	-.697	.4224	-1.525	.131	2.724	1	.099	.498	.218	1.140
[Culatea123=3.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

3. Sludge pond area

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.648	1	.104
Q_Sludarea	7.549	3	.056

Dependent Variable: concase
Model: (Intercept), Q_Sludarea

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.452	.3419	-.218	1.122	1.748	1	.186	1.571	.804	3.071
[Q_Sludarea=1.00]	-.064	.4530	-.952	.824	.020	1	.887	.938	.386	2.279
[Q_Sludarea=2.00]	-.937	.4667	-1.852	-.023	4.034	1	.045	.392	.157	.978
[Q_Sludarea=3.00]	.290	.5143	-.718	1.298	.318	1	.573	1.336	.488	3.662
[Q_Sludarea=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

4. Number of ponds

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	3.839	1	.050
Q_pond2	1.672	1	.196

Dependent Variable: concase
Model: (Intercept), Q_pond2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.693	.3873	-.066	1.452	3.203	1	.074	2.000	.936	4.273
[Q_pond2=1.00]	-.551	.4262	-1.387	.284	1.672	1	.196	.576	.250	1.329
[Q_pond2=2.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

5. Culture area/ water reservoir area ratio

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	.222	1	.638
CulREs	4.521	1	.033

Dependent Variable: concase
Model: (Intercept), CulREs

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.083	.1754	-.261	.426	.222	1	.638	1.086	.770	1.532
CulREs	.037	.0172	.003	.070	4.521	1	.033	1.037	1.003	1.073
(Scale)	1									

6. Caretaker

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	3.558	1	.059
Caretaker	4.445	2	.108

Dependent Variable: concase
Model: (Intercept), Caretaker

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.898	.3577	.197	1.599	6.302	1	.012	2.455	1.218	4.948
[Caretaker=1]	-.848	.4219	-1.675	-.021	4.040	1	.044	.428	.187	.979
[Caretaker=2]	-.847	.4802	-1.788	.094	3.109	1	.078	.429	.167	1.099
[Caretaker=3]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

7. Water sources

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	.803	1	.370
WaterSo	7.757	2	.021

Dependent Variable: concase
Model: (Intercept), WaterSo

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.875	.5323	-1.919	.168	2.705	1	.100	.417	.147	1.183
[WaterSo=1]	.613	.6784	-.717	1.943	.817	1	.366	1.846	.488	6.978
[WaterSo=2]	1.382	.5655	.273	2.490	5.971	1	.015	3.982	1.314	12.062
[WaterSo=3]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

8. Owner of multiple farms

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	4.291	1	.038
OwnerMul	3.121	1	.077

Dependent Variable: concase
Model: (Intercept), OwnerMul

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.055	.1916	-.321	.431	.083	1	.774	1.057	.726	1.538
[OwnerMul=1]	.638	.3612	-.070	1.346	3.121	1	.077	1.893	.933	3.842
[OwnerMul=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

9. Water recycling

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	.394	1	.530
Culsys2	1.466	1	.226

Dependent Variable: concase
Model: (Intercept), Culsys2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.351	.1846	-.011	.713	3.608	1	.058	1.420	.989	2.039
[Culsys2=1]	-.462	.3815	-1.210	.286	1.466	1	.226	.630	.298	1.331
[Culsys2=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

10. Presence of adjacent farm(s)

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	.000	1	.995
Adjfarm	1.668	1	.197

Dependent Variable: concase
Model: (Intercept), Adjfarm

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.318	.4647	-1.229	.592	.470	1	.493	.727	.293	1.808
[Adjfarm=1]	.640	.4956	-.331	1.611	1.668	1	.197	1.897	.718	5.010
[Adjfarm=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

11. Limited accessed

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.130	1	.144
LimAcc	.001	1	.981

Dependent Variable: concase
Model: (Intercept), LimAcc

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.246	.2036	-.153	.645	1.462	1	.227	1.279	.858	1.906
[LimAcc=1]	-.008	.3320	-.658	.643	.001	1	.981	.992	.518	1.902
[LimAcc=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

12. Fence

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	3.388	1	.066
Fence	1.214	1	.271

Dependent Variable: concase
Model: (Intercept), Fence

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.138	.1861	-.227	.503	.551	1	.458	1.148	.797	1.654
[Fence=1]	1	.412	.3739	-.321	1.145	1.214	1	.271	1.510	.726	3.141
[Fence=2]	2	0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

13. Pet

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.536	1	.111
Pet	.567	1	.452

Dependent Variable: concase
Model: (Intercept), Pet

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.137	.2137	-.282	.555	.408	1	.523	1.146	.754	1.743
[Pet=1]	1	.245	.3252	-.393	.882	.567	1	.452	1.277	.675	2.416
[Pet=2]	2	0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

14. Vehicle disinfectant

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.198	1	.138
Farmdis	.820	1	.365

Dependent Variable: concase
Model: (Intercept), Farmdis

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.205	.1658	-.120	.530	1.525	1	.217	1.227	.887	1.699
[Farmdis=1]	1	.643	.7097	-.749	2.034	.820	1	.365	1.901	.473	7.641
[Farmdis=2]	2	0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

15. Separate workers

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	1.776	1	.183
Sepwork	.003	1	.958

Dependent Variable: concase
Model: (Intercept), Sepwork

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.238	.1854	-.125	.602	1.653	1	.199	1.269	.882	1.825
[Sepwork=1]	.019	.3724	-.710	.749	.003	1	.958	1.020	.491	2.115
[Sepwork=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

16. Continuous culture

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.807	1	.094
ConCu	2.807	1	.094

Dependent Variable: concase
Model: (Intercept), ConCu

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	2.667E-17	.2157	-.423	.423	.000	1	1.000	1.000	.655	1.526
[ConCu=0]	.549	.3274	-.093	1.190	2.807	1	.094	1.731	.911	3.288
[ConCu=1]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

17. Sludge removal

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.373	1	.123
Sluremov	.398	1	.528

Dependent Variable: concase
Model: (Intercept), Sluremov

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.350	.2345	-.109	.810	2.230	1	.135	1.419	.896	2.247
[Sluremov=1]	-.204	.3225	-.836	.429	.398	1	.528	.816	.434	1.535
[Sluremov=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

18. Lime application

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	.572	1	.449
Lime	6.925	1	.009

Dependent Variable: concase
Model: (Intercept), Lime

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.575	.2083	.167	.984	7.627	1	.006	1.778	1.182	2.674
[Lime=1]	-.894	.3397	-1.560	-.228	6.925	1	.009	.409	.210	.796
[Lime=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

19. Water filter

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.664	1	.103
Filter	.400	1	.527

Dependent Variable: concase
Model: (Intercept), Filter

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.389	.2826	-.164	.943	1.899	1	.168	1.476	.848	2.569
[Filter=1]	1	-.218	.3439	-.892	.456	.400	1	.527	.804	.410	1.578
[Filter=2]	2	0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

20. Animal waste

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.138	1	.144
Aniwaste	.010	1	.921

Dependent Variable: concase
Model: (Intercept), Aniwaste

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.232	.1974	-.155	.619	1.378	1	.240	1.261	.856	1.857
[Aniwaste=1]		.034	.3403	-.633	.701	.010	1	.921	1.034	.531	2.015
[Aniwaste=2]		0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

21. Inorganic fertilizer

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.255	1	.133
Inorgani	.027	1	.870

Dependent Variable: concase
Model: (Intercept), Inorgani

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.278	.2651	-.242	.797	1.096	1	.295	1.320	.785	2.220
[Inorgani=1]	-.054	.3335	-.708	.599	.027	1	.870	.947	.493	1.821
[Inorgani=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

22. Insecticide

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	1.505	1	.220
Insectic	.072	1	.788

Dependent Variable: concase
Model: (Intercept), Insectic

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.167	.2897	-.401	.735	.333	1	.564	1.182	.670	2.085
[Insectic=1]	.094	.3487	-.590	.777	.072	1	.788	1.098	.554	2.175
[Insectic=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

23. Copper

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	1.975	1	.160
Copper	.355	1	.552

Dependent Variable: concase
Model: (Intercept), Copper

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.343	.2788	-.203	.889	1.513	1	.219	1.409	.816	2.433
[Copper=1]	-.204	.3428	-.876	.468	.355	1	.552	.815	.416	1.596
[Copper=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

24. Tea seed

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	4.709	1	.030
Saponin	3.172	1	.075

Dependent Variable: concase
Model: (Intercept), Saponin

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	1.179	.5718	.058	2.299	4.249	1	.039	3.250	1.060	9.967
[Saponin=1]	-1.063	.5967	-2.232	.107	3.172	1	.075	.346	.107	1.113
[Saponin=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

25. PE-lined pond

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.323	1	.127
PE2	.627	1	.429

Dependent Variable: concase
Model: (Intercept), PE2

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.121	.2199	-.310	.552	.301	1	.583	1.128	.733	1.736
[PE2=1]	.261	.3294	-.385	.906	.627	1	.429	1.298	.681	2.475
[PE2=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

26. Bird-proof netting

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	1.380	1	.240
Birdscar	.158	1	.691

Dependent Variable: concase
Model: (Intercept), Birdscar

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.283	.1892	-.088	.653	2.231	1	.135	1.327	.916	1.922
[Birdscar= 1]	-.143	.3595	-.847	.562	.158	1	.691	.867	.428	1.754
[Birdscar= 2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

27. Crab-proof fencing

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.084	1	.149
Crabfenc	.105	1	.746

Dependent Variable: concase
Model: (Intercept), Crabfenc

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.288	.2118	-.127	.703	1.844	1	.174	1.333	.880	2.020
[Crabfenc= 1]	-.105	.3256	-.743	.533	.105	1	.746	.900	.475	1.704
[Crabfenc= 2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

28. Hand- and foot- baths

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	4.512	1	.034
FGbath	2.437	1	.118

Dependent Variable: concase
Model: (Intercept), FGbath

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.136	.1738	-.205	.476	.608	1	.436	1.145	.815	1.610
[FGbath= 1]	.752	.4816	-.192	1.696	2.437	1	.118	2.121	.825	5.450
[FGbath= 2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

29. Vitamin C

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	1.601	1	.206
Vituse	.096	1	.757

Dependent Variable: concase
Model: (Intercept), Vituse

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.223	.1732	-.116	.563	1.660	1	.198	1.250	.890	1.755
[Vituse= 1]	.145	.4669	-.771	1.060	.096	1	.757	1.156	.463	2.886
[Vituse= 2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

30. Probiotics mix in feed

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	5.619	1	.018
Probio	3.742	1	.053

Dependent Variable: concase
Model: (Intercept), Probio

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	1.163	.5123	.159	2.167	5.154	1	.023	3.200	1.172	8.735
[Probio=1]	-1.045	.5404	-2.104	.014	3.742	1	.053	.352	.122	1.014
[Probio=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

31. Source of shrimp PL

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	4.499	1	.034
SoPL2	5.071	2	.079

Dependent Variable: concase
Model: (Intercept), SoPL2

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.767	.3356	.109	1.425	5.226	1	.022	2.154	1.116	4.158
[SoPL2=]	1	-.892	.4188	-1.713	-.072	4.541	1	.033	.410	.180	.931
[SoPL2=]	2	-.305	.4566	-1.200	.590	.445	1	.505	.737	.301	1.805
[SoPL2=]	3	0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

32. Virus detection of PL

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.292	1	.130
FryAna	.011	1	.916

Dependent Variable: concase
Model: (Intercept), FryAna

Parameter Estimates

Parameter		B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
				Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)		.262	.2428	-.214	.738	1.167	1	.280	1.300	.808	2.092
[FryAna=]	1	-.034	.3241	-.669	.601	.011	1	.916	.966	.512	1.824
[FryAna=]	2	0[a]	.	.	.	.	.	.	1	.	.
(Scale)		1									

33. Stocking density

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	5.968	1	.015
Q_Stock	1.792	2	.408

Dependent Variable: concase
Model: (Intercept), Q_Stock

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.636	.4122	-.172	1.444	2.380	1	.123	1.889	.842	4.237
[Q_Stock=1.00]	-.464	.4770	-1.399	.471	.947	1	.331	.629	.247	1.601
[Q_Stock=2.00]	.057	.5656	-1.051	1.166	.010	1	.920	1.059	.349	3.208
[Q_Stock=3.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

34. Distance to coastline

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.169	1	.141
Q_Coast	8.659	3	.034

Dependent Variable: concase
Model: (Intercept), Q_Coast

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.470	.3291	-1.115	.175	2.039	1	.153	.625	.328	1.191
[Q_Coast=1.00]	.624	.4599	-.277	1.526	1.842	1	.175	1.867	.758	4.598
[Q_Coast=2.00]	1.404	.4847	.454	2.354	8.393	1	.004	4.073	1.575	10.531
[Q_Coast=3.00]	.833	.4629	-.074	1.740	3.237	1	.072	2.300	.928	5.699
[Q_Coast=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

35. Distance to nearest national highway

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.311	1	.128
Q_Road	8.023	3	.046

Dependent Variable: concase
Model: (Intercept), Q_Road

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	1.065	.3667	.346	1.783	8.429	1	.004	2.900	1.413	5.950
[Q_Road=1.00]	-1.219	.4875	-2.174	-.263	6.251	1	.012	.296	.114	.768
[Q_Road=2.00]	-.807	.4886	-1.765	.151	2.727	1	.099	.446	.171	1.163
[Q_Road=3.00]	-1.219	.4875	-2.174	-.263	6.251	1	.012	.296	.114	.768
[Q_Road=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

36. Distance to nearest public canal

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.171	1	.141
Q_STRE_M	4.349	3	.226

Dependent Variable: concase
Model: (Intercept), Q_STRE_M

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.258	.3229	-.375	.891	.637	1	.425	1.294	.687	2.437
[Q_STRE_M=1.00]	-.309	.4549	-1.201	.582	.462	1	.497	.734	.301	1.790
[Q_STRE_M=2.00]	.553	.4740	-.376	1.482	1.362	1	.243	1.739	.687	4.402
[Q_STRE_M=3.00]	-.309	.4549	-1.201	.582	.462	1	.497	.734	.301	1.790
[Q_STRE_M=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

37. Distance to nearest mangrove forest

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
(Intercept)	2.416	1	.120
Qman	5.294	3	.152

Dependent Variable: CoCa
Model: (Intercept), Qman

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test	Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.539	.3363	-.120	1.198	2.569	1	.109	1.714	.887	3.314
[Qman=1]	-.488	.4645	-1.398	.423	1.103	1	.294	.614	.247	1.526
[Qman=2]	.154	.4780	-.783	1.091	.104	1	.747	1.167	.457	2.977
[Qman=3]	-.797	.4662	-1.711	.117	2.921	1	.087	.451	.181	1.124
[Qman=4]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

APPENDIX G

Univariate logistic regression analysis of farm management risk factors of WSD

Case Processing Summary

	N	Percent
Included	156	99.4%
Excluded	1	0.6%
Total	157	100.0%

Correlated Data Summary

Number of Levels	Subject Effect Farm ID	156
Number of Subjects		156
Number of Measurements per Subject	Minimum	1
	Maximum	1
Correlation Matrix Dimension		1

Categorical Variable Information

			N	Percent
Dependent Variable	concase	0	69	44.2%
		1	87	55.8%
		Total	156	100.0%
Factor	WaterSo	Ocean	23	14.7%
		River or Canel	116	74.4%
		Underground	17	10.9%
		Total	156	100.0%
	Lime for pond bottoom	Yes	57	36.5%
		No	99	63.5%
		Total	156	100.0%
	Probiotic used during culture	Yes	135	86.5%
		No	21	13.5%
		Total	156	100.0%
	OwnerMul	Yes	47	30.1%
		No	109	69.9%
		Total	156	100.0%
	ConCu	0	71	45.5%
		1	85	54.5%
		Total	156	100.0%
	Q_Road	1.00	39	25.0%
		2.00	39	25.0%
		3.00	39	25.0%
		4.00	39	25.0%
		Total	156	100.0%

Goodness of Fit[a]

	Value
Quasi Likelihood under Independence Model Criterion (QIC)[b]	200.971
Corrected Quasi Likelihood under Independence Model Criterion (QICC)[b]	201.047

Dependent Variable: concase
Model: (Intercept), WaterSo, Lime, Probio, OwnerMul, ConCu, Q_Road[a]

a. Information criteria are in smaller-is-better form.

b. Computed using the full log quasi-likelihood function.

Tests of Model Effects

	Type III		
Source	Wald Chi-Square	df	Sig.
(Intercept)	.695	1	.405
WaterSo	5.620	2	.060
Lime	5.455	1	.020
Probio	3.922	1	.048
OwnerMul	4.460	1	.035
ConCu	4.102	1	.043
Q_Road	5.729	3	.126

Dependent Variable: concase
Model: (Intercept), WaterSo, Lime, Probio, OwnerMul, ConCu, Q_Road

Parameter Estimates

			95% Wald Confidence Interval		Hypothesis Test		Hypothesis Test		95% Wald Confidence Interval for Exp(B)	
Parameter	B	Std. Error	Lower	Upper	Wald Chi-Square	df	Sig.	Exp(B)	Lower	Upper
(Intercept)	.804	.9321	-1.023	2.631	.744	1	.388	2.234	.360	13.885
[WaterSo=1]	.103	.8054	-1.475	1.682	.017	1	.898	1.109	.229	5.376
[WaterSo=2]	1.169	.6617	-.128	2.466	3.123	1	.077	3.220	.880	11.779
[WaterSo=3]	0[a]	.	.	.	.	.	.	1	.	.
[Lime=1]	-.932	.3992	-1.715	-.150	5.455	1	.020	.394	.180	.861
[Lime=2]	0[a]	.	.	.	.	.	.	1	.	.
[Probio=1]	-1.100	.5554	-2.188	-.011	3.922	1	.048	.333	.112	.989
[Probio=2]	0[a]	.	.	.	.	.	.	1	.	.
[OwnerMul=1]	.912	.4319	.066	1.759	4.460	1	.035	2.490	1.068	5.805
[OwnerMul=2]	0[a]	.	.	.	.	.	.	1	.	.
[ConCu=0]	.820	.4047	.026	1.613	4.102	1	.043	2.270	1.027	5.017
[ConCu=1]	0[a]	.	.	.	.	.	.	1	.	.
[Q_Road=1.00]	-1.067	.5491	-2.143	.010	3.772	1	.052	.344	.117	1.010
[Q_Road=2.00]	-.813	.5320	-1.855	.230	2.334	1	.127	.444	.156	1.259
[Q_Road=3.00]	-1.194	.5363	-2.246	-.143	4.959	1	.026	.303	.106	.867
[Q_Road=4.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1									

APPENDIX H

WSSV isolates used for molecular typing

- WSSV isolates and the results of PCR targeting the 5 variable loci

isolate	Province	Year	Indel-I	Indel-II	ORF75	ORF125	ORF94	rema
Ch1/07	Chanthaburi	2007	650	1500	5(3,2)	11	3	Pond
Ch2/07	Chanthaburi	2007	NA	NA	NA	NA	NA	Pond
Ch3/07	Chanthaburi	2007	650	2000	5(3,2)	7	5	Pond
Ch4/07	Chanthaburi	2007	650		5(3,2)	6	6	Pond
Ch5/07	Chanthaburi	2007	650	1500	5(3,2)	9	3	Pond
Ch1/08	Chanthaburi	2008	NA	NA	NA	NA	NA	Pond
Ch2/08	Chanthaburi	2008	650	1500	NA	NA	NA	Pond
Ch3/08	Chanthaburi	2008	650	2000	NA	NA	NA	Pond
Ch4/08	Chanthaburi	2008	650	2000	NA	NA	NA	Pond
Ch1/09	Chanthaburi	2009	650	1500	5(3,2)	5	10	Pond
Ch2/09	Chanthaburi	2009	650	2000	5(3,2)	8	6	Pond
Ch3/09	Chanthaburi	2009	650	2000	5(3,2)	7	14	Pond
Ch4/09	Chanthaburi	2009	650	NA	NA	NA	NA	Pond
Ch1/11	Chanthaburi	2011	650	1500	0	5	14	Pond
Ch2/11	Chanthaburi	2011	650	1500	5(3,2)	4	8	Pond
Ch3/11	Chanthaburi	2011	650	NA	NA	NA	NA	Pond
Ch4/11	Chanthaburi	2011	650	NA	NA	NA	NA	Pond
Ch5/11	Chanthaburi	2011	650	2000	5(3,2)	9	6	Pond
Ch6/11	Chanthaburi	2011	650	NA	5(3,2)	4	11	Pond
Ch7/11	Chanthaburi	2011	650	2000	5(3,2)	6	7	Pond
Ch8/11	Chanthaburi	2011	650	2000	5(3,2)	8	7	Pond
Ch9/11	Chanthaburi	2011	650	2000	NA	NA	NA	Pond
Ch10/11	Chanthaburi	2011	650	NA	NA	NA	NA	Pond
Ch11/11	Chanthaburi	2011	650	NA	NA	NA	NA	Pond
Ch12/11	Chanthaburi	2011	650	1500	NA	NA	NA	Pond
Ch13/11	Chanthaburi	2011	650	NA	NA	NA	NA	Pond
Ch14/11	Chanthaburi	2011	650	NA	NA	NA	NA	Pond
Ch15/11	Chanthaburi	2011	NA	NA	NA	NA	NA	Pond
Ch1/12	Chanthaburi	2012	650	2000	NA	NA	NA	Pond
Ch2/12	Chanthaburi	2012	650	NA	NA	NA	NA	Pond
Ch3/12	Chanthaburi	2012	650	NA	NA	NA	NA	Pond
Ch4/12	Chanthaburi	2012	650	NA	NA	NA	NA	Pond
Ch5/12	Chanthaburi	2012	650	NA	NA	NA	NA	Pond
Ch6/12	Chanthaburi	2012	650	NA	NA	NA	NA	Pond
Ch7/12	Chanthaburi	2012	650	NA	NA	NA	NA	Pond
Ch8/12	Chanthaburi	2012	650	2000	0	5	4	Pond
Ch9/12	Chanthaburi	2012	650		5(3,2)	6	8	Pond
Ch10/12	Chanthaburi	2012	650		5(3,2)	6	8	Pond
Ch11/12	Chanthaburi	2012	650		5(3,2)	4	6	Pond
Ch12/12	Chanthaburi	2012	650		5(3,2)	3	8	Pond
Ch1/13	Chanthaburi	2013	650	2000	5(3,2)	7	4	Pond
Ch2/13	Chanthaburi	2013	650	NA	NA	NA	NA	Pond
Ch3/13	Chanthaburi	2013	650	NA	NA	NA	NA	Pond
Ch4/13	Chanthaburi	2013	650	NA	NA	NA	NA	Pond
Ch5/13	Chanthaburi	2013	650	NA	NA	NA	NA	Pond
Ch1/14	Chanthaburi	2014	650	2000	5(3,2)	8	7	Pond

APPENDIX H (cont)

isolate	Province	Year	Indel-I	Indel-II	ORF75	ORF125	ORF94	remark
Ch2/14	Chanthaburi	2013	650	2000	5(3,2)	7	5	Pond
Ch3/14	Chanthaburi	2013	650	NA	NA	NA	NA	Pond
Ch4/14	Chanthaburi	2013	650	NA	NA	NA	NA	Pond
R1/12	Rayong	2012	650		0	6	10	Pond
R2/12	Rayong	2012	650	NA	5(3,2)	4	8	Pond
R3/12	Rayong	2012	650	2000	11(8,3)	5	4	Pond
R4/12	Rayong	2012	650	2000	11(8,3)	5	4	Pond
R5/12	Rayong	2012	650	1500	0	5	8	Pond
R6/12	Rayong	2012	650	2000	3(1,2)	4	4	Pond
R7/12	Rayong	2012	650	2000	0	5	4	Pond
R8/12	Rayong	2012	650	2000	0	5	4	Pond
R9/12	Rayong	2012	650	1500	0	5	8	Pond
R10/12	Rayong	2012	650	2000	0	5	4	Pond
R11/12	Rayong	2012	650	2000	11(8,3)	5	4	Pond
R12/12	Rayong	2012	650	NA	NA	NA	NA	Pond
R13/12	Rayong	2012	650	1500	0	5	8	Pond
R14/12	Rayong	2012	650	2000	0	5	8	Pond
R15/12	Rayong	2012	650	2000	0	5	4	Pond
R16/12	Rayong	2012	650	1500	5(3,2)	5	10	Pond
R17/12	Rayong	2012	650	2000	0	5	12	Pond
R18/12	Rayong	2012	650	1500	5(3,2)	5	10	Pond
T1/12	Trat	2012	650	2000	5(3,2)	7	6	Pond
T2/12	Trat	2012	650	1500	0	5	5	Pond
T3/12	Trat	2012	NA	NA	NA	NA	NA	Pond
T4/12	Trat	2012	NA	NA	NA	NA	NA	Pond
T5/12	Trat	2012	NA	NA	NA	NA	NA	Pond
T6/12	Trat	2012	650	2000	5(3,2)	7	8	Pond
T7/12	Trat	2012	NA	NA	NA	NA	NA	Pond
Su1/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su2/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su3/09	Surat Thani	2009	650		3(1,2)	9	11	Plant
Su4/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su5/09	Surat Thani	2009	650		3(1,2)	9	11	Plant
Su6/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su7/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su8/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su9/09	Surat Thani	2009	650	NA	NA	NA	NA	Plant
Su1/10	Surat Thani	2010	650	NA	NA	NA	NA	Plant
Su2/10	Surat Thani	2010	NA	NA	NA	NA	NA	Plant
Sk1/09	Songkhla	2009	650	NA	NA	NA	NA	Pond
Sk2/09	Songkhla	2009	650	2000	0	5	4	Pond
Sk3/09	Songkhla	2009	NA	NA	22(18,4)	6	7	Pond
Sk4/09	Songkhla	2009	650	NA	3(3,0)	9	11	Pond
Sk5/09	Songkhla	2009	650	NA	0	5	5	Pond
Sk1/12	Songkhla	2012	NA	NA	22(18,4)	6	7	Pond
Sk2/12	Songkhla	2012	650	NA	22(18,4)	6	7	Pond

APPENDIX H (cont)

isolate	Province	Year	Indel-I	Indel-II	ORF75	ORF125	ORF94	remark
Sk3/12	Songkhla	2012	650	NA	22(18,4)	6	7	Pond
Sk4/12	Songkhla	2012	NA	NA	NA	NA	NA	Pond
Sk5/12	Songkhla	2012	650	NA	22(18,4)	6	7	Pond
Sk6/12	Songkhla	2012	NA	NA	22(18,4)	6	7	Pond
Sk7/12	Songkhla	2012	NA	NA	NA	NA	NA	Pond
Sk8/12	Songkhla	2012	NA	NA	22(18,4)	6	7	Pond
Sk9/12	Songkhla	2012	NA	NA	NA	NA	NA	Pond
Sk10/12	Songkhla	2012	650	NA	5(3,2)	5	8	Pond
Sk11/12	Songkhla	2012	650	NA	22(18,4)	6	7	Pond
Sk12/12	Songkhla	2012	NA	NA	22(18,4)	6	7	Pond
Sk13/12	Songkhla	2012	650	NA	22(18,4)	6	7	Pond
Sk14/12	Songkhla	2012	NA	NA	22(18,4)	6	7	Pond
Sk15/12	Songkhla	2012	NA	NA	22(18,4)	6	7	Pond
Phu1/12	Phuket	2012	650	2000	0	5	5	Pond
Phu2/12	Phuket	2012	650	2000	0	5	5	Pond
Phu3/12	Phuket	2012	650	2000	0	5	5	Pond
Phu4/12	Phuket	2012	650	2000	5(3,2)	6	10	Pond
Phu5/12	Phuket	2012	650	NA	5(3,2)	4	8	Pond
Phu6/12	Phuket	2012	650	NA	5(3,2)	4	8	Pond
Phu7/12	Phuket	2012	650	NA	5(3,2)	4	8	Pond
Phu8/12	Phuket	2012	650	2000	5(3,2)	6	7	Pond
Phu9/12	Phuket	2012	NA	2000	0	5	7	Pond
Phu10/12	Phuket	2012	650	2000	0	5	7	Pond
Phu11/12	Phuket	2012	650	2000	0	5	7	Pond
Phu12/12	Phuket	2012	650	NA	5(3,2)	4	7	Pond
Phu13/12	Phuket	2012	650	2000	5(3,2)	4	7	Pond
K1/12	Krabi	2012	650	2000	0	3	7	Pond
K2/12	Krabi	2012	550	NA	3(1,2)	6	4	Pond
K3/12	Krabi	2012	650	2000	5(3,2)	8	6	Pond
K4/12	Krabi	2012	650	2000	0	5	4	Pond
K5/12	Krabi	2012	650	2000	5(3,2)	3	11	Pond
K6/12	Krabi	2012	550	2000	NA	NA	NA	Pond
K7/12	Krabi	2012	NA	NA	NA	NA	NA	Pond
K8/12	Krabi	2012	NA	NA	NA	NA	NA	Pond
K9/12	Krabi	2012	NA	NA	NA	NA	NA	Pond
K10/12	Krabi	2012	NA	NA	NA	NA	NA	Pond
K11/12	Krabi	2012	650	2000	0	3	7	Pond
Pac1/08	Prachuap Khiri	2008	650	NA	NA	NA	NA	Plant
Pac2/08	Prachuap Khiri	2008	650	NA	NA	NA	NA	Plant
Chu1/08	Chumporn	2008	650	NA	NA	NA	NA	Plant
Chu2/08	Chumporn	2008	650	NA	NA	NA	NA	Plant
Ran1/08	Ranong	2008	650	2000	NA	NA	NA	Plant
Ran2/08	Ranong	2008	650	NA	NA	NA	NA	Plant
Pat1/08	Pattani	2008	650	NA	5(3,2)	4	7	Plant
Pat2/08	Pattani	2008	650	NA	5(3,2)	4	7	Plant

VITA

Mr. Patharapol Piamsomboon was born on May 15, 1987 in Bangkok, Thailand. He graduated Doctor of Veterinary Medicine (Hons.) from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in March 2011. He enrolled as a Ph.D. candidate in the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University in June 2011. Currently, he holds a position as a lecturer in the Faculty of Veterinary Science, Prince of Songkla University, Songkhla Thailand.