On the epidemiology and evolution of white spot syndrome virus of shrimp

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This research was conducted under the auspices of the Graduate School of Production Ecology and Resource Conservation.

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Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof.dr. M.J. Kropff
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 1 June 2010
at 1.30 pm in the Aula

Bui Th.M.D.
Whispovirus epidemiology and evolution
142 pages

Thesis, Wageningen University, Wageningen, NL (2010)
With references, with summaries in Dutch, English and Vietnamese

ISBN 978-90-8570-564-2

Abstract

White Spot Syndrome Virus (WSSV) causes a devastating disease in shrimp aquaculture that has spread worldwide and probably increased in virulence over time. Understanding WSSV epidemiology and evolution is therefore important for developing novel intervention and management strategies. Both of these goals require finding suitable molecular markers to identify and discriminate WSSV strains, and hereby help infer their origin and track their spread. Five major variable WSSV genomic loci were evaluated as markers for virus identification and virus spread on different spatiotemporal scales. In this thesis the genetic variation between WSSV isolates from the key shrimp production regions in Vietnam was analyzed. A statistically supported model of spread suggests that multiple introductions of WSSV occurred in central Vietnam, and that the virus radiated out over time to the south and the north. Spurious variation was generated during molecular cloning of WSSV VNTR sequences, while no variation occurred in multiple replicates of amplification of VNTRs by Polymerase Chain Reaction. Moreover, VNTR sequences were stable over two passages of infection *in vivo*, indicating that *in vivo* cloning can be applied to study heterogeneity within WSSV isolates originating from a single shrimp. Genetic deletion of variable region variants appear to be more stable in extensive farms compare to intensive farms over time, indicating that farm practices affect the evolutionary dynamics of WSSV. Genetic variation between Asian WSSV isolates provides support for evolution of genome size according to a geometric model of adaptation, where incrementally smaller genomic deletions are substituted over time. The relationship between the molecular data and the time of first disease occurrence implies that shrimp transportation played an important role in the quick, long range spread of WSSV. Overall, the thesis results show that WSSV variable loci can be effectively employed as molecular markers to study WSSV spread and evolution on different spatiotemporal scales. However, the markers have different properties and the choice of a suitable marker for a pertinent question is critical.

Abstract

White spot syndrome virus là yếu tố gây bệnh với tác động hủy diệt đối với nghề nuôi tôm, bệnh đã lan truyền khắp thế giới với độc tính ngày càng tăng. Hiểu về dịch tễ học và tiến hóa của WSSV vì vậy rất quan trọng để phát triển các chiến lược phòng ngừa và quản lý bệnh. Mục đích nầy yêu cầu xác định các yếu tố đánh dấu di truyền phân tử để xác định và phân biệt các chủng WSSV, nhờ đó giúp phỏng đoán được nguồn gốc và biết được đường lan truyền bệnh của chúng. Luận án nầy đã đánh giá khả năng thích hợp làm yếu tố đánh dấu di truyền phân tử cho sự lan truyền của virus của năm vị trí biến đổi di truyền chủ yếu trong bộ gen WSSV ở các phạm vi không gian và thời gian khác nhau. Chúng tôi đã phân tích sự biến đổi di truyền ở các vị trí gen nầy giữa các chủng WSSV từ những vùng sản xuất tôm chủ yếu của Việt Nam. Số liệu nghiên cứu với sự hổ trợ của phân tích thống kê đã đề nghị một mô hình lan truyền của virus với nhiều đường xâm nhập đầu tiên vào Miền Trung Việt Nam và lan truyền đến miền Nam và miền Bắc. Các đoạn gen chứa các đơn vị lập lại liên kề thể hiện sự biến đổi di truyền thông qua qui trình tạo dòng phân tử nhưng lại không biến đổi trong quá trình nhiễm qua hai thế hệ liên tiếp của tôm cũng như qua nhiều lần lập lại với kỹ thuật PCR. Kết quả nầy cho thấy sự tạo dòng trên cá thể sống có thể được ứng dụng để nghiên cứu sự đa dạng di truyền trong quần thể của một chủng WSSV có nguồn gốc từ một cá thể tôm. Sự mất đoạn gen của các chủng virus ở các ao theo mô hình nuôi quảng canh thể hiện sự ổn định di truyền qua thời gian so với mô hình nuôi thâm canh. Điều nầy cho thấy mô hình nuôi với cách quản lý khác nhau đã anh hưởng đến động năng tiến hóa của WSSV. Sự biến đổi di truyền giữa các chủng WSSV từ các nước khác nhau ở Châu Á hổ trợ cho giả thiết là sự tiến hóa của kích thước bộ gen của virus tuân theo một mô hình hình học của sự thích nghi với những sự mất đoạn di truyền có kích thước tăng dần theo thời gian. Mối quan hệ giữa dữ liệu phân tử và thời điểm xuất hiện bệnh lần đầu tiên ở các nước cho thấy rằng sự vận chuyển tôm qua giao dịch thương mại đóng vai trò quan trọng trong việc lan truyền rộng rãi và nhanh chóng của WSSV. Tóm lại, kết quả nghiên cứu của luận án cho thấy rằng các đoạn gen biến đổi có thể được ứng dụng hiệu quả như những yếu tố đánh dấu di truyền để nghiên cứu sự lan truyền và tiến hóa của WSSV. Tuy nhiên các đoạn gen nầy với các đặc điểm khác nhau có độ hữu dụng khác nhau theo các phạm vi không gian và thời gian khác nhau. Vì vậy sự lựa chọn yếu tố đánh dấu di truyền thích hợp cho từng phạm vi là rất quan trọng.

Contents

Chapter 1

General Introduction

Shrimp aquaculture

Shrimp is one of the most traded seafood products worldwide, occupying about 14% of the total value of fishery products. Shrimp farming is one of the main forms of global shrimp production with an increase in contribution from 6% in 1970 to 69% in 2004 and has become a 'booming' business (Rosenberry, 2002; FAO 2006). Shrimp farming provides a major source of income for small farmers and plays a potential role in the poverty alleviation in developing countries including that in small coastal villages (Adger, 1998; Lewis *et al*., 2003). Moreover, shrimp aquaculture has been and still is significantly contributing to national economies in South-East Asia and Central and South America (Walker & Mohan, 2009).

Shrimp production has been sustainable for centuries in many countries. However, it has quickly become an important export sector for foreign exchange earnings and this success has encouraged many countries to enter into export-oriented industrial shrimp farming. The local farmers cannot fulfill the high standards and safety requirement of the production, pricing, marketing and processing chain. This practice then with its negative social and environmental impact has been proven to be unsustainable (Bardhan, 2006; FAO, 2006). Nearly all high quality shrimp was exported and the high prices paid by processing factories have made shrimp unaffordable for local consumers (Abila, 2003). Besides social problems, destruction of coastal areas (mangroves) and pollution of surface water and land, shrimp farming has also led to a conflict or competing claims with rice farmers (Whitmarsh & Palmieri, 2008). The requirements for large areas of land in extensive and semi-intensive shrimp farming practices have led to significant natural habitat loss through conversion of wetlands into shrimp ponds. In industrial shrimp farms with poor environmental management, shrimp feed and excrement, sometimes combined with antibiotics and fertilizer, are often discharged without any treatment directly into nearby waterways and onto surrounding lands. In addition, resulting plankton (a natural food source for shrimp) explosions deplete the oxygen for shrimp and other sea life (Lebel *et al*., 2002). Although there are many other reasons as well, industrial shrimp farming is considered to be the most important cause of coastal mangrove destruction. Major shrimp crop failures have occurred and, in many cases, ponds were abandoned. Therefore, the economic growth due to high profitability and foreign exchange from shrimp exports does not simply translate into a reduction of poverty or improving food security for the country and the local population (Pradhan & Flaherty,

2008). It is also clear that two of the top shrimp producing countries in the world (Bangladesh, Myanmar) still belong to the High Indebted Poor Countries group (CIA, 2007) and three of these (Vietnam, Myanmar, Honduras) belong to the Least Developed Countries list (Rivera-Ferre *et al*., 2009). Many new strategies have been suggested and explored in efforts to achieve sustainable shrimp production (Consortium Program, 2006).

In order to fulfill export-oriented industrial shrimp farming, farmers have been developing diversified approaches, including rice-shrimp and mangrove-shrimp farming, with various levels of shrimp production intensity such as extensive, improved extensive and intensive systems. Coastal rice fields have been converted into rice-shrimp farms and the mangrove forests have also been destroyed to make shrimp ponds. Extensive shrimp farming is a farming practice that typically requires very large ponds – up to hundreds of hectares – with low-stocking density (2-3 shrimps/m^2). Spring tides are used to seed and harvest the ponds and the shrimp feed on naturally-occurring food (i.e. plankton consisting of zooplankton with copepods, jellyfish, crustaceans, larvae of fish, starfish and crab, and microscopic plants) produced by the mangrove forest. Semi-extensive shrimp farming is an improvement of the extensive system, requiring (i) the stocking of ponds with post larvae (PL) to obtain higher stocking densities (10-15 shrimps/m^2), and (ii) the limited use of shrimp feed. Modern intensive farming uses dry feeds, antibiotics, and pumping aerator systems with high stocking densities of shrimp (from 20-30 PL/m^2 to 50-60 PL/m^2). The pond water must be repeatedly or continually treated to remove or discharge the resulting pollution. The semi-extensive farming system requires considerably less input than the intensive farming with lower shrimp stocking densities (Lebel *et al.*, 2002). Rice-shrimp farming is an attractive rotation system in the seawater intrusion zone, where the land is less suitable for conventional rice farming. During the dry season, brackish water is used to flood the rice fields for shrimp culture. In the beginning of the wet season, remaining salts are flushed from farmers' fields by rain and inundation with fresh river water, allowing for the planting of rice. One rice and two shrimp crops are typically grown every year. Some farmers nurse shrimp fry in a small pond next to the field releasing the nursed shrimp into the field after sowing rice. Shrimp consume naturally occurring food from the field (Vuong, 2001). Many *Penaeus* species are cultured on shrimp farms. The giant black tiger shrimp (*P. monodon*) was previously the leading shrimp species in terms of production compared to *Penaeus vannamei* (also known as *Litopenaeus vannamei*), *Penaeus chinensis* and *Litopenaeus stylirostris* (Figure 1). However, at present *P. vannamei* is the most produced shrimp species in the world (FAO, 2006), primarily because *P. vannamei* replaced *P. monodon* in South-East Asia.

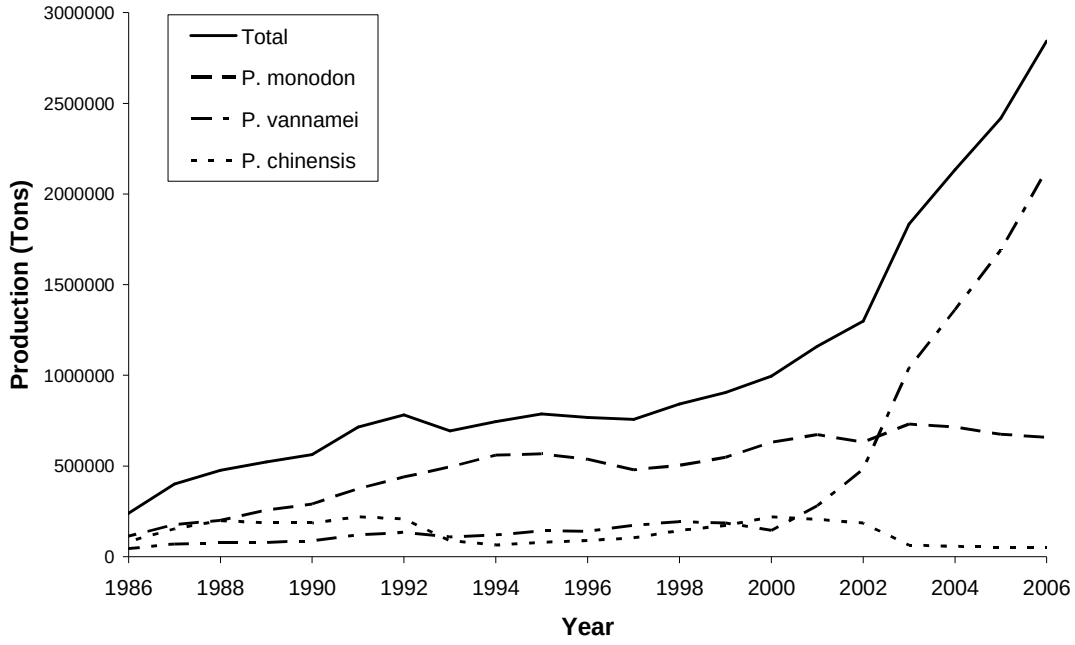


Figure 1. World production of cultured shrimp species (1986–2006)

Shrimp aquaculture in Vietnam

Vietnam has great potential for shrimp aquaculture given its natural features: a 3,260 km long coastline, a large number of bays, lagoons, straits and numerous river systems. Shrimp farming has rapidly developed in Vietnam, with unplanned farms expanding due to high profitability and supportive government policies. Shrimp production increased dramatically since the early 1990s and especially after the year 2000 because of the impacts of Resolution 09/2000/NQ-CP (http://vbqppl.moj.gov.vn/law/en/1991_to_2000/2000/200009/200009060002_en).

Over time Vietnam became one of the top five shrimp producing countries in the world, having the largest area for shrimp culture (Nhuong *et al.*, 2006). Shrimp aquaculture also became an important economic sector, earning significant foreign currency for Vietnam. In 2003 shrimp contributed 52% of the national fisheries export value (World Bank, 2004). The export value of shrimp increased 33-fold from 1999 to 2006, corresponding to 3.86% of GDP and 354,600 tons of cultured shrimp produced in 2006 (Dan, 2007). However, shrimp farming in Vietnam is based mainly on individual households: a small production scale that is often associated with poor pond management practices. High intensity of production with poor management has led to negative environmental impacts with mangrove degradation, ecological imbalance, pollution and, consequently, disease outbreaks (FAO, 2005). Most of the shrimp culture area in Vietnam is concentrated in the Mekong delta, responsible for 82% of the shrimp production, followed by 15% produced in the central coastal regions, and 3% in the northern provinces (Dan, 2007). The traditional extensive farming practice

has been reduced and replaced by the improved extensive system. Nowadays, semi-extensive farming has become the dominant aquaculture cultivation method in Vietnam (MOFI, 2004). The rice-farming rotation model is mostly concentrated in the Mekong delta. Whilst two crops are often harvested every year in the south of Vietnam, only one crop can be obtained in northern Vietnam.

Over the last ten years the technology for shrimp culture has improved significantly in Vietnam. The number of shrimp hatcheries has increased 2.5 fold and production levels reached 26 billion PL15-20 by 2004 (MOFI, 2005). Those hatcheries are mostly located in the central coastal area of Vietnam, although hatcheries are nowadays being set up in southern areas. These hatcheries supply shrimp seed for almost the whole country; even the northern provinces mainly use imported seed from the central provinces, although they also used seed from southern China (MOFI, 2001). The main shrimp species cultured in Vietnam has been for a long time the black tiger shrimp (*P. monodon*). White leg shrimp (*P. vannamei*) was introduced in 2001; however, this species can only be cultured legally in the northern and central provinces of Vietnam. The Vietnamese government has carried out several projects to translate the "International Principles for Responsible Shrimp Farming" (MOFI, 2006; NACA/SUMA, 2005) into practices to improve shrimp production, obtain higher product quality and target to environmental and socio-economic sustainability. However, many issues still remain to be solved to reach this goal, including the control of viral diseases (Khang, 2008).

Viral diseases

Industrial shrimp farming, in combination with poor management in shrimp aquaculture, has quickly led to severe pollution in shrimp ponds, thereby creating a suitable environment for development of bacterial (not discussed here) and virus diseases. Of the more than 20 viruses known to cause disease that reduces shrimp yields (Table 1), white spot syndrome virus (WSSV) is the most important one, followed by Taura syndrome virus (TSV) (OIE, 2003a). WSSV causes up to 100% mortality within 7-10 days in intensive shrimp farms, resulting in large economic losses to the shrimp farming industry (Lightner, 1996). Because of high susceptibility to WSSV and yellow head virus (YHV), *P. monodon* has gradually been replaced by *P. vannamei* or *P. chinensis* culture in South-East Asia (Briggs *et al*., 2004; Rosenberry, 2004; FAO, 2006). WSSV is a highly lethal, stress-related virus, usually accompanied by *vibriosis*, which is caused by *Vibrio* bacteria (Phuoc *et al*., 2009). These bacteria exist naturally in coastal waters and in shrimp as part of the natural bacterial flora in the gut, but become pathogenic when shrimps are stressed e.g. by poor water quality, disease or crowding (Horowitz & Horowitz, 2001).

Abbreviation full name	Key references.
BP-type = Baculovirus penaei-type viruses (PvSNPV type sp.):	
BP from the Gulf of Mexico	Couch, 1974a,b
BP from Hawaii	Brock *et al*., 1986
BP from the Eastern Pacific	Lightner *et al*., 1985
MBV-type = *Penaeus monodon*-type baculoviruses (PmSNPV type sp.):	
MBV from S.E. Asia	Lightner *et al*., 1983c
MBV from Italy	Bovo *et al*., 1984
PBV = *Penaeus plebejus* baculovirus	Lester *et al*., 1987
BMN-type = baculoviral midgut gland necrosis type viruses:	
BMNV = from *P. japonicus* in Japan	Sano *et al*., 1981
TCBV = type C baculovirus of *P. monodon*	Brock & Lightner, 1990a
WSSV-type = white spot syndrome baculoviruses (PmNOBII-type):	
SEMBV = systemic ectodermal and mesodermal baculovirus	Wongteerasupaya *et al*., 1995
RV-PJ = rod-shaped virus of *P. japonicus*	Takahashi *et al*., 1994
HHNBV = hypodermal and hematopoietic necrosis baculovirus	Huang *et al*., 1995
WSSV = white spot syndrome virus	Vlak *et al*., 2005
PHRV = hemocyte-infecting nonoccluded baculovirus	Owens, 1993
Other viruses:	
IHHNV = infectious hypodermal and hematopoietic necrosis virus	Lightner *et al*., 1983a,b; Bonami *et al*., 1990
HPV = hepatopancreatic parvovirus	Lightner & Redman, 1985
LPV = lymphoidal parvo-like virus	Owens *et al*., 1991
IRDO = shrimp iridovirus	Lightner & Redman, 1993
TSV = Taura syndrome virus	Lightner *et al*., 1995; Brock *et al*., 1995; Hasson *et al*., 1995
REO-III = type III reo-like virus	Tsing & Bonami, 1987
REO-IV = type IV reo-like virus	Adams & Bonami, 1991
LOVV = lymphoid organ vacuolization virus	Bonami *et al*., 1992
YHV/'YBV' = yellowhead virus of *P.monodon*	Boonyaratpalin *et al*., 1993; Flegel *et al*., 1995
RPS= rhabdovirus of penaeid shrimp	Nadala *et al*., 1992
GAV = Gill-associated virus	Spann et al. 1997; Munday & Owens 1998; Cowley *et al*.1999; Callinan & Jiang 2003; Callinan *et al*. 2003
IMNV = Infectious myonecrosis virus	Lightner *et al*. 2004; Senapin *et al*. 2007.

Table 1. The main viruses causing disease in *Penaeus* shrimp

WSSV

White spot syndrome virus was first reported at shrimp farms in northern Taiwan in 1992 (Chou *et al*., 1995) and has been found world-wide within a decade (Escobedo-Bonilla *et al.*, 2008). WSSV has also been found in wild shrimp stocks in the Americas and is held responsible for devastating Ecuadorian crops in 1999 and 2000, and hereby bringing this country to the brink of economic collapse (Rosenberry 2001; Lightner, 2003). The virus was given different names such as hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Durand *et al*., 1996), *P. monodon* non-occluded baculovirus (PmNOB III) (Wang *et al*., 1995), rod-shaped nuclear virus of *Marsupenaeus japonicus* (RV-PJ) (Inouye *et al*., 1994), penaeid rod-shaped DNA virus (Venegas *et al.*, 2000), systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya *et al*., 1995) or white spot baculovirus (WSBV) (Chou *et al*., 1995), as the viral agents causing disease in different regions were thought to be different. Finally, WSSV was approved as a new virus species by the International Committee on Taxonomy of Viruses (ICTV), when it was recognized that a novel viral agent with a unique genome and pathology was responsible for this disease (Vlak *et al*., 2005). WSSV is the most important disease agent causing outbreaks in various shrimp farming countries and has already costed the world-wide penaeid shrimp industry billions of dollars (OIE, 2003a). The impact of WSSV was disastrous on farmed shrimp production around the world (Fig. 2) (FAO 2006).

WSSV owes its name to the white spots of 0.5–3.0 µm in diameter embedded in the exoskeleton of infected shrimp (Lo *et al*., 1996a). It has been suggested that these spots are calcifications as a result of WSSV-induced dysfunctioning of the integument, although the exact mechanism of white spot formation is not known (Wang *et al*., 1999a). WSSV infected shrimp can also show a reddish coloration and reduced feed intake (Lightner *et al*., 1998), a lethargic response to stimulus (Durand *et al*., 1997), loose cuticle (Lo *et al*., 1996b), swelling of branchiostegites because of accumulation of fluid (Otta *et al*., 1999), enlargement and yellowish discoloration of the hepatopancreas (Sahul-Hameed *et al*., 1998), and thinning and delayed clotting of haemolymph (Wang *et al*., 2000). In the field stress factors such as environment, pond conditions (salinity, shading and temperature) and food could enhance the development of the WSSV disease in juvenile shrimp of all ages and sizes, but massive mortality usually occurs 1 or 2 months after stocking (Kasornchandra *et al*., 1998). WSSV-infected shrimp often gather near the pond edge and present clinical signs 1 or 2 days before the first mortalities occur (Kou *et al*., 1998).

Host range of WSSV

WSSV has a wide host range among decapod crustaceans: at least eighteen cultured and wild penaeid shrimp, eight caridean species, seven species of lobster, seven species of freshwater crayfish and thirty-eight crab species (Durand *et al*., 1997; Chou *et al*., 1998; Lightner *et al*., 1998; Lo *et al*., 1996b; Sahul-Hameed *et al*., 2003) have been found to contain WSSV. In addition, WSSV has been detected in six non-decapod crustacean species (Supamattaya *et al*., 1998; Hossain *et al*., 2001), *Chaetognata* predatory worms, rotifers, polychaete worms and some aquatic insect larvae. In experimental conditions WSSV replication has been confirmed to occur in many of these species. However, some wild species such as polychaete worms may function as mechanical carriers, which only showed WSSV positive by polymerase chain reaction (PCR) (Escobedo-Bonilla *et al*., 2008). WSSV can also be transmitted from instars to reproductive *Artemia* cysts, but is lost during hatching as it was undetectable in nauplii stage (Li *et al*., 2003).

Natural epizootics

The rapid growth of the *penaeid* aquaculture industry, together with increased international movement of live and dead infected shrimps, have contributed to the quick spread of WSSV and threatens the development of shrimp aquaculture world-wide (Durand *et al*., 2000). High opportunity of horizontal transmission of the virus through cannibalism and the waterborne route offers an explanation for the quick spread of the virus in shrimp populations (ponds), to the extent that WSSV is considered a pandemic disease (Wu *et al*., 2001). Epidemics of this disease have been reported throughout Asia, after first discovery of the virus in Taiwan in 1992 (Chou *et al*., 1995; Escobedo-Bonilla *et al*., 2008). In 1993 WSSV caused massive mortalities among the *penaeid* shrimp in Japan and Korea and this virus strain was suggested to originate from the same ancestor as the Taiwan, Thailand and China isolates (Inouye *et al*., 1994; Moon *et al*., 2003). WSSV was found in cultured *P. monodon* in Malaysia in 1994 (Wang *et al*., 1999b), while the Indian subcontinent outbreaks have occurred in both PL and cultured *P. monodon* (Manivannan *et al*., 2002; Selvin & Lipton, 2003). WSSV was detected in Texas in 1995 (Rosenberry, 1996). Since 1999 it also induced mortality of cultured *P. vannamei* in Ecuador (Rodriguez *et al*., 2003) and Mexico (Galaviz-Silva *et al*., 2004), causing severe damage to the shrimp industries of both central and south America (Global Aquaculture Alliance, 1999a,b). The import of frozen shrimp has been suggested to be the source that spreads WSSV from Asia to the Americas (Lightner *et al*., 1997). WSSV is thought to have reached shrimp farms in Australia and Spain in 2000-2001 by introductions of frozen infected shrimps that

were used as fresh food for broodstock, although this has not been confirmed (OIE 2003a).

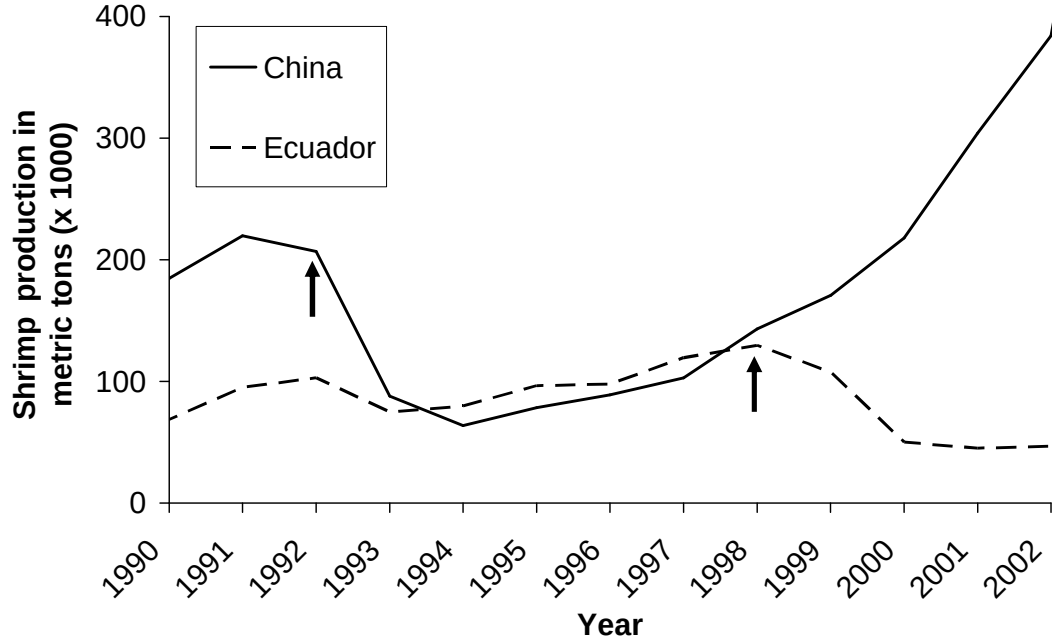


Figure 2. Impact of white spot syndrome virus on farmed-shrimp production in China and Ecuador (FAO, 2006). Arrows indicate year of first outbreak reported for each country.

WSSV was also reported to cause shrimp disease in France and Iran in 2002 (Rosenberry, 2002). WSSV was also shown to be present in wild populations of Atlantic blue crab (Chang *et al*., 2001).

WSSV is a reportable disease by the OIE and diagnostic tests are available to test for the virus (OIE, 2003a). The first test was developed by Lo *et al*. (1996a), based on specific nucleotide sequence amplification (PCR). Commercial kits are now available to test for the presence of WSSV in feed, broodstock, fry and PLs.

Transmission

WSSV can be transmitted vertically and horizontally in shrimp (Wu *et al.*, 2001; Lotz & Soto, 2002) and other cultured and wild crustacean species and aquatic organisms, such as crayfish, crabs, copepods etc., causing different levels of mortality depending on the host species (Jesus *et al*., 2007). Infection of shrimp with WSSV arises from many sources; rapid transmission of WSSV in culture systems may occur from infected shrimp, through water and by cannibalism of moribund shrimp (Chang *et al*., 1996, Wu *et al.*, 2001). However, infected spawners and post larvae are also major sources of infection for shrimp farms. Potential sources for WSSV transmission include human activities, seabirds or other animal's immigration, infected frozen food

products, infected pond sediments, contaminated aquaculture tools or instruments and untreated infected shrimp by-products from processing plants (Lightner *et al*., 1997). PCR results show that different arthropods, including copepods and insects, can act as vectors of WSSV (Lo *et al*., 1996b; Flegel, 1997; Hameed *et al*., 2003). Furthermore, some of these arthropods, such as *Portunus pelagicus* and *Acetes* sp., are common in shrimp culture areas and may transmit WSSV between ponds and farms (Supamataya *et al*., 1998). Other vectors can also get into shrimp ponds through pumped water (Hameed *et al*., 2003). Several species of crabs have been shown to carry WSSV, implicating them as asymptomatic carriers (Lo *et al*., 1996b; Otta *et al*., 1999; Chen *et al*., 2000; Chang *et al*., 2001).

WSSV genome

WSSV is one of the largest animal DNA viruses sequenced so far (Filee & Chandler, 2008) and has been assigned as a species of the genus Whispovirus, family Nimaviridae by the ICTV (ICTV, 2008). WSSV is the sole member of the genus (Whispovirus) and the family (Nimaviridae). WSSV virions are ellipsoid to bacilliform in shape and of considerable size (80-120 x 250-380 nm). They have a unique, tail-like appendage when in solution; hence the family name (nima = thread, appendage). Virions contain a large double-stranded DNA molecule wrapped into a rod-shaped nucleocapsid with an envelope. The nucleocapsids have a striated appearance. The WSSV genome encodes about 180 open reading frames (ORFs) (depending on the variant) and so-called nine homologous regions containing direct repeats, inverted repeats and palindromes dispersed along the genome (van Hulten *et al*., 2001a; Yang *et al.*, 2001).

There is variation in the WSSV genome size, ranging from 292,967 bp (WSSV-TH, GenBank accession No. AF369029, van Hulten *et al*., 2001a) to 305,107 bp (WSSV-CN, GenBank accession No. AF332093, Yang *et al*., 2001) to 307,287 bp (WSSV-TW, GenBank accession No. AF440570) for different geographical isolates originating from Thailand, China and Taiwan, respectively. However, the sequences shared by these genomes are almost identical; nucleotide identity is 99.3%. The size differences are mostly due to several small insertions/deletions in regions with repetitive DNA, to a genetically variable in a region of about 750 bp and one large approximately 13 kbp deletion (Marks *et al*., 2004; Figure 3). Recently, a new WSSV isolate was discovered with the largest WSSV genome on record, containing approximately 5 kbp more DNA than the largest so far sequenced isolates. This isolate may be the potential common ancestor of the characterized WSSV isolates up to date (Marks *et al*., 2005).

Virions comprise at least forty-five structural proteins that are arranged in three morphologically distinct layers (Tsai *et al*., 2004; Li *et al*., 2007). Most ORFs have a low, if any, level of amino acid identity compared to the proteins of other viruses or organisms (Lightner, 2003), underscoring the unique taxonomic position of WSSV (Vlak *et al*., 2005).

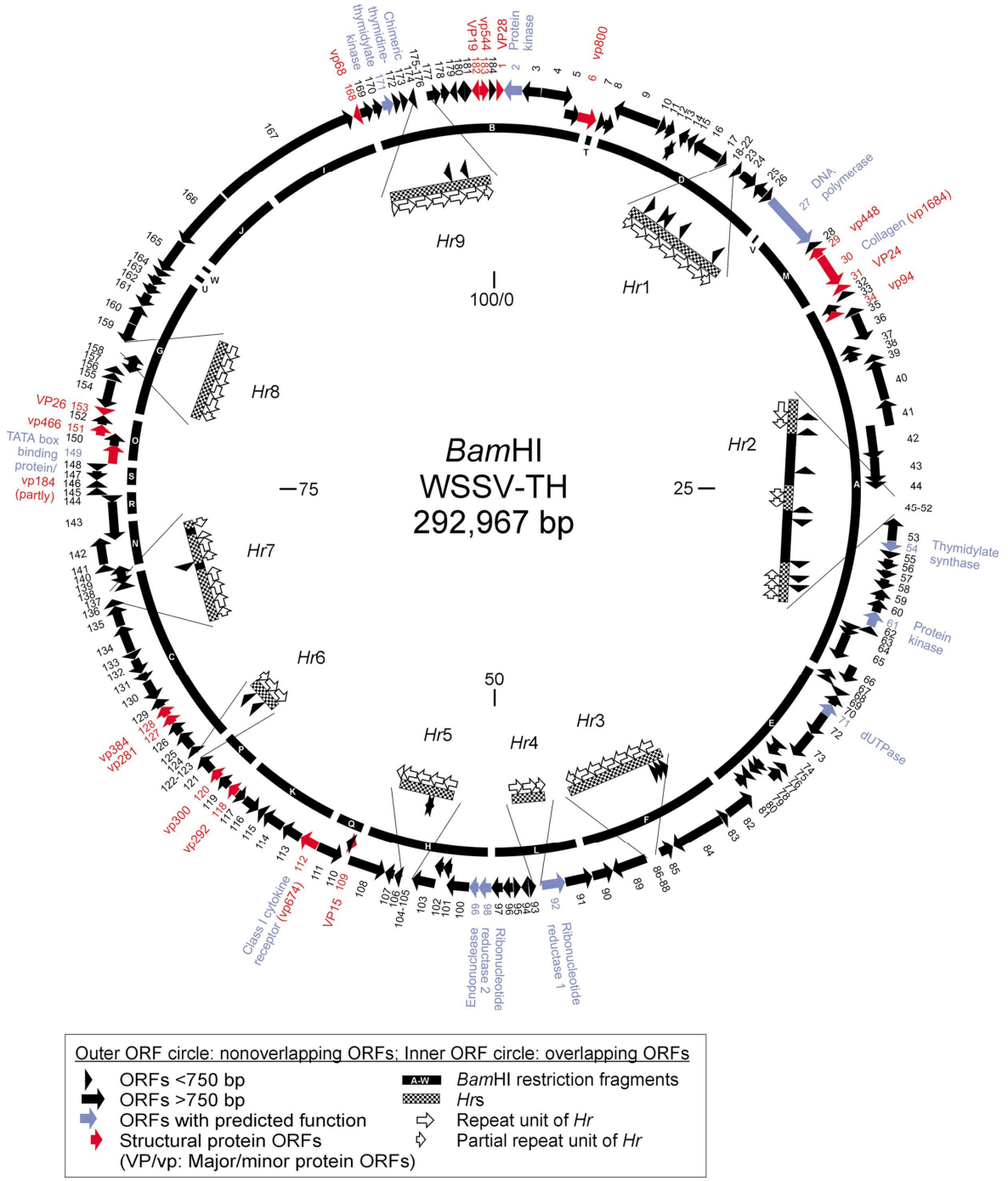


Figure 3. Genome of the WSSV isolate originating from Thailand (1996) and completely sequenced by van Hulten *et al*. (2001a).

Up to now WSSV isolates have shown little genetic and biological variation in terms of sequence and host range, suggesting that they are closely related and emerged from a single source (Lightner, 2003). On the other hand differences in virulence have been noted and it remains to be seen to what extent the genetic differences are related to virulence.

Control of WSSV

WSSV-induced economic losses in the aquaculture industry have prompted researchers to develop strategies to control or manage WSSV infection and disease occurrence. Several studies on WSSV control have been done and control measures showed some efficacy against WSSV under experimental conditions. Chemotherapy with STEL (STerility and ELectrochemistry) water, when used continuously, showed effective prevention of WSSV infection in shrimp (Park *et al.*, 2004). Mytilin, an antimicrobial peptide abundant in mussel's haemocytes, could prevent replication of the viral DNA (Dupuy *et al*., 2004). Addition of β-1,3-glucan to the shrimp feed also showed significant improvement of the immunity and survival of *P. monodon* after WSSV challenge (Chang *et al*., 2003). Crude fucoidan (CF) could inhibit WSSV infection and the growth of *Vibrio harveyi*, *Staphylococcus aureus* and *E. coli* through oral administration in *P. monodon* (Chotigeat *et al*., 2004). The peptides derived from some antiviral genes in *P. monodon* could specifically bind to WSSV and block virus infection, suggesting their potential to be exploited as an antiviral peptide drug (Luo *et al*., 2003; Yi *et al*., 2003).

Modulating environment factors has also been suggested as a method to control WSSV disease, although the effectiveness of various measures varies greatly. Low water temperatures (12-21°C) reduced WSSV pathogenicity and inhibited mortality in crayfish and shrimp (Dupuy *et al*., 2004; Jiravanichpaisal *et al*., 2004). Hyperthermia also increases the survival of infected shrimp, possibly through the facilitation of apoptosis in WSSV-infected *P. vannamei* (Granja *et al*., 2003). Mortality reduction was observed in *P. vannamei* juveniles at 33°C, but only in the early stages of infection (Rahman *et al*., 2006).

Although invertebrates lack a genuine adaptive immune response as with vertebrates, several products have shown potential to non-specifically stimulate the invertebrate immune system in experiment. The use of probiotic bacteria (Solano & Soto, 2006; Li *et al*., 2007; Balcázar *et al*., 2007; Balcázar & Rojas-Luna, 2007; Gómez & Shen, 2008; Mathieu *et al*., 2008), based on the principle of competitive exclusion, and the use of immunostimulants (Chotigeat *et al*., 2004) are two preventive methods explored against WSSV disease during the last few years. Probiotic strains isolated from shrimp culture water or from the intestine of different penaeid species

have been shown to significantly enhance tolerance or resistance to WSSV (Jiqiu *et al.*, 2009). The successful strains belong to the genus *Vibrio* and *Bacillus* and the species *Thalassobacter utilis*. Production or induction of digestive enzymes in the gut was proposed as the protective mechanism of probiotics, improving shrimp digestion, health and stress resistance. However, the lack of solid evidences on their successful use and action mechanisms *in vivo* makes application of probiotics in aquatic environments a controversial concept. Effects of certain probiotic bacterial strains were shown to be highly sensitive to many environmental factors. Therefore, development of optimum fermentation technologies is required to avoid bacterial strain misidentification, which has been a problem in commercial probiotic cultures (see Ninawe & Selvin, 2009).

Vaccination has also been suggested as one of the methods to control WSSV (see Johnson *et al.*, 2008). It was shown that there is possibly a humoral neutralizing factor in convalescent shrimp after challenge with WSSV (Venegas *et al.*, 2000; Wu *et al.*, 2002), which could potentially be developed as a treatment against WSSV. Viral accommodation at low levels, providing "specific memory" of previous infection, was proposed to explain this protection (Flegel, 2007). However, molecular evidence of this hypothesis remains elusive. Intramuscular injection (Namikoshi *et al.*, 2004) or oral feeding (Singh *et al.*, 2005) of formalin inactivated virus showed significant but only short protection against WSSV up to 10 days post vaccination. Recombinant expressed WSSV envelope proteins, which were used as subunit vaccines revealed enhanced tolerance and partial protection against WSSV in one crayfish species and various shrimp species. The protection was achieved from both purified protein and protein delivered in inactivated bacteria expressing the protein (administered by injection, immersion or orally), but only up to 14 days post vaccination (Witteveldt *et al.*, 2004; Witteveldt *et al.*, 2006; Vaseeharan *et al.*, 2006; Xu *et al.*, 2006; Du *et al.*, 2006; Jha *et al.*, 2007; Rout *et al.*, 2007). The mechanism involved in this defense response is still unclear. Recently envelope protein VP28 expressed in eukaryotic expression systems has been shown to protect crayfish from WSSV infection. Interestingly the eukaryotically expressed protein gave better protection compared to bacterially expressed product (Du *et al.*, 2006). This may reflect efficiency of the correct folding and/or posttranslational modification of this protein, which possesses several putative glycosylation and phosphorylation sites when expressed in a eukaryotic system. DNA vaccines, consisting of recombinant DNA plasmids that express envelope proteins VP28 or VP281 under the control of the CMV promoter, have been shown to protect *P. monodon* from WSSV for up to 50 days post vaccination. However, the protective response could not be achieved with the nucleocapsid proteins VP15 and VP35 (Rout *et al.*, 2007). All of these evidences

suggest that the protective mechanism requires the continuous presence of specific viral surface proteins for recognition of virus particles prior to cell entry. In conclusion, a better understanding of the mechanisms underlying protection is essential for developing a feasible vaccination strategy (Johnson *et al.*, 2009).

Genetic markers and molecular epidemiology

"Molecular epidemiology" is an approach that incorporates molecular, cellular, and other biological measurements into epidemiology research (Schulte & Perera, 1993). Genetic markers were suggested as "a quantum leap in the evolution of epidemiological ideas" (Schulte & Perera, 1993). They can be used as vital tools to link molecular and epidemiological studies. Quantification of marker variations can be performed with greater precision, and hence provide better data for statistical comparisons than many other types of ecological measurements. Polymorphic genetic markers can be used to track movement of individuals between populations and infer population structures. However, not all sections of the genome are useful as molecular markers. On the other hand, no genetic marker is ideal for all applications. Organisms with differences in polymorphism level require different markers. Some markers are sufficiently variable to detect differences among populations, but are rarely sufficiently polymorphic to distinguish between specific individuals. Protein polymorphism and allozymes have been used as powerful markers for several decades to address ecological and evolutionary questions (May, 1992). However, this type of marker only reveals sufficient variation among species at some protein-coding loci and is not suitable in case of low variation between populations within the same species. DNA markers, on the other hand, are advantageous because of their ease of extraction and use, and given that they can be extracted from almost every cell type of an organism throughout its entire life cycle.

Development of the polymerase chain reaction (PCR) has created powerful molecular-based tracking methods for epidemiological study. This led to techniques such as Restriction Fragment Length Polymorphism (RFLP) followed by Amplified Fragment Length Polymorphism (AFLP) as powerful marker techniques to examine the historical origins and geographic distribution of eukaryotes (Vitic & Strobeck, 1996) as well as viruses (Gouvea *et al.*, 1998; Sammels *et al.*, 1999). However, restriction fragment analysis of total genomic DNA is problematic for discriminating genotypes when there are low levels of genetic diversity and is limited because they require a large amount of DNA. As a general point PCR-based markers have the important advantage of requiring minimal amounts of tissue. In molecular microbial ecology, ribosomal gene sequences dominated as markers in the literature. Nevertheless, distinguishing closely related microorganisms proved to be problematic

with these markers. In traditional molecular epidemiology of viral infections, genetic markers are also the key tools to assist epidemiological investigation. However, there are no universal viral gene sequences (i.e. viruses have no ribosomal genes of their own). DNA polymerases are often used as genetic markers as their PCR amplicons often give diverse electrophoretic profiles. Many viruses do not have these genes and some contain RNA rather than DNA. Hence, most of the techniques designed for microbial cells can therefore not be relied upon when working with viruses. Currently separate analysis of all components of biological systems dominates molecular research. However, integrative molecular epidemiology, with supporting mathematical and computational modeling, can result in more comprehensive coherent studies of pathogen molecular evolution, phylogeny and population genetics, leading to better insight into disease occurrence and dynamics (Lara *et al.*, 2008).

Although many studies, mentioned above, have shown that shrimp can partly be protected against WSSV under experimental conditions, no adequate treatments are available yet to effectively control WSSV in the field. Therefore, at present, excluding pathogens from shrimp farms (sanitation) is the most effective approach for WSSV control. Methods to exclude WSSV include disinfecting ponds and water, preventing entrance of potentially infected animals by screening for the presence of WSSV, stocking SPF shrimp, correct monitoring of shrimp samples and evaluation of newly introduced broodstock, eggs, post larvae and juvenile shrimp for the presence of WSSV. Combination of these preventive measures and other control methods mentioned above is the best way to reduce the risk of a WSSV outbreak up to date.

WSSV prevention and management requires an integrated approach, in which understanding of WSSV transmission, epidemiology and evolution is one of the important issues. In the context of an emerging problem such as white spot disease, insights into WSSV origins and spread patterns can be achieved by linking predicted evolutionary histories with epidemiology using a molecular approach. However, the current level of understanding of the spread of WSSV – on both small and large scale – is insufficient: many possible transmission routes are known, but what are the actual routes taken by the virus? Moreover, viruses rapidly evolve like most other infectious disease agents, meaning that an evolutionary perspective on WSSV is required (e.g. Grenfell *et al.*, 2004) to predict future developments. WSSV has shown marked variation in genotypic (Marks *et al.*, 2004, 2005a) and biological characteristics such as virulence (Marks *et al.*, 2005a), but insight into the evolutionary processes leading to (a) virulence is lacking. Therefore we need tools to track the genetic structure of WSSV populations in order to infer patterns of spread and follow virus evolution over time and space (phylogeography; Avise, 2000). A phylogeographic approach has been

taken for many diseases that threaten human health, such as influenza, dengue, rabies, influenza and HIV to understand the origin and spread of the virus (Holmes, 2004).

Aim of the thesis

Industrial shrimp farming is associated with uncontrolled movement of broodstock and PL, high-density monoculture, transport of infected frozen shrimp, etc. These factors, together with poor sanitation and management in shrimp aquaculture, provide convenient conditions for many viral diseases – including white spot disease – to become pandemic. However, many questions about the origin and spread of the virus and how it evolved genetically and biologically over time and space remain unanswered. Patterns of WSSV distribution are poorly understood because conventional methods for WSSV epidemiology, based on farmer reports, met difficulties in monitoring disease outbreaks (Corsin *et al*., 2002). The role of intermediate or carrier hosts in the rapid emergence of the disease is also obscure. In addition, the current PCR tests for WSSV are very generic, do not allow for the discrimination of different variants and are therefore unsuitable for tracking the evolution of the virus.

Finding suitable methods to identify and discriminate WSSV strains – and infer their origin – is therefore important for WSSV forensics and understanding WSSV epidemiology and evolution. Moreover, the genetic relatedness between WSSV isolates and the history of shrimp sources could help infer virus origins and identify other factors involved in spread of WSSV. Molecular epidemiology with suitable genetic markers is a potential approach, in which identification and validation of informative molecular sequences are essential steps in this process. Five major variable loci in the WSSV genome were identified by Marks *et al*. (2004) based on the alignment of three completely sequenced WSSV isolates originated from Taiwan (WSSV-TW), China (WSSV-CN) and Thailand (WSSV-TH). These variable loci were suggested to be promising genetic markers for the study of WSSV diversity. This thesis analyzes these loci in detail and tests their suitability as molecular markers to study WSSV epidemiology and evolution.

The availability of well-defined genetic markers, access to well-recorded longitudinal sample sources of WSSV and a relatively undisturbed, regionally organized shrimp industry are very important for a successful study of the molecular epidemiology of WSSV. These requirements are met in Vietnam, where semi-intensive and intensive shrimp culture developed relatively late (2000), with limited shrimp introductions from abroad and government-controlled shrimp practices. This situation and these conditions provide a unique opportunity to use molecular markers in combination with epidemiological models to explain the evolutionary path of a

virus, in this case a virus in an aquatic environment: WSSV. This largely retrospective study will provide a basis for future experimental studies on this aquatic virus-host system to further understand the epidemiology of WSSV and to design novel intervention strategies.

Outline of the thesis

Chapter 1: In this chapter a short account is given on shrimp culture and shrimp production systems and on the consequences for the emergence of diseases. Among the virus diseases, WSSV is the most important one and an account is given on the taxonomic status of the virus, its molecular characteristics and pathology and its current epidemic status

Chapter 2: An eclectic selection of WSSV isolates from various regions in Vietnam, but mainly the central and southern regions of the country (the earliest regions for industrial shrimp production), were used in this chapter for a preliminary evaluation of whether the major variable loci of WSSV genome are suitable as genetic markers to study the epidemiology of WSSV and to devise a model on the spread of the virus in Vietnam.

Chapter 3: In this chapter the spread model was further validated by extending the number of samples representing key areas of shrimp production, covering all of Vietnam from the north to the south. A statistical analysis was performed to test which variable loci are appropriate markers for WSSV epidemiology at this spatiotemporal scale. Moreover, a statistically supported model of WSSV spread in Vietnam could be inferred.

Chapter 4: The WSSV variable loci that are the most appropriate molecular markers for the small geographical scales (local) are variable number tandem repeat (VNTR) loci (Chapters 2 and 3). In this chapter it was tested whether VNTRs could be used to study genetic heterogeneity within WSSV populations (i.e. from individual shrimp).

Chapter 5: The sample source is very important in epidemiology. Using molecular markers it is evaluated whether shrimp farming practices (intensive / extensive) affect the genetic structure of WSSV populations over time. Moreover, the data allow for inferences on suitable sample sources for retrospectively studying the spread of WSSV.

Chapter 6: The variable locus that proved to be an appropriate molecular marker for large geographical scales (Chapter 2) was used to describe the spread and evolution of WSSV on the continental scale, i.e. spread through Asia. This chapter also shows the relationship between the genomic deletion sizes and WSSV evolution: a simple geometric model can describe the adaptive trajectory of WSSV genome size evolution.

Chapter 7: In this chapter the outcome of the previous chapters is discussed in the broader context of the use of molecular markers in understanding virus epidemiology in general. Furthermore an outlook is given on the potential implications of the acquired knowledge and the future research that is required to better understand the epidemiology and evolution of WSSV.

CHAPTER 2

Molecular epidemiology of White Spot Syndrome Virus within Vietnam

Abstract

WSSV, sole member of the virus family *Nimaviridae*, is a large dsDNA virus infecting shrimp and other crustaceans. By alignment of three completely sequenced isolates originating from Taiwan (WSSV-TW), China (WSSV-CN) and Thailand (WSSV-TH), the variable loci in the genome were mapped. The variation suggests the spread of WSSV from a common ancestor originating from either site of the Taiwan Strait to Thailand, but support for this hypothesis through analysis of geographic intermediates is sought. RFLP analysis of eight Vietnamese WSSV isolates, of which six were collected along the central coast (VN-central) and two along the south coast (VN-south), showed apparent sequence variation in the variable loci identified previously. These loci were characterized in detail by PCR amplification, cloning and sequencing. Relative to WSSV-TW, all six VN-central isolates showed a approximately 8.5 kb deletion in the major "variable region ORF23/24" (ORF = Open Reading Frame), whereas the two VN-south isolates contain a deletion of approximately 11.5 kb and approximately 12.2 kb, compared to a approximately 1.2 kb and approximately 13.2 kb deletion in WSSV-CN and WSSV-TH, respectively. The minor "variable region ORF14/15" showed deletions of various sizes compared to WSSV-TH for all eight VN isolates. The data suggest that the VN isolates and WSSV-TH have a common lineage, which branched off from WSSV-TW and WSSV-CN early on, and that WSSV entered Vietnam by multiple introductions. We present a model for the spread of WSSV from either site of the Taiwan Strait into Vietnam based on the gradually increasing deletions of both "variable regions". The number and order of repeat units within ORF75 and ORF125 appeared to be suitable markers to study regional spread of WSSV.

Key words: White spot syndrome virus, molecular epidemiology, WSSV variable loci, strain identification, PCR genotyping, Vietnam, shrimp culture

This chapter has been published as: Dieu, B. T. M., Marks, H., Siebenga, J. J., Goldbach, R. W., Zuidema, D., Duong, T. P. & Vlak, J. M. (2004). Molecular epidemiology of White Spot Syndrome Virus within Vietnam. *Journal of General Virology* **85**, 3607 – 3618.

Introduction

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

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

Except for South-East Asia, WSSV also has been reported from the United States in 1995 (Rosenberry, 1996), and from central- and south- America since early 1999 (Rosenberry, 2000). In 2002, WSSV was also detected in France and Iran (Rosenberry, 2002). The various geographical isolates of WSSV identified thus far are very similar in morphology and proteome. Limited differences in restriction fragment length polymorphism (RFLP) patterns have been reported, suggesting either a high degree of genomic stability or a recent emergence (Nadala & Loh, 1998; Lo *et al.,* 1999; Wang *et al.,* 2000a, b; Marks *et al.,* 2004). Preliminary studies indicated that there is also little difference in virulence between various WSSV isolates, although direct comparisons were not made (Wang *et al.,* 1999b; Lan *et al.,* 2002). After the complete sequencing of three different WSSV isolates originating from Taiwan (WSSV-TW; Wang *et al.,* 1995), China (WSSV-CN; Yang *et al.,* 2001) and Thailand (WSSV-TH; van Hulten *et al.,* 2001a), the major variable loci in the WSSV genome were mapped by alignment of these sequences (Marks *et al.,* 2004). Roughly, the variable loci can be divided into deletions, variable number of tandem repeats (VNTRs), single nucleotide indels and single nucleotide polymorphisms (SNPs). The variation within these loci, in particular in the large genomic deletions, suggested a geographical spread from a common ancestor from either site of the Taiwan Strait to Thailand (Marks *et al.,* 2004), but genetic intermediates were missing to support this hypothesis.

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

Materials and methods

Infected shrimp sampling

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

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

Name of the pond	Place (district)	Province	Date of collection	Origin of postlarvae*	Abbreviation
central WSSV-VN isolates					
Khanh	Hoi An	Da Nang	18/03/2003	Local	K
Thanh	Son Tinh	Quang Ngai	19/03/2003	Da Nang	T
Luong	Qui Nhon	Binh Dinh	20/03/2003	Local	L
Xu	Tuy Hoa	Phu Yen	21/03/2003	Local	X
Suu					S
Anh	Ninh Hai	Ninh Thuan	22/03/2003	Local	A
south WSSV-VN isolates					
C. Thanh A	Long Hoa	Tra Vinh	10/01/2004	Unknown	Tv
T. Sang	Kien Luong	Kien Giang	04/03/2003	Local	Kg

*All ponds obtained their post larvae from hatcheries; the location of this hatchery is mentioned in this column.

DNA extraction

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

PCR analysis of WSSV-infected shrimp

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

PCR analysis for WSSV variable loci

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

Table 2a. Primers used during PCR analysis for WSSV screening

Primer pair name	Primer name	Sequence (5'-3')	Annealing temperature (^{0}C) / elongation time (s)	WSSV-TH sequence coordinates	Size (bp) of PCR product
16s rRNA	16S-FW	GTGCGAAGGTAGCATAATC	52 / 50		414
	16S-RV	CTGCTGCAACATAAGGATAC			
VP26	VP26-FW	ATGGAATTTGGCAACCTAACAAACCTG	52 / 50	228835-228809	304
	VP26-RV	GGGCTGTGACGGTAGAGATGAC		228532-228553	

Table 2b. Primers used during PCR analysis for WSSV variable loci

Primer pair name	Primer orientation	Sequence (5'-3')	Annealing temperature (^{0}C) / elongation time (s)	Sequence coordinates	Size (bp) of PCR product
VR23/24-1	Forward	ATGGGCTCTGCTAACTTG	50 / 360	4359-4376*	10833*
	Reverse	ATGATTGTATTCGTCGAAGG		15191-15172*	
VR23/24-screen	Forward	CACACTTGAAAAATACACCAG	49 / 65	5503-5523*	9088*
	Reverse	GTAAGTTTATTGCTGAGAAG		14590-14571*	
VR23/24-south	Forward	CTACAACGGCCAAGTCAT	49 / 100	30701-30718†	1555†
	Reverse	CGCAATTCTCCTCGCAGTT		32255-32237†	
VR14/15-screen	Forward	GAGATGCGAACCACTAAAAG	49 / 75	22904-22923†	1254†
	Reverse	ATGGAGGCGAGACTTGC		24157-24141†	
Transposase	Forward	GTGGATAATATTCGTCTTCAAC	55 / 120	253988-254009†	1489* (151†)
	Reverse	CTCAAAGACAACGACATTAG		254138-254119†	
ORF75-flank	Forward	GAAGCAGTATCTCTAACAC	49 / 80	107875-107893†	868†
	Reverse	CAACAGGTGCGTAAAAGAAG		108742-108723†	
ORF94-flank	Forward	GTGCCGCAGGTCTACTC	51 / 80	142656-142672†	682†
	Reverse	CATACGACTCTGCTTCTTG		143337-143319†	
ORF125-flank	Forward	CGAAATCTTGATATGTTGTGC	52 / 100	187791-187811†	652†
	Reverse	CCATATCCATTGCCCTTCTC		188442-188423†	
Polymerase	Forward	CAATATTACACGCCCTTCAG	49 / 60	35867-35886†	504*,†
	Reverse	GCTTGCATGATTTTTCTCC		36370-36352†	

*WSSV-TW sequence coordinates or WSSV-TW sizes

† WSSV-TH sequence coordinates or WSSV-TH sizes

Cloning of PCR products

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

Virus production and purification

The virus isolate WSSV-TH used in this study originated from infected *P. monodon* imported from Thailand in 1996 and was obtained as described before (van Hulten *et*

al., 2000a). The virus WSSV-VN isolate T (Table 1) originated from a single infected *P. monodon*. Tissue of a WSSV-VN-T infected *P. monodon* was homogenized in 330mM NaCl. After centrifugation at 1,700 x g for 10 min the supernatant was filtered (0.45 µm filter; Schleicher & Schuell) to obtain the virus. Crayfish *Orconectes limosus* or *Astacus leptodactylus* were injected intramuscularly with a lethal dose of WSSV (WSSV-TH or WSSV-VN-T), using a 26-gauge needle (Microfine B&D). Virus was isolated and processed according to published procedures (van Hulten *et al.*, 2000a).

Purification of viral DNA and restriction enzyme analysis

Viral DNA was isolated from purified virions as described by van Hulten *et al.* (2000a). WSSV DNA was digested with *Bam*HI (Invitrogen) and fragments were separated by electrophoresis in a 0.6% agarose gel at 40 V (1.3 V cm^{-1}) for 20 h. After separation, the gels were stained with ethidiumbromide (0.5 µg ml^{-1} in Tris-Acetate-EDTA (TAE)).

Sequencing and computer analysis

Plasmid clones were sequenced using universal T7 and/or Sp6 primers, and by primer walking in case inserts were >1.5 kb (BaseClear, the Netherlands). Sequence data were analyzed using the software package DNASTAR 4.2 (DNASTAR Inc.) and the output was edited in GeneDoc, version 2.6.000 (Nicholas *et al.* 1997). Complete WSSV sequences were obtained from the NCBI databank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) using the accession numbers for WSSV-TW (AF440570), WSSV-CN (AF332093) and WSSV-TH (AF369029). Dotplot analysis was performed using PIPmaker (http://bio.cse.psu.edu/pipmaker/).

Results

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

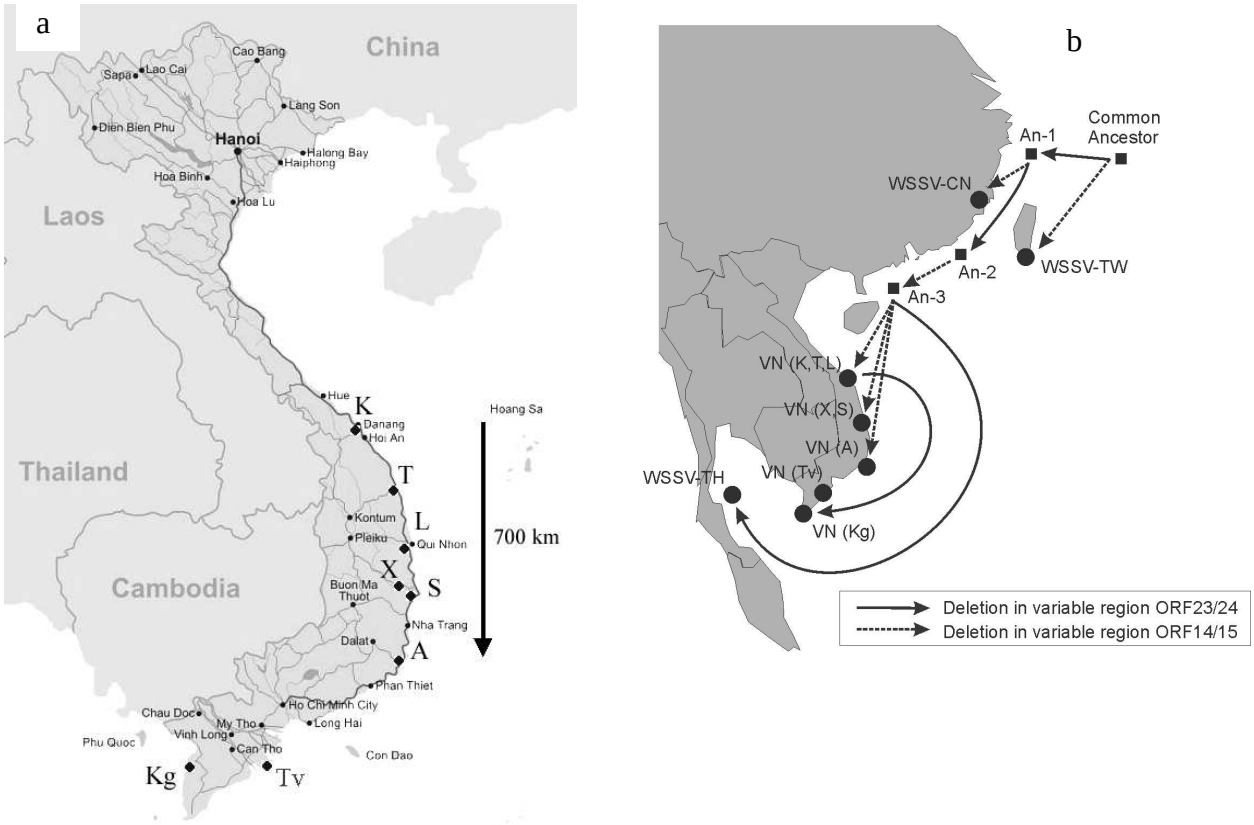


Figure 1. (a) Map of Vietnam, showing the geographical origins of the isolates used for this study, indicated by K, T, L, X, S, A, Tv and Kg, respectively, according to Table 1. **(b)** Model of spread of WSSV in South-East Asia from either site of the Taiwan Strait towards the West. Circular dots represent identified isolates, while square dots represent hypothetical isolates. Each line represents a single-step deletion. Ancestor is abbreviated as An, WSSV-VN isolates are abbreviated as VN.

The WSSV genomic loci, which were shown to be variable in their genetic make-up among different WSSV isolates (Marks *et al.*, 2004), were used for our analysis. These loci were studied in detail for each of the VN isolates by PCR amplification, cloning and sequence analysis. The variable loci screened for can be divided into (*i*) a genomic region prone to large deletions, referred to as "variable region ORF23/24" (this region is called "13 kb deletion" by Marks *et al.* (2004)), (*ii*) a genetic variable region, which will be referred to as "variable region ORF14/15", (*iii*) a genomic region encoding a putative transposase and (*iv*) the VNTRs located in ORF75, ORF94 and ORF125. Furthermore, we analyzed a conserved genomic fragment encoding part of WSSV DNA polymerase (*v*). The data for each of these loci will be dealt with separately. To reduce the possibility that the VN isolates have major genetic differences at loci which were not screened for, a detailed restriction enzyme analysis (RFLP) was performed for one of the VN isolates (WSSV-VN-T) and the result was compared to WSSV-TH.

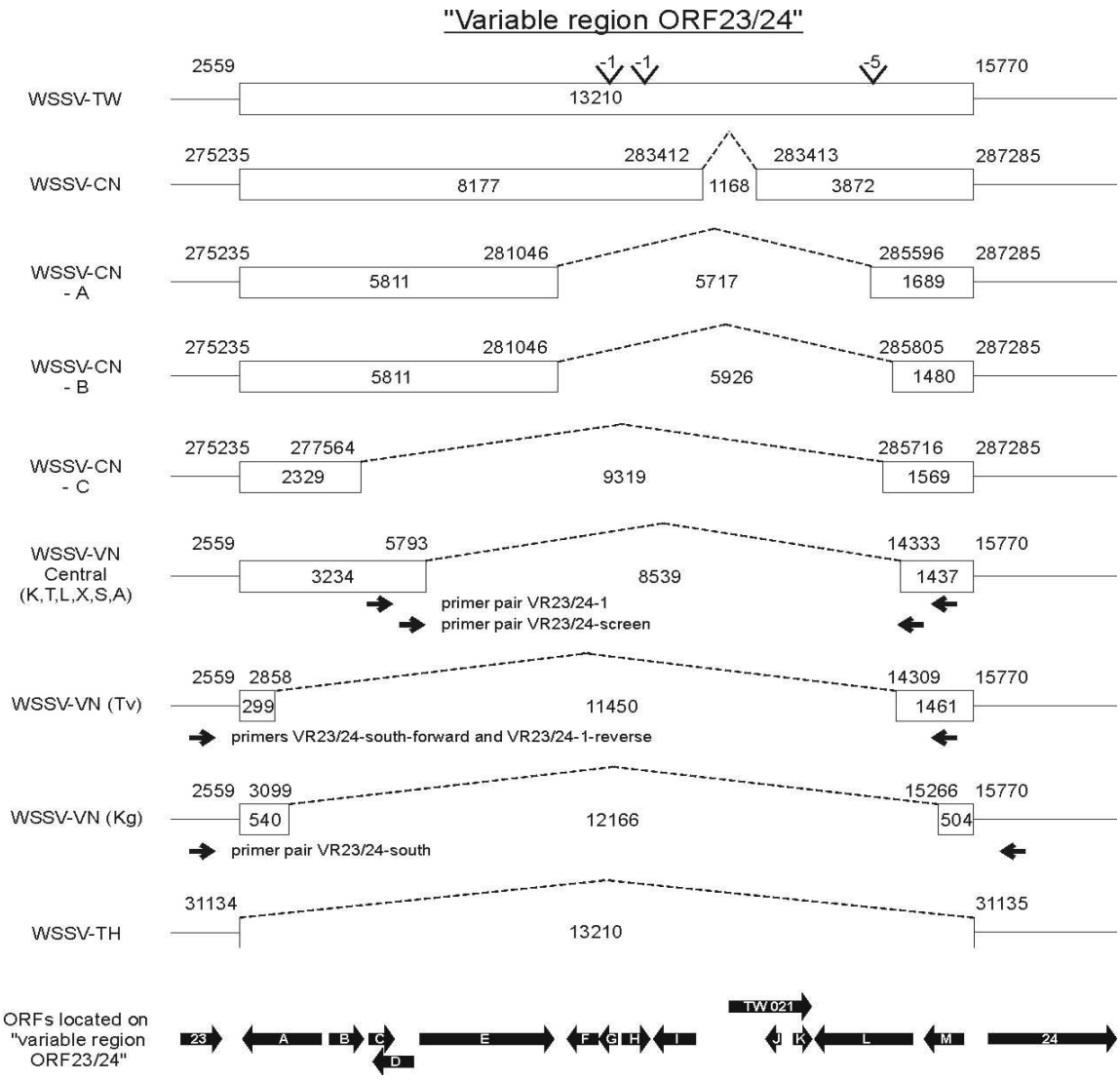


Figure 2a. Schematic representation of the "variable region ORF23/24" of WSSV-TW, WSSV-CN, WSSV-TH, three different isolates from China 2001 (WSSV-CN - A, - B and - C, respectively: map numbers are in accordance with WSSV-CN; Lan *et al.*, 2002) and the VN isolates. The map numbers, indicated above each isolate, are in accordance with the numbers in the NCBI databank for the genomic sequence of each isolate. The coordinates of the WSSV-VN isolates are according to the WSSV-TW annotation. The length of the fragments is indicated within boxes or sequences. The positions of the ORFs located in this region are indicated by closed arrows, which also represent the direction of transcription. ORFs are numbered in accordance with the numbering used by Marks *et al.* (2004). Open arrows represent primers.

(i) Variable region ORF23/24

Previously, this genomic region was shown to contain deletions of approximately 1.2 kb and approximately 13.2 kb in WSSV-CN and WSSV-TH, respectively, compared to WSSV-TW (Fig. 2a; Marks *et al.,* 2004). Three other unique deletions in this region were reported in Chinese isolates collected in Tong'an and Anhui in South-East China (2001) (Fig. 2a: WSSV-CN -A till -C; Lan *et al.,* 2002).

We mapped this locus initially in WSSV-VN isolate K by performing PCR reactions with primer pairs equally distributed over the genomic region 2332-15861 (WSSV-TW coordinates), which harbors the "variable region ORF23/24". Based on the primer pairs that failed to give a product, the flanking primer set "VR23/24-1" (Table 2b; Fig. 2a) was used to exactly pinpoint the coordinates of the deletion. Cloning and sequencing of the approximately 2.3 kb fragment obtained in the PCR revealed that a deletion of 8,539 bp existed in WSSV-VN isolate K compared to WSSV-TW (Fig. 2a). The flanking sequences present in the approximately 2.3 kb fragment were 100% homologous to the sequences of WSSV-TW and WSSV-CN. Based on this result, a new PCR reaction was performed with primer set "VR23/24-screen" (Table 2b; Fig. 2a) flanking this deletion to specifically detect this deletion in all VN isolates. The amplified fragment had a similar size of 548 bp for the six VN-central WSSV isolates, indicating that they have a deletion of about 8,539 bp in this locus compared to WSSV-TW (Fig. 2a). The VN-south isolates (Tv and Kg) failed to give a product in this PCR reaction.

We mapped the "variable region ORF23/24" in isolates Tv and Kg using a similar method used to map the deletion for WSSV-VN isolate K. Cloning and sequencing of the approximately 1.6 kb PCR product obtained with primers VR23/24-south-forward and VR23/24-1-reverse (Table 2b; Fig. 2a) showed that isolate Tv has a deletion of 11,450 bp relative to the WSSV-TW genome (Fig. 2a). A PCR with the primer pair "VR23/24-south" (Table 2b; Fig. 2a) for isolate Kg resulted in a approximately 2.6 kb PCR product, which after cloning and sequencing showed that this isolate contains a deletion of 12,166 bp relative to the WSSV-TW genome (Fig. 2a). We previously mapped 5 SNPs and a 1 bp deletion within WSSV-TW coordinates 16447-16773 (flanking the deletion) compared to WSSV-CN and WSSV-TH (Marks *et al.,* 2004). With respect to these genetic differences, isolate Kg is identical to WSSV-CN and WSSV-TH, suggesting that this isolate is more closely related to these isolates than to WSSV-TW.

Dot plot analysis showed that, except for the *hr*s (van Hulten *et al.,* 2001a), the genomic region in WSSV-TW in which these deletions occur contains the most direct and inverted repeats of the entire WSSV genome (Fig. 2b). However, for the deletion

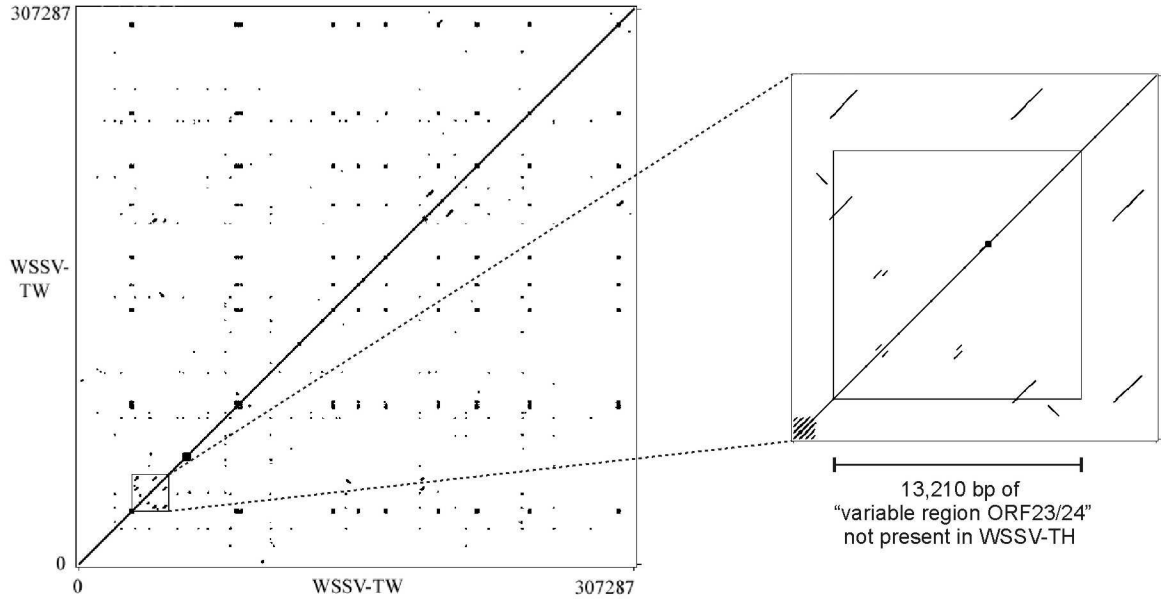


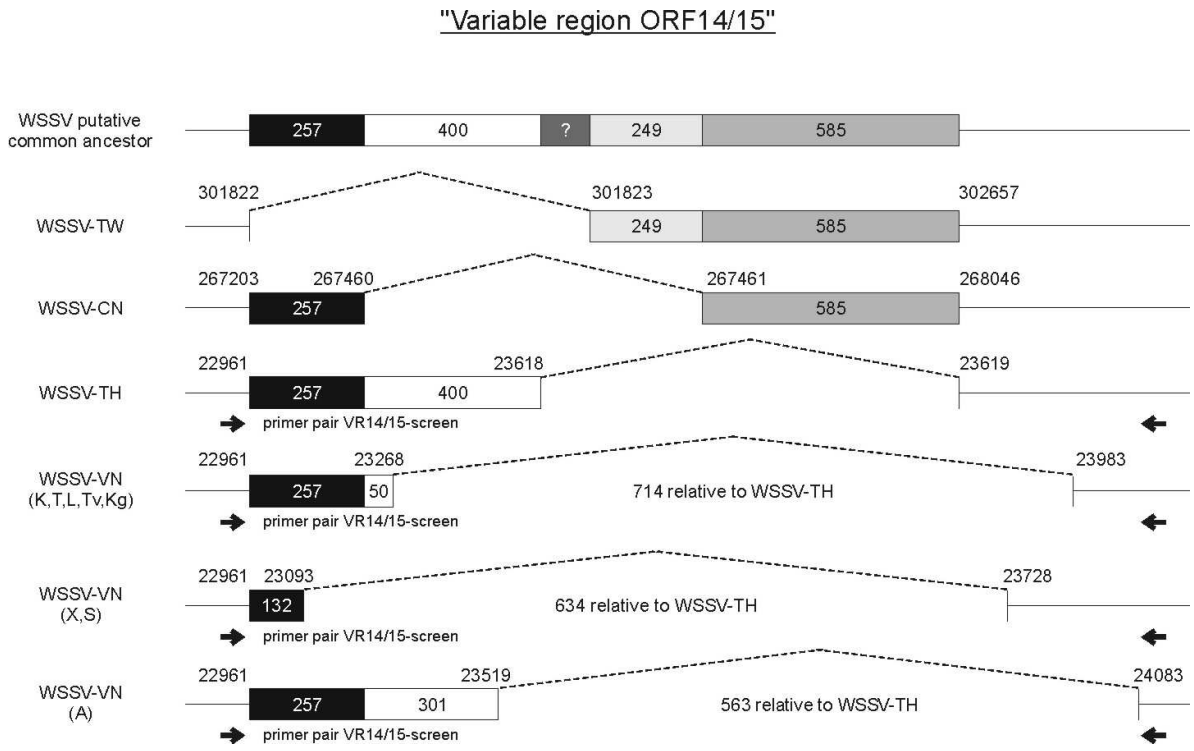
Figure 2b. Dot plot comparison of the nucleotide sequences of WSSV-TW to itself (the adenine residue at the translation initiation codon of VP28 was designated as starting point for the numbering of WSSV-TW in this dot plot), including an enlargement (of original WSSV-TW coordinates 425-20425).

in the VN-south isolates Tv and Kg as well as in the six VN-central isolates no direct repeats, which could be involved in recombination, were identified within 300 bp flanking the putative recombination sites in WSSV-TW. (Fig. 2b; sequence data not shown).

(ii) Variable region ORF14/15

The ”variable region ORF14/15” is centered in a region of 842 bp in size in WSSV-CN, of which 257 bp of its 5’ end is only present in WSSV-TH, while the remaining 585 bp of its 3’ end is only present in WSSV-TW (Fig. 3a; Marks *et al.,* 2004). This locus was thought to be a variable region prone to recombination (Marks *et al.,* 2004). However, a partly characterized isolate recently studied by our laboratory, contains at least all unique sequences present in this locus, suggesting that WSSV-TW, WSSV-CN and WSSV-TH are derived from a common ancestor by deletions of various sizes (Fig. 3a). Because WSSV-TW, WSSV-CN and WSSV-TH each contain unique sequences, these isolates seem to be distinct and probably evolved separately. Using the same strategy as used for the “variable region ORF23/24”, this locus was mapped for all VN isolates using primer set “VR14/15-screen” (Table 2b; Fig. 3a). WSSV-TH DNA, taken as positive control for the PCR, showed the expected fragment of 1,254 bp, whereas the VN isolates showed fragments of different sizes ranging from approximately 500 bp to approximately 700 bp (Fig. 3b). Cloning and sequencing of these fragments revealed that all VN isolates seem to have deletions between 563 bp

(a)



(b)

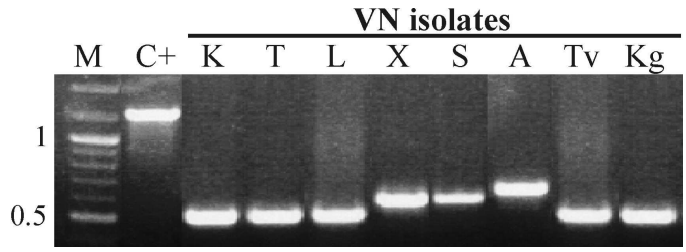


Figure 3. (a) Schematic representation of the "variable region ORF14/15" of the WSSV putative common ancestor, WSSV-TW, WSSV-CN, WSSV-TH, and the VN isolates. Different gray tints represent unique sequences within the WSSV putative common ancestor, WSSV-TW, WSSV-CN, WSSV-TH. The map numbers, indicated above each isolate, are in accordance with the numbers in the NCBI databank for the genomic sequence of each isolate. The coordinates of the WSSV-VN isolates are according to the WSSV-TH annotation. The length of the fragments is indicated within boxes or sequences. Open arrows represent primers. **(b)** PCR on the "variable region ORF 14/15" using genomic DNA of the WSSV-VN isolates as template. The lanes indicate the respective VN isolate used. C+ is the same PCR on genomic DNA of WSSV-TH, used as positive control for the PCR. M represents a 100 bp DNA marker of which some of the sizes are indicated next to the gels (in kb).

and 714 bp relative to WSSV-TH (Fig. 3a). The flanking sequences of the deletions present in the approximately 500 bp to approximately 700 bp fragment were identical to the sequences of WSSV-TH. The VN isolates K, T, L, Tv and Kg had the same deletion of 714 bp, VN isolates X and S had a deletion of 634 bp, while VN isolate A had the smallest deletion of 563 bp compared to WSSV-TH (Fig. 3a).

(iii) A genomic region coding for a putative transposase

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

(iv) Genetic variation in VNTR loci

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

To study the VN-central isolates for each of these loci, we performed a PCR reaction with a specific primer set (Table 2b) flanking the non-*hr* unidirectional tandem repeats. The results for ORF75, ORF94 and ORF125 are shown in Fig. 4. For all three loci a major band was observed for each isolate, often different in size among isolates. The PCR fragments of all VN isolates were cloned, sequenced and aligned. The sequenced regions flanking the tandem repeats (between the primers used and the actual repeats) on both the 5' and the 3' end showed 99.6-100% nucleotide identity with the corresponding sequences of WSSV-TW, WSSV-CN and WSSV-TH. This indicates that the correct fragment had been amplified for each of the three loci of the VN-central isolates, eliminating the possibility of false annealing of the primers.

Table 3a. Number of repeat units present within the non-*hr* unidirectional repeats of ORF75, ORF94 and ORF125

WSSV isolate	ORF75 (45 bp and 102 bp*) / 107965-108675†	ORF94 (54 bp*) / 142744-143067†	ORF125 (69 bp*) / 187899-188312†
TW	21 (16 and 5‡)	6	8
CN	15 (11 and 4‡)	12	8
TH	12 (9 and 3‡)	6	6
VN-central:			
K	5 (3 and 2‡)	10	6
T	5 (3 and 2‡)	17	5
L	5 (3 and 2‡)	10	6
X	5 (3 and 2‡)	7	7
S	14 (10 and 4‡)	7	7
A	6 (4 and 2‡)	10	6

*Length of RUs

†WSSV-TH coordinates of total repeat

‡Number of 45 bp and 102 bp RUs, respectively

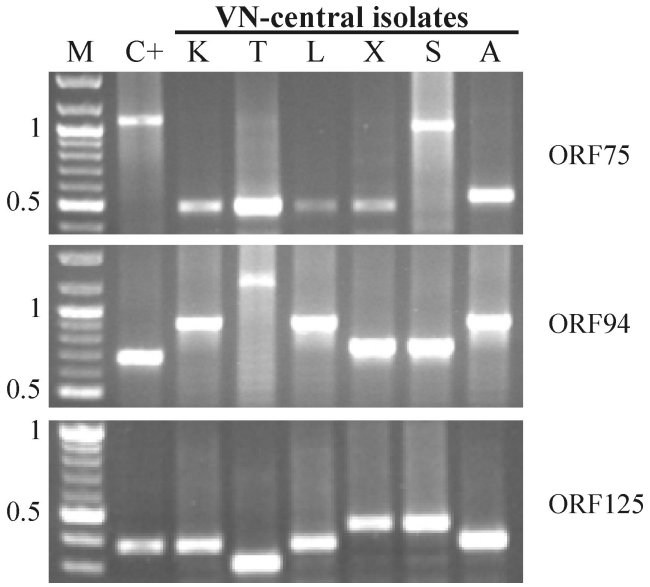


Figure 4. PCR on the non-*hr* unidirectional repeats of ORF75, ORF94 and ORF125, using genomic DNA of the WSSV VN-central isolates as template. The lanes indicate the respective VN isolate used. C+ is the same PCR on genomic DNA of WSSV-TH, used as positive control for the PCR. M represents a 100 bp DNA marker of which some of the sizes are indicated next to the gels (in kb).

ORF75: For all WSSV isolates characterized thus far, ORF75 has two types of RUs with a length of 102 bp and 45 bp, respectively (Table 3b). The first 45 nucleotides of the 102 bp RUs are identical to the RUs of 45 bp. Comparison of all RUs within one isolate showed that they contain SNPs at position 3, 15, 30, 40, 42 and 44, the RUs of 102 bp have an extra SNP at position 83. Each of the RUs can be recognized by its specific SNPs.

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

ORF94: ORF94, in all WSSV isolates characterized thus far, has tandem RUs of 54 bp with a SNP at position 48 (either guanine or thymine) when comparing the RUs mutually within one isolate (Table 3c). The number of RUs was highly variable between the various isolates for which this locus has been characterized: WSSV-TW, WSSV-CN, WSSV-TH, and 55 other isolates originating from Thailand. The number of RUs varied from 6 to 20 repeat units (van Hulten *et al.,* 2000a; Wongteerasupaya *et al.,* 2003; Marks *et al.,* 2004).

Table 3b. Number and position of the RUs located within the non-*hr* unidirectional repeat of ORF75**.**

WSSV isolate	Number of repeat units	Positioning of 45 bp and 102 bp RUs[†]										
		1	2	3	4	5	6	7	8	9	10	11
TW	21	45	102	4*45	102	3*45	102	2*45	102	4*45	102	2*45
CN	15	45	102	4*45	102	2*45	102	2*45	102	2*45		
TH	12	45	102	4*45	102	2*45	102	2*45				
VN-central												
K	5	102	45	102	2*45							
T	5	102	45	102	2*45							
L	5	102	45	102	2*45							
X	5	102	45	102	2*45							
S	14	45	102	4*45	102	45	102	2*45	102	2*45		
A	6	102	2*45	102	2*45							

[†]Number of successive tandem repeat units of 45 bp are summarized as x*45

The WSSV-VN-central isolates contained between 7 and 17 RUs (Table 3a), corresponding to the respective sizes of their PCR fragments (Fig. 4). The identity of the nucleotide at position 48 of each of the VN isolates is shown in Table 3c. Isolates X and S are identical, while the other isolates, although some having the same number of RUs, all have a unique pattern of the nucleotides at position 48. The VN isolates K, T and L had a thymine deletion at position 143149 (WSSV-TH coordinates), located in the 3' end flanking the repeat. As this is outside the coding region, it will not cause a frameshift in ORF94.

ORF125: ORF125 contains tandem RUs of 69 bp, of which the first two as well as the last can be recognized by their specific SNPs when comparing the RUs mutually within one isolate (Table 3d). The other RUs (the 3rd till the penultimate) contain SNPs at position 8, 18, 25, 66 and 69 (Marks *et al.*, 2004). The WSSV-VN-central isolates contained between 5 and 7 RUs (Table 3a), corresponding to the respective sizes of their PCR fragments (Fig. 4). VN isolates X and S, as well as VN isolates A and L, are identical in this locus (Table 3d). The genotype of the VN isolates A and L is identical to the genotype of WSSV-TH (Table 3d).

Table 3c. Genotype of the SNP of each RU within the non-*hr* unidirectional repeat of ORF94

WSSV isolate	Number of repeat units	Successive RU*																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
TW	6	T	T	T	G	T	T											
CN	12	T	T	G	G	G	G	G	G	T	T	T	T					
TH	6	T	G	G	G	T	T											
†Thai:																		
Sur #1	9	T	T	T	T	G	T	T	G	T								
Sur #2	8	T	T	G	T	T	G	G	T									
Chu #2	7	T	T	T	G	T	G	T										
Chu #3	8	T	T	G	T	T	G	G	T									
Chu #4	6	T	T	T	G	G	G											
VN-central:																		
K	10	G	G	G	T	T	T	G	G	T	T							
T	17	G	T	T	T	T	G	T	T	T	G	T	G	G	G	G	T	T
L	10	G	G	G	G	G	G	G	G	G	G							
X	7	T	T	T	T	G	T	T										
S	7	T	T	T	T	G	T	T										
A	10	G	G	G	T	G	G	T	T	T	T							

*Genotype of each of the successive RU at position 48 (guanine (G) or thymine (T)) is shown

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

Table 3d. Genotype of the SNPs of each RU within the non-*hr* unidirectional repeat of ORF125

WSSV isolate	Number of repeat units	Successive RU*†						
		C	D	E	F	G	H	I
TW	8	TGGTC	-	TGGTC	TGGTC	-	TTGGT	CGAGT
CN	8	TGGTC	TTGGT	TGGTC	-	TTGGT	TTGGT	-
TH	6	TGGTC	-	-	-	-	TTGGT	CGAGT
VN-central:								
K	6	TGGTC	-	TGGTC	-	-	TTGGT	-
T	5	TGGTC	-	-	-	-	TTGGT	-
L	6	TGGTC	-	-	-	-	TTGGT	CGAGT
X	7	TGGTC	-	TGGTC	-	-	TTGGT	CGAGT
S	7	TGGTC	-	TGGTC	-	-	TTGGT	CGAGT
A	6	TGGTC	-	-	-	-	TTGGT	CGAGT

* The order of the RUs is kept, but the RUs are categorized (C-I) by genotype, starting from the 3rd RU (C) to the penultimate RU (I). In case no RU is present, it is indicated by -

†Genotype of each successive RU at position 8, 18, 25, 66 and 69, respectively, is shown.

(v) Fragment encoding part of DNA polymerase

To further classify the WSSV-VN isolates, a PCR was performed on a conserved genomic fragment encoding part of WSSV DNA polymerase using primer set "Polymerase" (Table 2b). Within this genomic fragment, a single nucleotide deletion occurs in WSSV-CN (WSSV-TH coordinates 36030) compared to WSSV-TW and WSSV-TH, causing a frameshift in the polymerase gene (Chen *et al.*, 2002; Marks *et al.*, 2004). The WSSV-VN isolates gave a PCR fragment of the similar size as the positive control WSSV-TH. Cloning and sequencing of the 8 PCR fragments of the central and south VN isolates failed to detect an adenine deletion as is present in WSSV-CN. The PCR fragments showed 100% nucleotide identity with the respective fragments of WSSV-TW and WSSV-TH.

(vi) Restriction enzyme analysis of VN isolate T

The RFLP analysis between WSSV-TH and WSSV-VN-T is shown in Fig. 5. The *Bam*HI restriction pattern of WSSV-TH exactly matches the expected pattern based on the complete nucleotide sequence (van Hulten *et al.*, 2001), except for the 3 smallest fragments which are not visible due to their estimated size of <1 kb. Two clear polymorphisms (shifts) are visible between WSSV-TH and VN isolate T, indicated with A and B, respectively. Shift A, in which a fragment of approximately 27.5 kb for VN isolate T shifts to approximately 24.5 kb for WSSV-TH, can be explained by the observed sequence diversity in "variable region ORF 14/15" and "variable region ORF 23/24", which are both located on this large fragment. The approximately -3 kb

discrepancy is the sum of the observed differences in PCR mapping of both "variable regions" of approximately 0.7 kb and approximately -3.7 kb, respectively (Fig. 3a & 2a). Shift B, in which a corresponding fragment has a size of approximately 11.2 kb for WSSV-TH and of approximately 11.8 kb for VN isolate T, can be explained by the sequence variation of the repeat in ORF94 (Table 3a). The difference of 11 RUs of each 54 bp results in a shift of 594 bp. The differences in the repeats in ORF75 and ORF125 are not clearly visible. ORF75 is located on a large fragment (approximately 20 kb) for which the 350 bp difference in size will only show a minor shift, whereas the difference in the repeats of ORF125 between WSSV-TH and VN isolate T is marginal (138 bp).

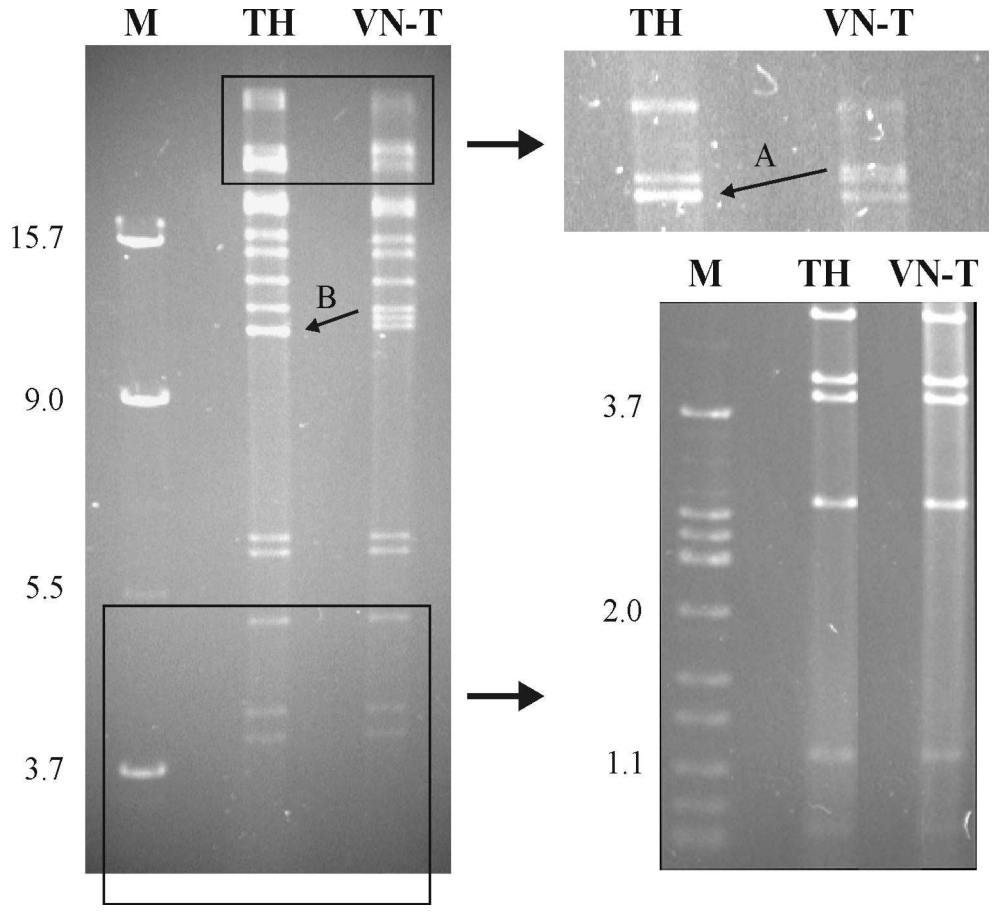


Figure 5. WSSV genomic DNA of WSSV-TH (TH) and WSSV-VN isolate T (VN-T) digested with *Bam*HI. M represents a molecular size standard (lambda digested with *Bam*HI-*Eco*RI-*Hin*dIII), of which some of the sizes are indicated next to the gels (in kb). The enlargements focus on the major (>20 kb) and minor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

Discussion

Genomic analyses of WSSV showed that conserved genes, often used in molecular epidemiological studies to unravel evolutionary relationships by phylogenetic analysis, are too homologous to use for this purpose in case of WSSV (Marks *et al.*, 2004). For example, the complete DNA polymerase gene of WSSV only contains 3 SNPs and a 1 bp and 3 bp deletion when comparing this gene for the three completely sequenced WSSV isolates (Chen *et al.,* 2002; Marks *et al.* 2004). Similar high homologies were found for other conserved WSSV genes (Chang *et al.,* 2001; Marks *et al.,* 2004). Moreover, also the major structural protein genes, which for some virus families show a relative high number of mutations due to antigenic drift or adaptation to different hosts, show 99.5%-100% nucleotide identity between several geographical WSSV isolates (Moon *et al.,* 2003; Marks *et al.,* 2004). These data indicate that the isolates of WSSV identified thus far are very closely related and probably evolved recently from a common ancestor. The DNA polymerase sequences obtained from all VN isolates, showing 100% identity with WSSV-TW and WSSV-TH, further confirmed this observation. Therefore, we chose the most variable loci of WSSV to classify new WSSV isolates from Vietnam (Marks *et al.,* 2004). The RFLP analysis between WSSV-TH and WSSV-VN-T (Fig. 5) confirmed the high degree of homology among WSSV isolates, but indeed identified the major genomic insertions and deletions in WSSV-VN (isolate T).

Based on both “variable region ORF23/24” and “variable region ORF14/15”, we propose a model to explain the genotypic changes of WSSV during its geographical spread from either site of the Taiwan Strait towards the west to Thailand between 1992 and 1995 (Fig. 1b). In this model, both loci evolved independently, and both deletions in the “variable regions” showed a progressive increase in length during the spread of WSSV. The WSSV common ancestor (Fig. 1b) contains a genotype similar to WSSV-TW in the “variable region ORF23/24” (Fig. 2a) and a genotype similar to the putative common ancestor in “variable region ORF14/15” (Fig. 3a). WSSV-TW evolved from this common ancestor by a deletion in “variable region ORF14/15”, while WSSV-CN evolved by a deletion of approximately 1.2 kb in “variable region ORF23/24” (Fig. 1b: An-1) followed by a deletion in “variable region ORF14/15”. Based on the observation that the genotypes of the VN isolates seem to have evolved from a genotype similar to WSSV-TH in “variable region ORF14/15” by separate unique deletions of different sizes, the VN isolates and WSSV-TH probably have a common lineage, which branched off at an early stage from WSSV-TW and WSSV-CN. However, the extra sequences in the “variable region ORF23/24” present in the VN isolates compared to WSSV-TH exclude the possibility that the WSSV-VN isolates are derived from WSSV-TH. Therefore, WSSV-TH and the WSSV-VN isolates probably have a

common ancestor An-3 (Fig. 1b), which could contain the genotype of WSSV-TH in "variable region ORF14/15", but the approximately 8.5 kb deletion similar to the VN-central isolates in "variable region ORF23/24". Within the three different WSSV-VN genotypes in "variable region ORF14/15", each contains unique sequences, and thus probably evolved separately. Therefore, WSSV entered Vietnam by multiple introductions from the common ancestor An-3, from where it further spread within Vietnam (VN isolate Kg; Fig. 1b). WSSV isolates, collected along the further coast of South-East Asia (i.e. isolates from north-Vietnam, China (Hainan) and Cambodia), are genotyped to confirm and further detail this model.

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

Based on the genetic make-up in both "variable regions" and the thymine deletion shared by the isolates K, T and L in the 3' flanking region of the repeat located in ORF94, three groups of VN-central isolates can be distinguished ((K, T, L) and (X, S) and (A); Fig. 1b). Within these groups, each of the non-*hr* unidirectional tandem repeats located in ORF75, ORF94 and ORF125 seem to have their own, independent genesis in terms of insertion or deletion of repeat units (Table 3). Possibly, insertion or deletion of repeat units are generated during homologous recombination or replication slippage, as is proposed for repeats such as the baculovirus homologous repeats (*hr*s) (Garcia-Maruniak *et al.,* 1996) and the herpesvirus direct repeats (DRs) (Umene, 1991).

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

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

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

Acknowledgements

This work was supported by Intervet International BV, Boxmeer, the Netherlands. We thank NUFFIC for sponsoring the internship of Bui Thi Minh Dieu at Wageningen University in the Netherlands, and Dr. Rommert C. van den Bos and Bert M. Wennekes for their help and hospitality. We thank Arnold Kuźniar for preparation of the dotplots. We thank Dr. Mariëlle C.W. van Hulten for her continued interest and advice.

Chapter 3

Valuation of White Spot Syndrome Virus variable DNA loci as molecular markers of virus spread at intermediate spatiotemporal scales

Abstract

Variable genomic loci have been employed in a number of molecular epidemiology studies of white spot syndrome virus (WSSV), but it is unknown which loci are suitable molecular markers for determining WSSV spread on different spatiotemporal scales. Although previous work suggests that multiple introductions of WSSV occurred in central Vietnam, it is largely uncertain how WSSV was introduced and subsequently spread. Here, we evaluate five variable WSSV DNA loci as markers of virus spread on an intermediate (i.e. regional) scale, and develop a detailed and statistically-supported model for the spread of WSSV. The genotypes of seventeen WSSV isolates from along the coast of Vietnam – nine of which were newly characterized in this study – were analyzed to achieve sufficient samples on an intermediate scale and to allow statistical analysis. Only the ORF23/24 variable region is an appropriate marker on this scale, as geographically-proximate isolates show similar deletion sizes. The ORF14/15 variable region and variable number tandem repeat (VNTR) loci are not useful as markers on this scale. ORF14/15 may be suitable for studying larger spatiotemporal scales, whereas VNTR loci are probably suitable for smaller scales. For ORF23/24, there is a clear pattern in the spatial distribution of WSSV: the smallest genomic deletions are found in central Vietnam, and larger deletions are found in the south and the north. WSSV genomic deletions tend to increase over time with virus spread in cultured shrimp, and our data are therefore congruent with the hypothesis that WSSV was introduced in central Vietnam and then radiated out.

Key words: White spot syndrome virus, molecular epidemiology, VNTR, variable loci, PCR genotyping

This chapter has been published as: Dieu, B. T. M., Marks, H., Zwart, M.P. & Vlak, J. M. (2010). Valuation of White Spot Syndrome Virus variable DNA loci as molecular markers of virus spread at intermediate spatiotemporal scales. *Journal of General Virology* **94**: 1164-1172.

Introduction

White spot syndrome virus (WSSV), the major cause of large economic losses in shrimp farming, is a large, double stranded DNA (dsDNA) virus belonging to the family Nimaviridae, genus Whispovirus (Vlak *et al.*, 2005). WSSV was first discovered in 1992 in Taiwan and China, and the virus subsequently spread quickly to most countries in South-East Asia, the Indian subcontinent, and north and south America. In addition to shrimp, WSSV can infect a broad range of crustaceans including crabs and crayfish. This broad host range is thought to be a major cause of the rapid and extensive spread of WSSV (Flegel, 1997).

Molecular methods for genotyping WSSV isolates are powerful tools for understanding viral spread and epidemiology (Marks *et al.*, 2004; Dieu *et al.*, 2004; Pradeep *et al.*, 2008a,b). Initial studies using molecular methods to compare WSSV isolates suggested that genetic differences between various isolates were small, as these studies used insensitive techniques such as restriction fragment length polymorphism (RFLP; Lo *et al.*, 1996a,b; Nadala & Loh, 1998; Lo *et al.*, 1999, Wang *et al.*, 2002, Moon *et al.*, 2003). However, differences between WSSV isolates could be detected readily by using more sensitive methods, such as PCR. Lan *et al.* (2002) for example found host-dependent differences among WSSV isolates with a PCR-based method. Marks *et al.* (2004) aligned three completely sequenced WSSV isolates, originating from Taiwan (WSSV-TW) (Wang *et al.*, 1995), China (WSSV-CN) (Yang *et al.*, 2001) and Thailand (WSSV-TH) (Van Hulten *et al.*, 2001). Although the overall nucleotide identity was more than 99%, five variable loci were identified, consisting of two regions with genomic deletions (ORF23/24 and ORF14/15 variable regions) and three loci with a variable number tandem repeats (VNTR) (ORF75, ORF94 and ORF125) (Marks *et al.*, 2004).

A number of subsequent studies on WSSV epidemiology have used the regions with genomic deletions (Musthaq *et al.*, 2006; Waikhom *et al.*, 2006; Pradeep *et al.*, 2008b) or one or more VNTR loci (Hoa *et al.*, 2005; Kiatpathomchai *et al.*, 2005; Kang & Lu, 2007; Pradeep *et al.*, 2008a; Tan *et al.*, 2009), or both (Dieu *et al.*, 2004; Marks *et al.*, 2005; Pradeep *et al.*, 2009) as genetic markers to characterize WSSV variants. VNTRs appear to be more variable than the deletions (Dieu *et al.*, 2004). High degrees of polymorphism for VNTR-like loci have been reported in various other large dsDNA viruses such as Cytomegaloviruses (Davis *et al.*, 1999). This suggests that, whilst VNTRs may be useful for studying WSSV spread on small spatiotemporal scales, genomic deletions are more suitable for studying spread on intermediate and large scales. We use the following terms to describe different scales on which WSSV has spread: (i) very small: spread between ponds and farms (10 km), (ii) small: spread

between clusters of shrimp farms and villages (100 km), (iii) intermediate: regional spread within and between countries (1000km), and (iv) large: continental and global spread (10,000 km). A systematic comparison of the two approaches – using VNTRs or genomic deletions – to studying spread on these different spatial scales has not been reported.

The presence of WSSV in Vietnam (VN) was first confirmed by PCR analyses on samples collected in 1997 (Corsin *et al.*, 2001), but it is not clear how many times the virus was introduced from abroad. Also, it is unclear where WSSV originated and how it subsequently spread to other regions in Vietnam from the original introduction site(s). Epidemiological studies have been faced with numerous design and execution problems, making it difficult to infer WSSV spread based on farmer reports of shrimp health and screening ponds for WSSV (Corsin *et al.,* 2002). Finding suitable methods to identify and discriminate WSSV strains – and infer their origin – is therefore important for WSSV forensics and epidemiology. We previously reported a preliminary study of genomic variation in central Vietnam, based on the mapping of deletions and VNTRs of eight WSSV isolates. Vietnam is an ideal location to study the spatiotemporal spread and evolution of WSSV because of the relatively late introduction of large-scale shrimp culture, the moderate socio-economic development around the turn of the millennium, small-size farming operations and an accurate WSSV reporting system. Our previous results suggested that WSSV originated from a common ancestor – reported in Taiwan – and subsequently spread to Vietnam through multiple introductions (Dieu *et al.*, 2004). However, WSSV isolates from important shrimp production regions in northern and southern Vietnam were not available during the previous study. Isolates originating from these regions are important to develop a statistically-supported model of the introduction and spread of WSSV in Vietnam. This analysis is now even more relevant as WSSV has become more virulent over time, which could be attributed to the above genomic mutations (Marks *et al*., 2005a).
Here we study genomic variation in WSSV isolates obtained from all important shrimp-production regions in Vietnam. We performed genomic analysis on two northern, one central, and six southern WSSV isolates. For our analysis we used the five variable loci described by Marks *et al.* (2004). These loci were characterized for each Vietnamese isolate by PCR amplification, cloning and sequencing. We could further test and validate our previous hypothesis on the spread of WSSV in Vietnam (Dieu *et al.*, 2004). Finally, we could for the first time systematically evaluate the utility of each of the variable regions as genetic markers for studying WSSV spread and epidemiology on an intermediate spatiotemporal scale.

Materials and methods

Infected shrimp sampling

The origin of the WSSV infected shrimp (*Penaeus monodon*) analyzed in this study is shown in Table 1. The shrimp were cleaned with 70% ethanol and kept in 96% ethanol during transportation to Can Tho University (Vietnam). After transportation, the ethanol was removed and the samples were stored at -20° C until further processing.

Analysis of variable loci

DNA extracts of collected shrimp, primarily from gill tissue, were screened for the presence of WSSV with specific primers for *VP26*, as described by Dieu *et al.* (2004). PCR on the genomic variable loci of WSSV was performed with 250ng DNA extract using Taq DNA polymerase (Promega). The specific primer sets, PCR conditions used and sizes of the PCR products are shown in Table S1. PCR products were cloned, sequenced and analyzed according to published procedures (Dieu *et al.*, 2004).

Statistical Analysis

All statistical analysis was performed in SPSS 15.0 (SPSS Inc., Chicago, IL, USA). We considered the following quantitative traits for different loci: (i) the number of repeat units (RUs) for VNTR loci with one repeat type (ORF94 and ORF125), (ii) the total number of RUs, regardless of the identity of the repeat, for the VNTR locus with multiple repeat types (ORF75), or (iii) the size of the genomic deletion (ORF23/24 and ORF14/15 regions). WSSV isolates were given an ordinal code corresponding to their relative location along the Vietnamese coast, from north to south (e.g. VN-HP = 1, VN-ND = 2, […], VN-BL = 14, VN-CM = 15, VN-Kg = 16, VN-HT = 17), and a 'runs test' (Wald & Wolfowitz, 1940) was then performed. A 'run' is a series of consecutive samples with a trait value greater than or less than the cut off point, a threshold value for which we used the mean of a trait. This procedure tests whether the number of runs in a sample is greater than or lower than the number of runs expected if trait values are independent for each sample. If WSSV spread along the Vietnamese coast, a suitable genetic marker should give a significantly smaller numbers of runs than expected by chance, because geographically-proximate isolates (i.e. from consecutive locations along the coast) are likely to have similar trait values.

A Jonckheere-Terpstra test (see Bewick *et al.*, 2004) was used to determine whether median RU number or deletion size increased or decreased when the samples were ordered from north to south. We tested five variable loci, and a Šidák correction (Sokal & Rolff, 1995) was therefore made to the significance threshold α for both the

runs test and the Jonckheere-Terpstra test, such that the threshold *P*-value is $\alpha' = 1-(1-\alpha)^{1/n} = 1-(1-0.05)^{1/5} = 0.010$.

Results

Description of WSSV isolates

WSSV-infected *P. monodon* shrimp were collected from nine shrimp culture ponds in Vietnam in 2004 and subsequently genotyped (Table 1). The ponds from which samples we collected were distributed over nine different provinces, covering 2,500 km of the Vietnamese coast (Fig. 1). Juvenile shrimp were selected for WSSV testing if (i) there was a WSSV outbreak in the pond, or (ii) the shrimp showed reduced feeding. All chosen shrimp tested positive for the presence of WSSV using a single-step PCR for *VP26*. One WSSV-infected shrimp from each pond was chosen randomly for further analysis, and assumed to be representative of that pond and region.

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

Region in Vietnam	Name of the pond	Place (district)	Province	Origin of postlarvae	Date of collection	Abbre-viation
north	Hai Phong	Do Son	Hai Phong	central region	14/08/2004	HP
	Nam Dinh	Nam Dinh	Nam Dinh	central region	01/09/2004	ND
central	Hue	Phu Vang	Hue	central region	28/06/2004	H
couth	Ba Ria	Xuyen Moc	Ba Ria	Unknown	22/02/2004	BR
	Tra Vinh -b	Duyen Hai	Tra Vinh	Local	10/02/2004	Tv-b
	Soc Trang	My Xuyen	Soc Trang	Unknown	05/03/2004	ST
	Bac Lieu	Vinh Loi	Bac Lieu	Unknown	15/2/2004	BL
	Ca Mau	Tan Thanh	Ca Mau	Local	20/02/2004	CM
	Ha Tien	Thuan Yen	Kien Giang	central region	10/02/2004	HT

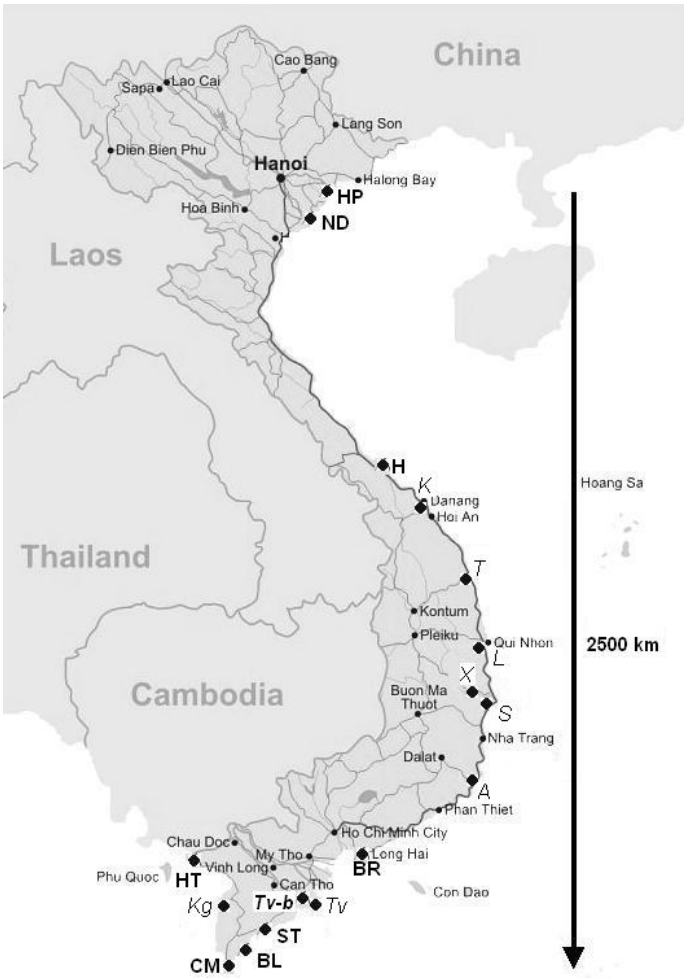


Figure 1. Map of Vietnam, showing the geographical origins of the isolates used for this study, indicated by HP, ND, H, BR, TV-b, ST, BL, CM and HT (in bold), respectively, according to Table 1. The isolates K, T, L, X, S, A, Tv and Kg, used in our previous study (Dieu *et al*., 2004), are also in indicated (in italics).

Variable region ORF23/24

In order to map the ORF23/24 locus, we first performed a PCR with the "VR23/24-screen" primers on all samples (Table S1). These primers were previously used to detect deletions in six WSSV isolates from central Vietnam (Dieu *et al.*, 2004). Only isolate H - from central Vietnam - tested positive, rendering a 548 bp amplicon (Fig. 2). Cloning and sequencing of this PCR fragment indicated that isolate H was identical to the other VN-central WSSV isolates, with a deletion of about 8,539 bp compared with WSSV-TW. New primers were developed to genotype "variable region ORF23/24" for the other isolates from across Vietnam. VN isolate ND gave a product of approximately 3.8 kb with primer set VR23/24-ND; HP gave a product of approximately 850 bp with primer set VR23/24-HP. Both PCR products were

sequenced to reveal the exact identity of the deletions up to the nucleotide level (Fig. 2). Overall the data indicate increasing deletion size of the isolates from central Vietnam relative to the ND and HP isolates from the north (Fig. 2). Isolates from southern Vietnam produced unique PCR amplicons with a different set of primers (VR23/24-south) indicating a larger deletion. ST, BL, CM, and HT all gave the same PCR product of approximately 400 bp which, after sequencing, indicated that they contained the same deletion of 11,866 bp, as compared with the WSSV-TW sequence. Isolate BR produced an approximately 400 bp amplicon with this primer set. Sequencing indicated a 13,210 bp deletion, identical to WSSV-TH (Dieu *et al*., 2004). PCR on the Tv-b isolate using the same primers resulted in an approximately 1.6 kb amplicon, similar to in size to Tv, a previously analyzed isolate but from a different district in Tra Vinh province. Restriction enzyme analysis of the PCR products confirmed that the amplified sequences are the same (data not shown). Together, the data indicated that WSSV isolates show an increasing deletion size from central to southern Vietnam (Fig. 2).

Table S1. Primers used in PCR analysis for the variable loci of WSSV, the size of the PCR products is dependent on the isolate to be genotypes and therefore not mentioned in this table.

Primer pair name	Primer orient.	Sequence (5'-3')	Anneal. Temp. (^{0}C) / elongation time (s)	WSSV-CN sequence coordinates
VR23/24 - ND	Forward	CACCCCTTCTCTAAATAATC	51 / 200	30402-30421*
	Reverse	ATGATTGTATTCGTCGAAGG		286706-286687
VR23/24 - HP	Forward	CAGATAATGCAAACACGAGACAC	51 / 120	275794-275816
	Reverse	ATGATTGTATTCGTCGAAGG		286706-286687
VR23/24 - screen	Forward	CACACTTGAAAAATACACCAG	49 / 75	278179-278199
	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086
VR23/24 - south	Forward	GTAGTGCATGTTTCTCTAAC	49 / 100	275032-275051
	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086
VR23/24 -Tv	Forward	CTACAACGGCCAAGTCAT	49 / 100	274802-274819
	Reverse	ATGATTGTATTCGTCGAAGG		286706-286687
VR23/24 – BR	Forward	GAGTAGTCTTCAATGGCAATGT	55/80	275008-275029
	Reverse	GATGACTCGGTACGCTTTAG		287376-287357
VR14/15-screen	Forward	GAGATGCGAACCACTAAAAG	49 / 75	22904-22923*
	Reverse	ATGGAGGCGAGACTTGC		24157-24141*
VR14/15-1	Forward	GAGATGCGAACCACTAAAAG	49 / 80	22904-22923*
	Reverse	GAAAAATAAATCACGGGCTAATC		23646-23624*

* According to the WSSV-TH sequence (van Hulten *et al*., 2001)

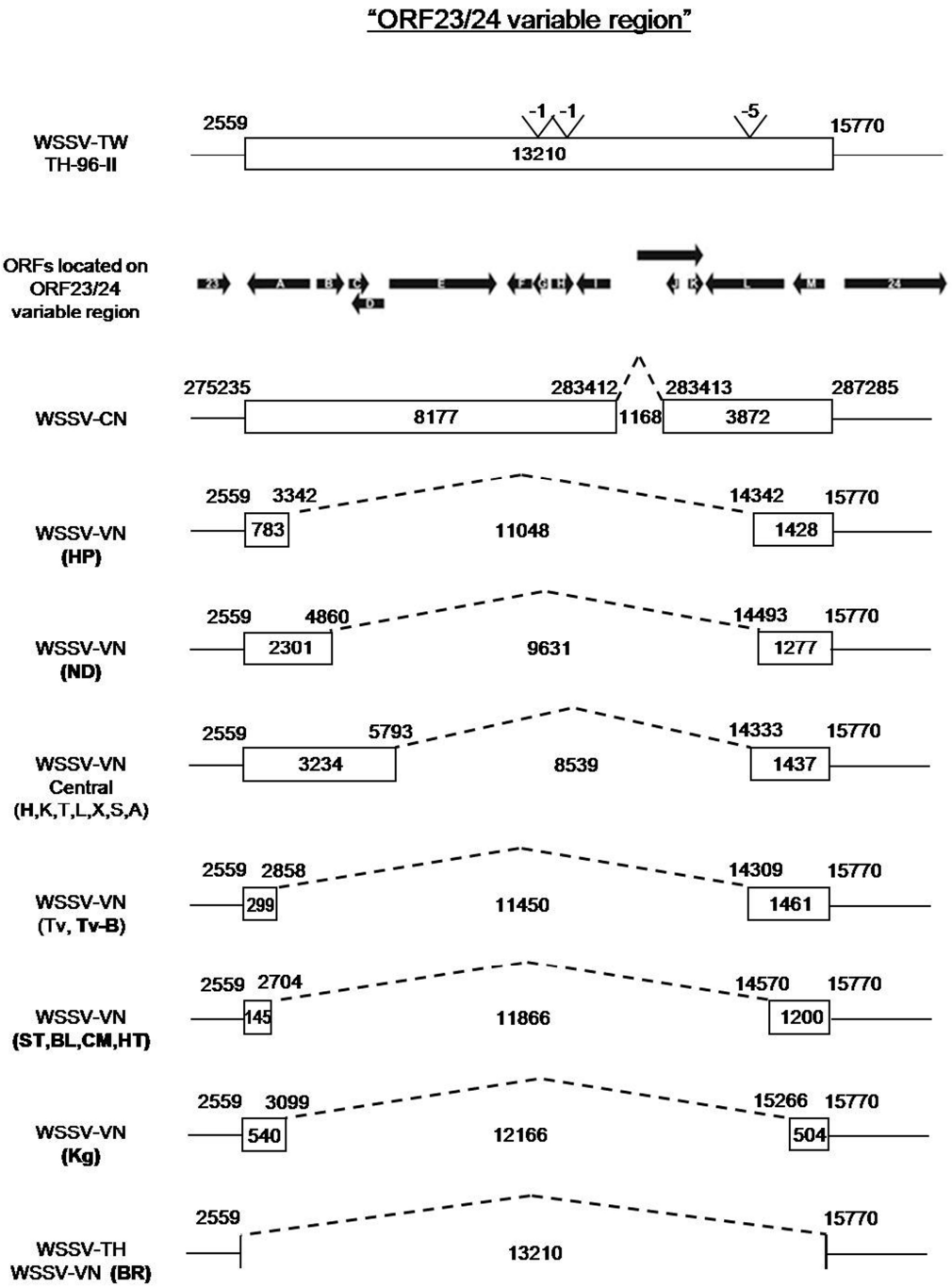


Figure 2. Schematic representation of the "variable region ORF 23/24" of WSSV-TW, WSSV-TH-96-II, WSSV-CN, WSSV-TH, and the VN isolates. The map numbers, indicated above each isolate, are in accordance with the numbers in the NCBI databank for the genomic sequence of each isolate. The coordinates of the WSSV-VN isolates are according to the WSSV-TW annotation. The length of the fragments is indicated within boxes or sequences. The positions of the ORFs located in this region are indicated by closed arrows, which also represent the direction of transcription. ORFs are numbered in accordance with the numbering used by Marks *et al*., 2004 and Dieu *et al*., 2004. Open arrows represent primers.

Variable region ORF14/15

The TH-96-II isolate has an additional 6,436 bp segment region in the ORF14/15 variable region compared with all other known WSSV isolates, and Marks *et al.* (2005) suggested that this genotype is representative of the common ancestor of WSSV in South-East Asia. The coordinates and size of the genomic deletions in ORF14/15 were determined for the new Vietnamese isolates using a similar approach as was used for the ORF23/24 variable region. TH-96-II was used as a reference sequence for determining the size of the deletion. A PCR reaction with the "VR14/15-screen" primers (Table S1; Fig. 3a) was performed. Almost all the new WSSV-VN isolates (ND, HP, H, ST, BL, CM and Tv-b) showed an approximately 500 bp amplicon, similar in length to that reported previously for isolate K (Fig. 3b). Restriction enzyme analysis of the PCR products confirmed that these isolates have the same 6,031 bp deletion as most WSSV-VN isolates analyzed previously (Fig. 3a; Fig. 3c; Dieu *et al.*, 2004). However, the HT and BR isolates failed to give a PCR product with this primer set. Using the VR14/15-1 primers, an approximately 900 bp product was obtained for the HT isolate, whilst an approximately 750 bp product was obtained for the BR isolate (Table S1). Cloning and sequencing of these PCR products showed that isolate HT had a genotype similar to that of WSSV-TW, whilst BR was similar to WSSV-TH (Fig. 3a).

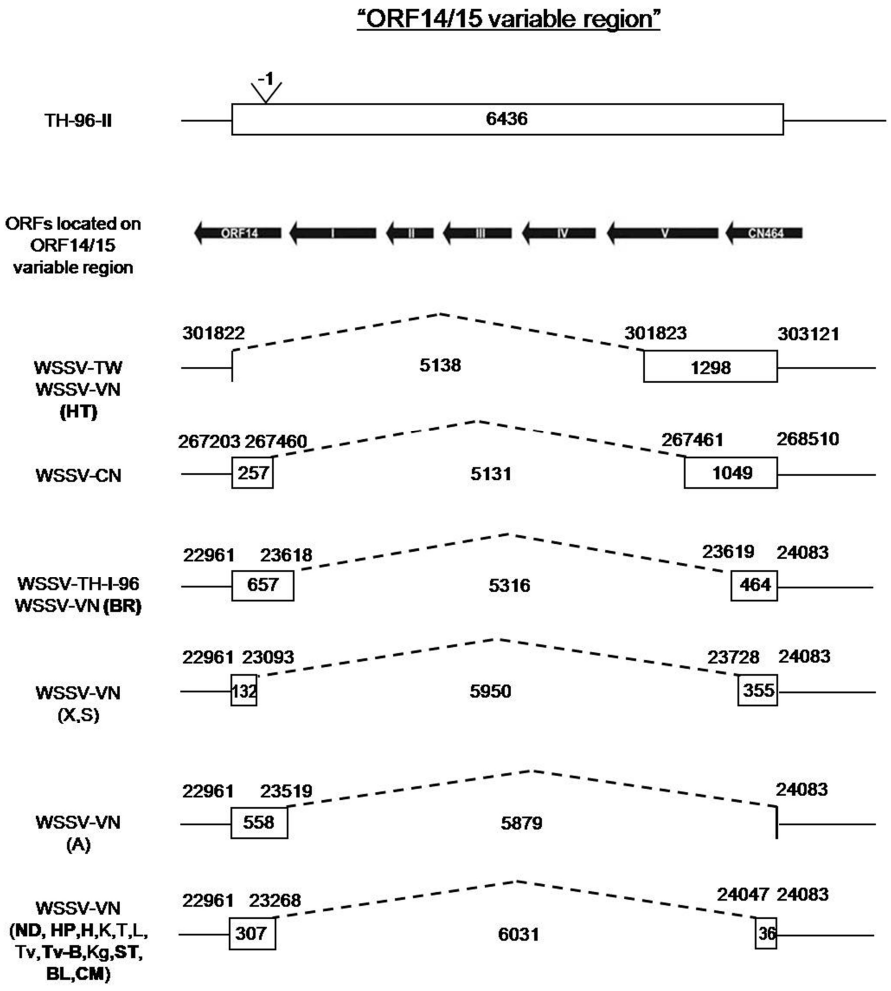
VNTR loci ORF75, ORF94 and ORF125

The known WSSV VNTR loci were also analyzed. ORF75 possesses two types of repeat units (RUs), with lengths of 102 and 45 bp. ORF94 and ORF125 have RUs with a single type of repeat sequence of 54 bp and 69 bp, respectively. These loci were analyzed for the nine WSSV-VN isolates (Table 2) by PCR and sequencing. The number of RUs present in ORF94 ranges from four to seventeen, whereas those from ORF125 range from four to ten. The RU unit of 102 bp appeared to be present at a frequency ranging from one to four, whereas the 45 bp repeat is present between five to forteen times (Table 2). This variation in RUs is very similar to what has been found previously in other Vietnamese WSSV isolates (Dieu *et al.*, 2004; Hoa *et al.*, 2005). Overall, however, there do not seem to be any trends in these data (Table 2).

Statistical Analysis

To test which loci are suitable as genetic markers for inferring WSSV spread on an intermediate scale, a runs test was performed on the number of repeat units (ORF75, ORF94 and ORF124) or the size of genomic deletion (ORF 23/24 and ORF14/15). The ORF23/24 variable region was the only one giving a significant outcome for this test

(a)



(b)

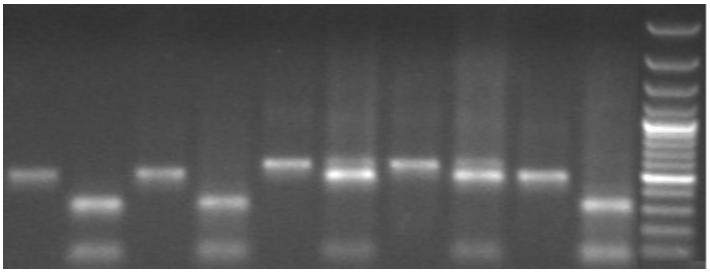


Figure 3. (a**)** Schematic representation of the "variable region ORF14/15" of the WSSV putative common ancestor (WSSV-TH96-II), WSSV-TW, WSSV-CN, WSSV-TH, and the VN isolates. The map numbers, indicated above each isolate, are in accordance with the numbers in the NCBI databank for the genomic sequence of each isolate. The length of the fragments is indicated within boxes or sequences. Open arrows represent primers. (b) Restriction enzyme analysis using *Nco*I and *Pst*I of PCR products of WSSV-VN isolates with primer VR14/15-screen.

Table 2. Number of repeat units (RUs) present within the non-*hr* unidirectional repeats of ORF75, ORF94 and ORF125 (Van Hulten *et al.*, 2001). WSSV isolates genotyped within the context of the current study are in bold, those genotyped by Dieu *et al.* (2004) are regular text.

Region	WSSV isolate	ORF75 (45 bp and 102 bp*) / 107965-108675†	ORF94 (54 bp*) / 142744-143067†	ORF125 (69 bp*) / 187899-188312†
north	**HP**	12 (9 and 3)	9	10
	ND	7 (6 and 1)	4	‡
central	**H**	5 (3 and 2)	12	5
	K	5 (3 and 2)	10	6
	T	5 (3 and 2)	17	5
	L	5 (3 and 2)	10	6
	X	5 (3 and 2)	7	7
	S	14 (10 and 4)	7	7
	A	6 (4 and 2)	10	6
south	**BR**	5 (3 and 2)	8	7
	Tv	§	§	§
	Tv-b	6 (4 and 2)	10	9
	ST	5 (3 and 2)	4	5
	BL	6 (4 and 2)	‡	9
	CM	‡	9	4
	Kg	5 (3 and 2)	15	7
	HT	5(4 and 1)	11	6

*Length of RUs
†WSSV-TH coordinates of total repeat
‡PCR reaction gave no product.
§: no data.

Table 3. Statistical analysis of data for all five genomic loci. The runs test determines whether the number of runs in a sample is greater or less than the number of runs expected if the outcomes for a trait were independent. A suitable genetic marker should give a significant *P*-value (indicated by an asterisk), indicating that repeat unit (RU) or deletion size values of consecutive geographic samples are not independent but related. The Jonckheere-Terpstra test was used to determine whether RU or deletion size values increase or decrease when the samples are ordered along the Vietnamese coast, from north to south. For all statistical tests, the significance threshold was adjusted ($\alpha' = 0.010$) because multiple comparisons are being made. Stand. *JT* = standard JT-value

Variable Locus	**Cases**	**Runs Test**			**Jonckheere-Terpstra Test**	
		Runs	*Z*-value	*P*-value	Stand. *JT*	*P*-value
ORF75	15	4	-1.139	0.255	-1.018	0.309
ORF94	15	6	-1.059	0.290	0.301	0.763
ORF125	15	10	0.556	0.578	0.051	0.959
ORF23/24	17	3	-3.002	0.003*	2.853	0.004*
ORF14/15	17	6	0.000	1.000	-1.290	0.197

(Table 3). A significantly lower number of runs than expected by chance was found, indicating that geographically-proximate isolates were related for this locus. For ORF 14/15, ORF75, ORF94 and ORF125 the *P*-value was higher than 0.010 and hence there was no evidence that deletion size (ORF14/15) and repeat unit number (ORF75, ORF94, ORF125) were spatially structured.

We then performed a Jonckheere-Terpstra test, to test for increasing or decreasing median trait value if the WSSV isolates from Vietnam were ordered sequentially from north to south. This test indicated that there was a significant increase in median deletion size in ORF23/24 if the samples were ordered along the Vietnamese coast from north to south (Table 3). For all other loci, there were no significant increases or decreases found with this test. The results from the runs test and Jonckheere-Terpstra test are therefore congruent: (i) the runs test indicates that only the ORF23/24 variable region is a suitable marker on an intermediate scale, and (ii) the Jonckheere-Terpstra test indicates that there is a significant trend in trait value, but only for this locus.

Discussion

The development of genetic markers for identifying and distinguishing WSSV isolates from different geographic origins is the first step in reconstructing, monitoring and ultimately controlling the spread of WSSV infection in shrimp farming. In a previous study we showed that two variable regions with large deletions (ORF23/24 and ORF14/15) and three VNTR loci (ORF75, ORF94 and ORF125) could be used as molecular markers for epidemiological studies (Dieu *et al.*, 2004). Others have also employed one or more of these loci as markers to characterize WSSV isolates (Hoa *et al.*, 2005; Kiatpathomchai *et al.*, 2005; Marks *et al.*, 2005; Musthaq *et al.*, 2006; Waikhom *et al.*, 2006; Kang & Lu, 2007; Pradeep *et al.*, 2008a,b; Pradeep *et al.*, 2009; Tan *et al.*, 2009), but a statistically supported model was not derived. Moreover, the degree of between-isolate variation for these loci appears to be very different. Therefore, a suitable WSSV marker locus should be selected depending on the spatiotemporal scale to be considered in a study – be it population structure in and around shrimp ponds, or global virus spread.

We found that the ORF23/24 variable region was a suitable marker locus for determining patterns of WSSV spread at an intermediate spatiotemporal scale. Geographically-proximate isolates had similarly-sized deletions in this genomic region, and there was a clear overall spatial pattern: deletion size tended to become larger along the coast of Vietnam, from north to south (Table 3). On the other hand, the other WSSV variable loci were not suitable markers at this scale. For the

ORF14/15 variable region, there may be too little between-isolate variation at an intermediate spatiotemporal scale. Although the runs test gave an insignificant outcome, twelve out of seventeen WSSV-VN isolates had the same 6,030 bp deletion (Table 3). Conversely, for VNTR loci ORF75, ORF94 and ORF125, there appears to be too much between-isolate variation at this scale, as geographically-proximate samples were unrelated (Table 3). Our data therefore suggest that the ORF14/15 variable region may be a suitable marker at larger scales (i.e. global spread), whereas VNTR loci may suitable markers at smaller scales (i.e. between-farm spread). These conclusions are congruent with reported levels of ORF14/15 variation at large scales (Marks *et al.,* 2004; Dieu *et al.,* 2004; Pradeep *et al.*, 2008b), and VNTR variation at small (Pradeep *et al.*, 2008a) and very small (e.g. Hoa *et al.*, 2005) scales.

To date, single or multiple locus sequences of conserved and functional genes (Greiser-Wilke *et al*., 2000; Uzcategui *et al*., 2001; Eyer-Silva & Morgado, 2006), RFLP (Eda *et al*., 2007) and amplified RFLP (ARFLP; Gouvea *et al*., 1998; Sammels *et al*., 1999; Hamano *et al*., 2005) have been used as genetic markers for virus molecular epidemiology, depending on level of between-isolate genomic variation. To our knowledge, we report here for the first time a systematic comparison between VNTR loci and variable genomic deletions as molecular markers to devise a model to explain the geographic spread of a DNA virus.

Our results indicate that a genomic deletion can be a suitable marker at an intermediate spatiotemporal scale, and probably also at large scales. The application of genomic-deletion markers is, however, probably both limited and transient, as it requires selection for the removal of redundant sequences from the viral genome. Such selection occurs only if a virus is introduced into specific novel environments – those allowing for adaptation in genome size – and for as long as size of the virus genome is evolving rapidly. In the case of WSSV, this means that whilst ORF23/24 is an excellent marker for studying spread early in the WSSV outbreak (i.e. until ~1998), it may be less useful for studying later WSSV spread (i.e. spread in the Americas). VNTRs are probably not useful markers on an intermediate spatiotemporal scale, regardless of whether the virus is adapting rapidly to a novel environment or not. This conclusion is more general because the occurrence of variation in VNTRs is not dependent on adaptation.

WSSV isolates from *P. monodon* in central and southern Vietnam showed deletions ranging from 8,539 to 12,166 bp in the ORF23/24 variable region (Dieu *et al.,* 2004). The nine new isolates characterized in this study also had a deletion size within this range, and deletion size become progressively larger from central Vietnam to either the north or the south (Fig. 2; Table 3). Isolates from central Vietnam (K, T, L, H) were similar, and these isolates have the smallest ORF23/24 deletion size of all

Vietnamese WSSV isolates (Fig. 2; Dieu *et al.,* 2004). We therefore propose that (i) the first introduction of WSSV in Vietnam was in central Vietnam, and (ii) that the virus then spread to the south, and probably the north of Vietnam from this site (see spread model in Fig. 4). This suggests that the spread of WSSV was concomitant with the spread of shrimp aquaculture in Vietnam, which was first introduced in central Vietnam and later in the south and the north of Vietnam (Nguyen, 2008). However, we cannot rule out the possibility that WSSV in northern Vietnam may have originated from China, which was reported to be a source of shrimp seed for northern Vietnam (MOFI, 2001). As more data on WSSV become available for this region (Tan *et al.,* 2009) it may become possible to evaluate and compare different spread models.

An alternative explanation of the patterns observed for the ORF23/24 locus (Fig. 2) is that they stem from environmental differences along the Vietnamese coast. As the Vietnamese coastline extends further than 2500 km, there are differences in temperature, salinity, rainfall, vector species and their densities, and aquaculture practices. Central Vietnam, for example, is a relatively hot and dry region, and this may explain why certain ORF23/24 variants only appear there. However, small ORF14/15 deletion variants typical of Vietnam (X, S and A; Fig. 3) are only found in this region (HT and BR are not typical of Vietnam; the presence of these variants probably stems from import of post larvae as discussed below). Although conditions in central Vietnam could result in selection for variants with smaller deletions at both loci, we think that the most parsimonious explanation is that these variants reflect the spread of WSSV.

We found two variants that are present as unique subgroups for Vietnam, represented as follows: (i) isolate HT, from the south of Vietnam, has the same genotype in ORF14/15 as WSSV-TW, which suggests that this isolate may have been generated through recombination between directly introduced WSSV-TW and existing Vietnamese variants (Fig. 4). (ii) Isolate BR, has the same genotype as WSSV-TH in both the ORF23/24 and ORF14/15 loci, suggesting that perhaps this WSSV variant was introduced from Thailand (Fig. 4).

The presence of these variants in Vietnam suggests that human activities – such as the transportation of post larvae and broodstock – have contributed to the long range spread of WSSV. However, these molecular data must be interpreted carefully, because there can be genetic diversity within WSSV populations (Hoa *et al.*, 2005; Pradeep *et al.*, 2008a; B.T.M. Dieu & J.M. Vlak, unpublished data) and the methods used here are expected to detect only the predominant genotype in an isolate.

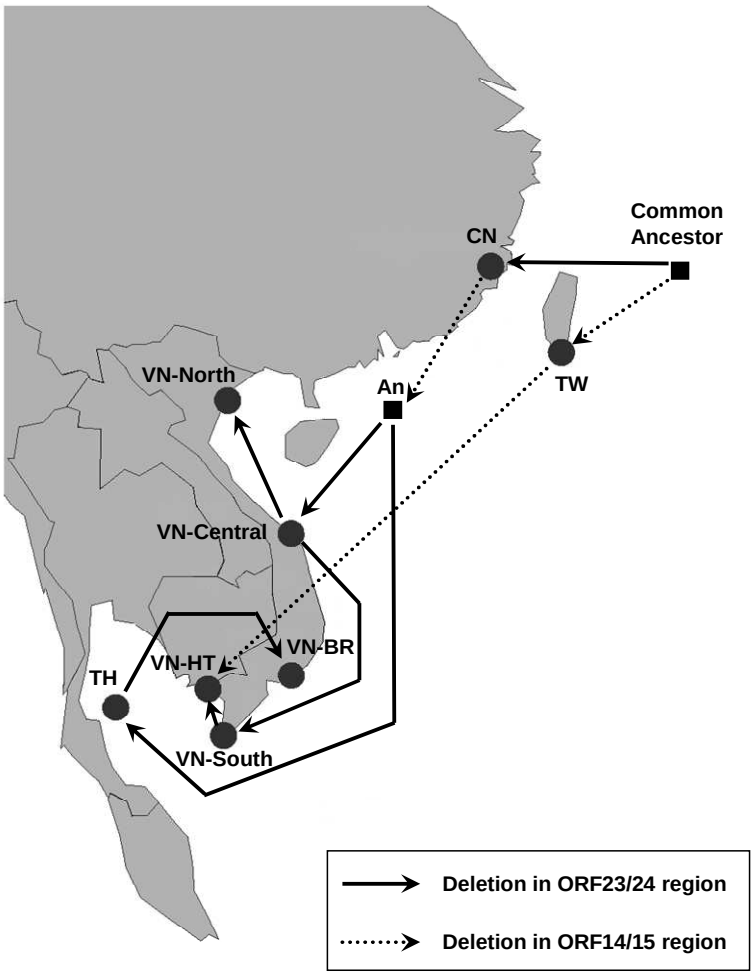


Figure 4. Model of spread of WSSV in Southeast Asia from Taiwan and China towards the West. Circular dots represent identified isolates, or groups of isolates, while square dots represent hypothetical isolates. An intermediate ancestor is abbreviated as An.

We collected WSSV isolates at different geographic locations at a single time point (i.e. 2003 / 2004). However, the word "spatiotemporal" is used to describe in the underlying spread process, to stress a temporal component implicit to our understanding of spread: WSSV molecular evolution during site-to-site transmission, and at each site where the virus has become established. The stability of WSSV genotypes at a geographic site will therefore influence how suitable these methods are for retrospective determination of the spread of WSSV. In other words, is the WSSV genotype(s) sampled at a location genetically representative of the WSSV strains first introduced into this area? Striking spatial patterns seen for WSSV isolates (Dieu *et al.*, 2004; Pradeep *et al.*, 2008b) suggest that this is the case, but solid empirical support – e.g. longitudinal studies of WSSV evolution – is missing. WSSV variable loci are considered key elements for understanding the rapid emergence and evolution of this

rampant virus in shrimp culture, but a complete, experimentally supported framework would be valuable for interpreting marker data and knowing the limitations of the conceptual model.

Acknowledgment

B. T. M. D. was supported by a WOTRO Ph.D. scholarship (Grant WB 83-207).

Chapter 4

Can VNTRs be used to study genetic variation within white spot syndrome virus isolates?

Abstract

Variable number tandem repeat (VNTR) loci are useful molecular markers for studying white spot syndrome virus (WSSV) epidemiology and spatiotemporal spread. To test whether VNTRs could also be used to study within-isolate WSSV genetic heterogeneity, clonal VNTR sequences from ORF75, ORF94 and ORF125 were cloned into a plasmid vector. Surprisingly, we found that a small percentage of clones had a lower number of repeat units than the original sequence. No variation could be detected in multiple replicates of PCR amplification, indicating that PCR-based genotyping alone is a suitable strategy. We then passaged WSSV twice in *Penaeus vannamei*, with 20 replicates, and found that VNTR sequences were stable in all replicates. WSSV VNTR loci are therefore sufficiently stable to be used as molecular markers on short time scales. As the WSSV dose-response relationship corresponds to theoretical predictions, we propose in vivo cloning and genotype discrimination by VNTRs to study within-isolate heterogeneity.

Key words: White spot syndrome virus, VNTR, genetic variation, Vietnam, PCR genotyping.

This chapter has been published as: Dieu, B. T. M., Zwart, M.P. & Vlak, J. M. (2010). Can VNTRs be used to study genetic variation within white spot syndrome virus isolates? *Journal of Fish Diseases* (in press).

Variable number tandem repeat (VNTR) loci are DNA elements consisting of adjacent repeat units (RUs) that are highly polymorphic (Pourcel *et al.*, 2003). VNTRs have been extensively used in forensics (Jeffreys *et al.*, 1985) and for strain typing bacteria (e.g., Liu *et al.*, 2003). Moreover, RU numbers have been linked to disease severity (Hummerich & Lehrach, 1995). VNTRs have also been used to demonstrate high levels of between- and within-population genetic variation (Davis *et al.*, 1999; Dieu *et al.*, 2004). The repetitive nature of VNTRs could facilitate DNA rearrangements through recombination, as proposed for baculovirus homologous repeats (Garcia-Maruniak *et al.*, 1996) and herpes virus direct repeats (Umene, 1991). Alternatively, DNA rearrangements could occur through 'slip-strand mispairing' (e.g., Levinson & Gutman, 1987).

White spot syndrome virus (WSSV) is an emerging, large dsDNA virus that adversely affects shrimp aquaculture worldwide (Walker & Mohan, 2009). Marks *et al.* (2004) identified non-hr unidirectional VNTR loci in ORF75, ORF94 and ORF125. High levels of genetic variation at these loci have been found between isolates (Dieu *et al*. 2004), whereas Tran *et al.* (2005) and Pradeep *et al.* (2008a,b) suggest that there may be variation within WSSV isolates, as some samples rendered major and minor PCR products. Here we define a virus isolate as a field sample collected from a single host. High levels of biologically significant, within-isolate variation have been found for some invertebrate dsDNA viruses (e.g., baculoviruses; Lee & Miller, 1978; Cory *et al.*, 2005). On the other hand, genetic variation within WSSV isolates has not been studied, but could have major implications for WSSV adaptation and virulence evolution.

We first evaluated a cloning-based approach to study WSSV within-isolate variation in VNTR sequences. The approach was to: (i) PCR amplify a VNTR locus with specific primers flanking the repeat region, (ii) clone PCR products into a bacterial plasmid vector, and (iii) PCR genotype and sequence clones. This approach could be both sensitive and possibly semi-quantitative (i.e. clone frequency could roughly indicate genotype frequency in the isolate). However, to measure variation in the virus isolate, the experimental procedure itself must not generate variation. Here, we test whether or not this procedure generates variation using clonal (i.e., a single number of RUs) starting material.

To evaluate this approach, we used WSSV isolates from Vietnam we previously characterized (Dieu *et al.*, 2004). Viral DNA was extracted (Dieu *et al.*, 2004) and the VNTR regions in ORF75, ORF94 and ORF125 were PCR-amplified with specific primers (Table 1). PCR products were run in a 1% agarose gel, and the major VNTR band was excised and purified by a gel band purification kit (GE Healthcare; Chalfont

St. Giles, UK) to give clonal starting material. This clonal DNA was ligated into the pGEM-T easy vector (Promega; Madison, WI) and electro-transformed into competent *Escherichia coli* DH5α cells. White-colony insert size was checked by PCR using the SP6 and T7 primers (binding up- and downstream of the cloning site). Plasmid DNA was purified (Fermentas Plasmid Miniprep Kit, Fermentas GMBH; St. Leon-Rot, Germany) and sequenced from individual colonies that showed variation in PCR amplicon size. Sequence data were analyzed using DNASTAR 4.2 and GeneDoc (version 2.6.000; Nicholas *et al.*, 1997).

Between-clone variation in RU number was found for all three ORFs (Table 2). Only variants with a lower number of RUs than the clonal starting material were found. Significant differences between loci in the frequency at which variants occurred were not found ($P > 0.1$ for all pair-wise comparisons of proportions, 'prop.test', R 2.7.0; R Foundation, Vienna, Austria). As a further test, DNA was PCR-amplified from a culture of single *E. coli* clone containing ORF94. The single-band PCR product was purified and re-cloned as described above. We again found between-clones variation in RU number, and again only clones with a lower number of RUs (Table 2). The frequency of variants was not significantly different to the previous experiment with ORF94 ($P = 0.384$). We therefore consistently found that variation in RU number was generated during the cloning procedure. The amount of variation generated did not differ significantly between experiments, regardless of VNTR locus and source of DNA for the PCR.

Table 1. Primers used for VNTR PCR of WSSV

Primer pair	Orientation	Sequence (5'-3')	Anneal. temp. (^{0}C) / elongation time (s)	WSSV-TH sequence coordinates
ORF75-flank	Forward	GAAGCAGTATCTCTAACAC	49 / 80	107875-107893
	Reverse	CAACAGGTGCGTAAAAGAAG		108742-108723
ORF94-flank	Forward	GTGCCGCAGGTCTACTC	51 / 80	142656-142672
	Reverse	CATACGACTCTGCTTCTTG		143337-143319
ORF125-flank	Forward	CGAAATCTTGATATGTTGTGC	52 / 100	187791-187811
	Reverse	CCATATCCATTGCCCTTCTC		188442-188423

Table 2. VNTR RU number for *E. coli* colonies with clonal DNA**.** The RU number in the clonal DNA source has been marked with an asterisk.

VNTR locus	DNA source	Number of clones analyzed(frequency)							
		Total	10 RU	7 RU	5 RU	4 RU	3 RU	2 RU	1 RU
ORF75	Isolate K	90 (1.00)	83* (0.92)	-	-	-	5 (0.06)	1 (0.01)	1 (0.01)
ORF125	Isolate T	72 (1.00)	-	-	71* (0.99)	1 (0.01)	-	-	-
ORF94	Isolate K	100 (1.00)	94* (0.94)	3 (0.03)	2 (0.02)	-	-	-	1 (0.01)
ORF94	*E. coli* clone (Isolate T)	74 (1.00)	-	66* (0.89)	2 (0.03)	3 (0.04)	-	2 (0.03)	1 (0.01)

These results demonstrate that our proposed approach is not suitable for detecting within-isolate variation in RU number, because variation is generated during the cloning procedure itself. The source of this variation could be mutation in *E. coli* (Vogler *et al.*, 2006) or a cloning artefact. We excluded variation in the starting material by purifying the single gel band from PCR product prior to cloning. However, others have reported PCR-induced artefacts when using VNTRs (Campbell *et al.*, 2001). To investigate whether this occurs for WSSV loci we performed 10 replicates of PCR amplification of the ORF94 VNTR on four WSSV isolates with different RU numbers, which previously only gave a single band (isolates ST, K, H, S; Dieu *et al*. 2004; Dieu *et al.*, 2010). Only the expected band was visible upon electrophoresis for all forty samples. As a further test, we performed 78 replicates of a PCR on the ORF94 VNTR of a single plasmid clone originating from isolate T (Dieu *et al*., 2004). Upon electrophoresis we found only the expected band for all replicates; PCR-induced variation (0%) was therefore significantly lower than mean cloning-induced variation (8%, P=0.003, two-sided exact binomial test, R 2.7.0). Because the identity (i.e., RU number) of the major band was conserved in all reactions, we conclude that PCR amplification does not appear to introduce variation in RU number and has no implications for the interpretation of WSSV VNTR data.

Our data support PCR-based methods for analyzing VNTRs, while showing complications with methods based on molecular cloning. To study WSSV within-isolate heterogeneity, other methods will therefore have to be considered. Possible approaches include (i) VNTR southern blot (Campbell *et al*. 2001), (ii) cloning-based methods on loci without repetitive elements (i.e., ORF14/15 and ORF23/24 variable regions; Dieu *et al*., 2004), (iii) ultra-deep pyrosequencing (Wang *et al.*, 2007), and (iv) *in vivo* cloning of genotypes: the infection of hosts with a low dose resulting in infection by one or a few virions (Smith & Crook, 1988). A benefit of *in vivo* cloning is that the cloned genotypes are available for further molecular and biological analysis.

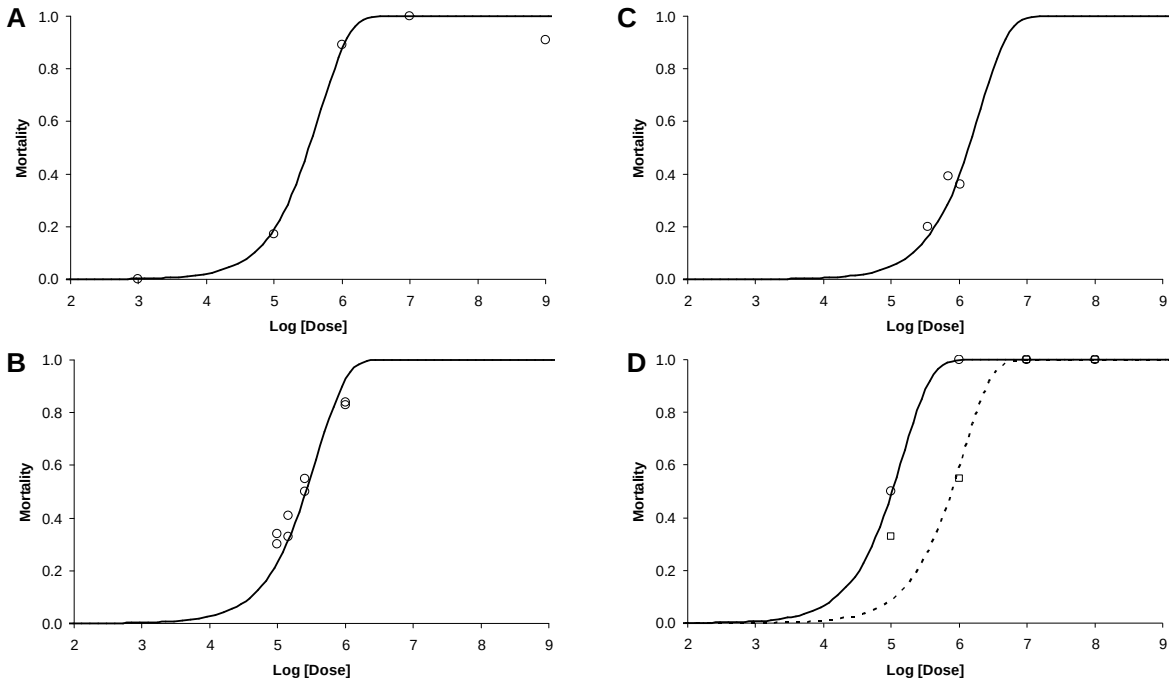
However, this method will only identify genotypes that are capable of autonomous infection, and a stable genetic marker is required.

We therefore tested the stability of WSSV VNTR sequences by passaging WSSV isolate T – amplified in *Orconectes limosus* (Rafinesque) – in SPF *Penaeus vannamei*. Isolate T was used because we did not detect genetic variation within this isolate (Dieu *et al.*, 2004). Post-larvae of 6-10 gram of weight were injected with a high dose ($10xLD_{50} = 10^{5.5}$ virions), and then individually caged. A second, un-injected shrimp was added to the cage of each injected shrimp. For 20 un-injected shrimps that died of WSSV infection, we determined the RU number of ORF94, which is the most variable WSSV VNTR (Dieu *et al.*, 2004; Tran *et al*., 2005; Pradeep *et al*., 2008a, b). All 20 shrimp gave the expected PCR-band (736 bp, corresponding to 7 RUs). Over two passages *in vivo* (i.e., infection of injected and subsequently un-injected shrimp), VNTR sequences were stable. We conclude that *in vivo* cloning on WSSV isolates that give multiple VNTR PCR bands may be a suitable method to investigate heterogeneity within WSSV isolates, because WSSV VNTR loci are sufficiently stable. All cloned genotypes should be detected in the original isolate by PCR, however, to exclude *de novo* variation.

If each WSSV virion can infect the host independently, virus genotypes can be efficiently cloned *in vivo*. We therefore tested whether WSSV dose-response data support independent action theory, i.e., the idea that one virion can cause infection and that virions act independent of each other (Druett, 1952; Smith & Crook, 1988). Independent action leads to a fixed-shape dose-response relationship, which has been shown to be a good indicator for determining applicability of the theory (Zwart *et al.*, 2009). We therefore fitted the independent-action dose-response equation (Druett, 1952):

(1) $$m = 1 - e^{-n \cdot p}$$

to published dose-response data (Figure 1) (van Hulten *et al.*, 2001a; Prior *et al.*, 2003; Marks *et al.*, 2005a) using non-linear regression (SPSS 15.0, SPSS Inc., Chicago, IL), where *m* is mortality, *p* is infection probability and *n* is viral dose. The data appear to be congruent with independent-action predictions, as evidenced by high r^2-values (Figure 1). This strongly suggests that *in vivo* cloning methods can be efficiently used to isolate autonomously replicating WSSV genotypes. Their *in vivo* stability qualifies VNTR sequences as suitable molecular markers for detecting and discriminating WSSV genotypes, provided analysis of PCR products does not require cloning.



Panel	Data	Virus isolate	Host	*P*	SEM	r^2
A	van Hulten *et al.*, 2001	TH-I-96	*P. monodon*	2.1×10^{-6}	3.0×10^{-7}	0.99
B	Prior *et al.,* 2003 - injection	USA '97, S.C.	*P. vannamei*	2.6×10^{-6}	2.3×10^{-7}	0.88
C	Prior *et al.,* 2003 - emersion	USA '97, S.C.	*P. vannamei*	5.0×10^{-7}	9.0×10^{-8}	0.44
D	Marks *et al.*, 2005 1 (circles)	TH-I-96	*P. monodon*	6.7×10^{-6}	1.0×10^{-8}	1.00
D	Marks *et al.*, 2005 2 (squares)	TH-II-96	*P. monodon*	8.9×10^{-7}	3.2×10^{-7}	0.82

Figure 1. Fit of dose-reponse equation derived from independent action (see Zwart *et al.*, 2009) to published WSSV data. Virus dilutions were converted to dose on a convenient, arbitrary scale prior for non-linear regression (the model is concerned only with relative doses). For all panels, log10 of dose is on the x-axis and the proportion mortality is on the y-axis. *P* is the infection chance (see equation 1), SEM is the standard error of the mean of $_p$, and r^2 is the co-efficient of determination from the non-linear regression. High r^2 values (close to 1) are used as indicators of good model fit. All the data found gave high r^2 values (> 0.80), with the exception of emersion data from Prior *et al.* (2003). The latter is a small data set with only three similar doses. Note that between-experiment comparisons of $_p$ values cannot be made.

Acknowledgements

We thank Tuyen Xuan Ngo and Hendrik Marks for valuable assistance and discussions. B.T.M.D. was supported by a WOTRO Ph.D. scholarship (Grant WB 83-207).

Chapter 5

Effects of extensive and intensive shrimp farming on the genetic composition of white spot syndrome virus populations

Abstract

White spot syndrome virus (WSSV) has a major negative impact on shrimp farming and industry. There are many different shrimp farming practices, and these may affect the genotypic composition of WSSV populations and possibly the virulence of the virus. Here we investigated whether extensive and intensive farming practices (1) result in selection of WSSV genotypes, and (2) affect genotypic composition over time in WSSV populations. WSSV samples were collected from Vietnamese farms on various sites over a period of several years and the samples were then genotyped. We found no significant effect of farm practice on the genotypic composition of WSSV populations. On the other hand, we found an effect of farm practice on change over time in the ORF23/24 variable region: this region was significantly more stable in extensive farming. This result is a first observation suggesting that farm practice may affect the evolutionary dynamics of WSSV. Moreover, these data also suggest that for retrospectively studying the spread of WSSV, it is better to sample from extensive farms than from intensive farms because WSSV populations in extensive farms will be more stable over a long period of time.

Keywords: White spot syndrome virus, shrimp farming, shrimp aquaculture, genetic marker, epidemiology

This chapter is accepted as: Dieu, B. T. M., Zwart, M.P. & Vlak, J. M. (2011). Effects of extensive and intensive shrimp farming on the genetic composition of white spot syndrome virus populations. In: *Diseases in Asian Aquaculture VII* (Jones et al., eds.), Fish Health Section, Asian Fisheries Society, Manila (in press).

Introduction

White spot syndrome virus (WSSV) has been a scourge on shrimp farming since the early 1990s, causing massive mortality and major damage to all sorts of shrimp farming operations, from extensive to high-intensity practices (Escobedo-Bonilla *et al.*, 2008). During this period of time the virus has undergone distinct genotypic change resulting in the occurrence of variants world-wide and in variants with increased fitness and virulence (Marks *et al.*, 2004, Marks, *et al.*, 2005a). This suggests an adaptive evolution to the novel environment and ecological niche provided by shrimp ponds. In shrimp ponds host density will typically be much higher than in natural habitats. The between- and within-species variation in hosts is also likely to be lower in intensive ponds than in natural environments, and hosts are likely to be stressed due to pond conditions. All of these factors probably contribute to generating a novel environment in which WSSV can thrive and further optimize its fitness.

Shrimp-farming operations are, however, highly varied. Extensive and improved extensive shrimp farms (i) stock shrimp larvae directly from the sea, in part or entirely, (ii) have a relatively low density of shrimp, and (iii) have overlapping generations of shrimp. On the other hand, intensive shrimp farms (i) stock shrimp post-larvae (PL) from hatcheries, and the broodstock can originate from geographic locations far from the farm, (ii) have a relatively high density of shrimp, and (iii) have non-overlapping generations of shrimp. Typically a pond is seeded with post-larvae (PL) and they are subsequently harvested together, after which the pond is drained and cleaned. Because extensive and intensive shrimp farms provide such differing environments, farming practice may lead to differential selection of WSSV genotypes. Moreover, in intensive farming operations, virus populations present in ponds may be largely discontinuous. This discontinuity will arise because (1) regular drainage and cleaning of the pond removes infectious host cadavers or debris, and (2) non-overlapping shrimp generations preclude between-cohort transmission of the virus by infectious shrimp. This may have implications for the genetic composition, evolutionary dynamics and epidemiology of WSSV populations.

Here, we hypothesize that different farming practices will have an effect on WSSV genotype composition and population structure. WSSV isolates were collected from Vietnamese improved extensive and intensive farms at different geographic locations and different time points. These isolates from each type of farm were genetically characterized, allowing us to test whether farming practice affected genotypic composition and change thereof over time.

Materials and methods

Classification of shrimp farms

WSSV infected shrimp were purposely collected from shrimp farms with different farming practices. We classified the farms based on farm organization and management, in a manner similar to Nhuong *et al.* (2002) using the following three categories:

(1) Intensive shrimp farming: Pond size varies from 0.2 to 0.6 ha, and stocking density from 15-30 post larvae per m^2. Shrimp are stocked only once for each crop, industrial shrimp food is used, and water oxygen supply is augmented by machinery. PLs are bought from local hatcheries, but the origin of the broodstock is typically unknown. Shrimp crops are harvested after about four months. If there are disease outbreaks, shrimps are quickly harvested and the pond is chemically treated and drained for cleaning prior to new stocking.

(2) Improved extensive farming: Pond size greatly varies, ranging from 1 to 15 ha, including ditches and surrounding dikes. Shrimp seed are trapped from wild stock by making use of tides, and sometimes farmers supplement wild stock with PL from hatcheries (approx. once a month, although this varies greatly between farms). This results in a low stocking density of 1-2 shrimp per m^2. No additional feed is required in this system as shrimp use natural feed in pond. Farmers typically harvest shrimp once or twice a month, based on tides. Large shrimp are trapped by nets as marketable harvest, although small shrimp suffering of disease are also often trapped and hereby removed from the pond.

(3) Shrimp-rice farming: similar to improved extensive farming in terms of farm management, although the plots used are somewhat smaller than improved extensive ponds. In the Mekong Delta of Vietnam, farmers use the same plot to cultivate rice in the rainy season, when freshwater is abundant, and shrimp in the dry season, when saline water is used to flood the plot.

Collection of virus isolates

Shrimp showing disease symptoms characteristic for WSSV were selected, cleaned on the outside with 70% ethanol, and stored in 96% ethanol during transportation to Can Tho University (Vietnam). The ethanol was then removed and samples were stored at -20° C until further processing. All shrimp selected were *Penaeus monodon*. Information on the WSSV infected shrimp collected is recorded in Table 1, and geographic locations are given in Figure 1.

Table 1: WSSV isolates:

Region	Province	Place (district)	Farming practice	Origin of post larvae	Date of collection	Abbreviation
central VN	Quang Nam	Nui Thanh	Intensive	central region	2003, 2008	QN
south VN	Tra Vinh	Duyen Hai	Intensive	Unknown	2004, 2006	TV
	Ha Tien	Thuan Yen	Intensive	Unknown	2004, 2005	HT
	Kien Giang	Kien Luong	Intensive	central region	2003, 2005	Kg
	Soc Trang	My Xuyen	Rice-shrimp	Unknown	2002, 2004, 2008	ST
	Bac Lieu	Vinh Loi	Extensive	Unknown	2004, 2008	BL
	Ca Mau	Tan Thanh	Extensive	Local	2002, 2004, 2005, 2006, 2007, 2008	CM

Figure 1: Location of the Vietnamese farms that were sampled for this study, indicated by QN, TV, ST, BL, CM, Kg and HT. See Table 1 for further details.

Genetic characterization of virus isolates

The characteristics of WSSV have been described by Vlak *et al.* (2005) and further ICTV updates (www.ictvonline.org). Marks *et al.* (2004) identified five variable regions (see Figure 2): three loci with variable number of tandem repeats (VNTR; ORF75, ORF94 and ORF125; ORF nomenclature according to Van Hulten *et al.*, 2001a), and two loci with large deletions (ORF23/24, ORF14/15). These variable loci have been employed as markers in different studies on different spatiotemporal scales

(e.g. Dieu *et al.*, 2004; Marks *et al.*, 2005; Waikhom *et al.*, 2006; Pradeep *et al.*, 2008a, 2008b). Here we employed these five variable regions as markers.

DNA was extracted from tissue behind the heads of collected shrimp, and screened for the presence of WSSV according to published procedures (Dieu *et al*. 2004). PCR on the genomic variable loci of WSSV was performed with 1µl DNA extract (approx. 250 ng DNA), using Taq DNA polymerase (Promega). Specific primers, PCR conditions used and and amplicon lengths are shown in Table 2. PCR for VNTRs is described elsewhere (Dieu et al., 2004). PCR products were analyzed, sequenced and computational analysis was done according to published procedures (Dieu *et al*., 2004). For analysis of the deletion loci (ORF14/15 and ORF23/24), two WSSV infected shrimp from each pond were PCR analyzed.

Statistical analysis

To analyze VNTR data we considered the number of repeat units (ORF94 and ORF125). For ORF75, we considered the total length of the repeat region, since this variable locus contains two types of repeats. To analyze data from genomic deletions (ORF14/15 and ORF23/24) we considered the length of the genomic deletion. We refer to these quantitative data as 'locus trait values'.

Table 2. Primers used in PCR analysis for the variable loci of WSSV (WSSV-TH sequence)

Primer pair name/ (Detected deletion)	Primer orient.	Sequence (5'-3')	Anneal. Temp. (°C) / elongation time (s)	WSSV–CN sequence coordinates	Size (bp) of PCR product for VN samples
VR23/24 –HTvar (10970 bp)	Forward Reverse	GAGTAGTCTTCAATGGCAATGT GTAAGTTTATTGCTGAGAAG	49 / 100	275008-275029 286105-286086	~1200
VR23/24 – CM (11045 bp)	Forward Reverse	CAGATAATGCAAACACGAGACAC GTAAGTTTATTGCTGAGAAG	49 / 75	275794-275816 286105-286086	~500
VR23/24 –screen (8539 bp)	Forward Reverse	CACACTTGAAAAATACACCAG GTAAGTTTATTGCTGAGAAG	49 / 75	278179-278199 286105-286086	~550
VR23/24 –south (11866 bp)	Forward Reverse	GTAGTGCATGTTTCTCTAAC GTAAGTTTATTGCTGAGAAG	49 / 100	275032-275051 286105-286086	~400
VR23/24 –TV (11450 bp)	Forward Reverse	CTACAACGGCCAAGTCAT ATGATTGTATTCGTCGAAGG	49 / 100	30701-30718* 286706-286687	~1600
VR23/24 –Kg (12166 bp)	Forward Reverse	CTACAACGGCCAAGTCAT CGCAATTCTCCTCGCAGTT	49 / 100	30701-30718* 32255-32237*	~2600
VR14/15-screen (6031bp and 5950 bp)	Forward Reverse	GAGATGCGAACCACTAAAAG ATGGAGGCGAGACTTGC	49 / 75	22904-22923* 24157-24141*	~500/600
VR14/15-HT (5138 bp)	Forward Reverse	GAGATGCGAACCACTAAAAG GAAAAATAAATCACGGGCTAATC	49 / 80	22904-22923* 23646-23624*	~900

Because the data set is limited, we grouped improved extensive and shrimp-rice farms together as being 'extensive', and compared them to intensive farms.

A simple test of whether farming practice had an effect on genotypic composition, we performed a Mann-Whitney *U*-test (SPSS 15.0; SPSS Inc., Chicago, IL, USA), with farming practice as the independent variable and locus trait value (RU number, total length of TRs or deletion size) as the dependent variable. We only included data from the years 2002, 2006 and 2008 from the CM site (Ca Mau, Table 1) to avoid biasing our analysis due to high number of samples from a single site and small time intervals (see Table 3). CM 2006 was chosen as the intermediate sample because for that year the VNTR data are complete.

To test whether farming practice had an effect on changes in genotypic composition over time, we first determined whether there was a change in locus trait value between samples from the same site. For sites at which more than two samples were available, we compared the earliest and the latest available samples only. Thus the genotypic data from two samples from different time points represent one event: locus trait values are either the same or they are not. We recorded the total number of locus trait value changes for each locus, and then tested whether intensive farms had more changes in locus trait value than extensive farms using a one-sided test of equal proportions (R 2.7.0; The R Foundation for Statistical Computing, Vienna, Austria).

Results

Genotyping of WSSV isolates

All shrimp samples tested positive for the presence of WSSV using a single step PCR. VNTR variable regions were analyzed for all the studied isolates (Table 3). In order to map deletions in the ORF14/15 and ORF23/24 variable regions, we first performed PCR with the "VR14/15-screen" and "VR23/24-screen" primers on all samples, respectively (Table 2). These primer sets were previously used to detect deletions in six VN-central WSSV isolates (Dieu *et al*., 2004). Those samples which failed to give a PCR product were then analyzed by means of a 'walking PCR' with different primer sets, starting from two ends of variable regions. Genotypes were detected with the corresponding primer set (Table 2). All PCR products were cloned and sequenced to confirm their identity and map the exact position of the deletion. The location and size of the genomic deletion was determined using WSSV-TH-96-II (acc. no. AY753327; Marks *et al.*, 2005) as a reference sequence for ORF14/15, and WSSV-TW (acc. no. AF440570; Wang *et al.*, 1995; see also Marks *et al.*, 2004) as a reference for ORF23/24.

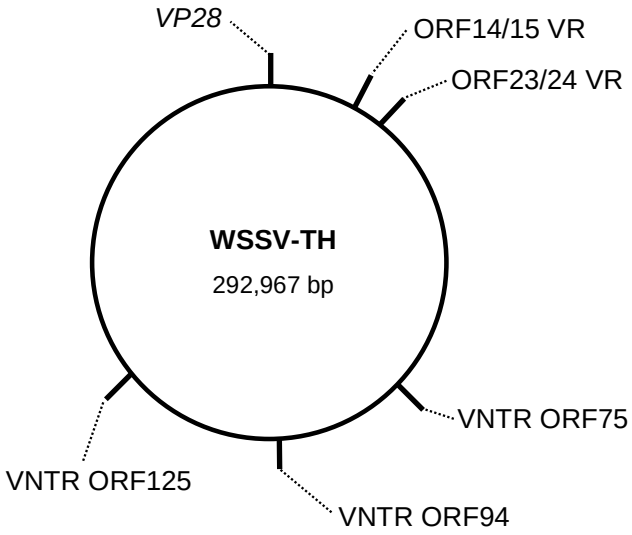


Figure 2: The location of the five variable regions used here to characterize WSSV samples, shown in the WSSV-TH genome (Van Hulten *et al*., 2001a; Marks *et al.*, 2004). VR stands for variable region, and VNTR for variable number of tandem repeats. The location of the major WSSV virion envelope protein gene *VP28* is given as a reference point only.

Effects of farm practice on genotypic composition

A Mann-Whitney *U*-test (Table 4) demonstrated that there was no significant effect of farming practice (extensive or intensive) on any locus trait values (RU number, total length of TRs or deletion size) for all geographic locations.

Effects of farm practice on changes in genotypic composition

A test of equal proportions (Table 5) demonstrated that for the ORF75, ORF94, ORF125 and ORF 14/15 variable regions, there was no effect of farming practice on change in locus trait value in consecutive samples from a single geographic location. However, for the ORF23/24 variable region, there was a significant effect of farming practice on change in locus trait value (i.e. deletion size). For the intensive farms a change in locus trait value for ORF23/24 was always observed (4 changes in 4 observed events). For the extensive farms no change in locus trait value was observed (no changes in 3 observed events included in statistical analysis, no changes in 8 observed events in total).

Although we analyzed changes in trait locus values over time, the interval between sampling at different locations was irregular (extensive farms: 5.33 ± 0.58 [mean ± SE]; intensive farms: 2.50 ± 0.87). The mean time interval was twice as long on the extensive farms meaning that our analysis of change in viral genotypes over time is probably conservative.

Table 3: Genotyping of five variable loci in the WSSV isolates. An asterisk (*) indicates the PCR reaction failed. A double dagger (‡) indicates that the data were excluded from statistical analysis to avoid biases. For ORF75, (a) stands for an RU with 102 bp, and (b) for RU with 45 bp.

Farming Practice	Location	Year	ORF75		ORF94	ORF125	ORF14/15	ORF23/24
			RUs (total)	TR Length				
Extensive	ST	2002	abbabb (6)	384	14	8	6031	11,866
		2004	abbab (5)	339	4	5	6031	11,866
		2008	babb (4)	237	*	4	5950	11,866
	BL	2004	babb (4)	237	*	*	6031	11,866
		2008	babb (4)	237	10	7	5950	11,866
	CM	2002	ababb (5)	339	3	4	6031	11,045
		2004‡	*	*	*	*	6031	11,045
		2005‡	abbabb (6)	384	9	*	6031	11,045
		2006	abbab (5)	339	7	5	6031	11,045
		2007‡	ababb (5)	339	*	7	6031	11,045
		2008	babb (4)	237	6	8	5950	11,045
Intensive	QN	2003	ababb (5)	339	10	6	6031	8,539
		2008	abbabb (6)	384	6	7	6031	11,866
	HT	2004	babbb (5)	282	11	6	5138	11,866
		2005	abbab (5)	339	10	5	5950	10,970
	Kg	2003	abbab (5)	339	15	6	6031	12,166
		2005	abbab (5)	339	12	9	6031	11,866
	TV	2004	abbab (5)	384	10	9	6031	11,450
		2006	abab (4)	294	6	7	5950	10,970

Table 4: Comparison of locus trait values between extensive and intensive farms. Note that analysis was performed on different measures (Analysis). A Mann-Whitney *U*-test was performed to test for significant differences in trait value between extensive and intensive farms. No significant *P*-values were found.

Locus	Analysis	Samples	Mean ± SE		Mann-Whitney *U*-test	
			Extensive	Intensive	*Z*	*P*
ORF75	Total length TRs (bp)	16	293.6 ± 22.0	337.5 ± 12.9	-1.441	0.149
ORF94	RU number	14	7.33 ± 1.67	10.00 ± 1.05	-1.377	0.169
ORF125	RU number	15	5.86 ± 0.67	6.88 ± 0.52	-1.173	0.241
ORF14/15	Deletion size (kb)	16	6.00 ± 0.00	5.89 ± 0.11	-0.185	0.854
ORF23/24	Deletion size (kb)	16	11.56 ± 0.15	11.19 ± 0.41	-0.507	0.613

Table 5: Comparison of change in locus trait values over time between extensive and intensive farms. A test of equal proportions was performed to test for significant differences in changes in trait value between extensive and intensive farms. Significant *P*-values are marked with an asterisk (*).

Locus	Changes / Total Observed (Proportion)		Test of equal proportions	
	Extensive	Intensive	X^2	*P*
ORF75	2 / 3 (0.67)	2 / 4 (0.50)	0.000	0.5000
ORF94	1 / 1 (1.00)	4 / 4 (1.00)	-	-
ORF125	2 / 2 (1.00)	4 / 4 (1.00)	-	-
ORF14/15	3 / 3 (0.00)	2 / 4 (0.50)	0.365	0.727
ORF23/24	0 / 3 (0.00)	4 / 4 (1.00)	5.405	0.030*

Discussion

We investigated the effect of extensive or intensive shrimp farming on WSSV genotypic composition and on changes in WSSV genotypic composition over time. We found no effect of shrimp-farming practice on WSSV genotypic composition for any of the five variable loci investigated (Table 4). This suggests that the environments associated with extensive and intensive farms are not divergent enough to impose differential selection for WSSV genotypes. On the other hand, the number of samples was relatively small, making it difficult to draw definitive conclusions from these data alone. More intensive sampling in the future followed by genetic analysis should substantiate this claim.

For four out of five variable loci (ORF75, ORF 94, ORF 125, ORF14/15) we found no effect of farming practice on changes in locus trait value over time (RU number, total length of TRs or deletion size; Table 5). For ORF23/24, however, we did find a significant effect of farming practice on change in locus trait value (deletion size); whereas deletion size never changed over time for an extensive farm, it always changed for intensive farms (Tables 3 and 5). Moreover, the average time between sampling was twice as long on the extensive farms as on intensive farms, meaning that viral populations on extensive farms had twice as much time to undergo genetic change. Overall, these data therefore suggest that our hypothesis that WSSV populations on intensive farms will be more variable is correct. An effect of farm practice on viral genotypic stability is probably due to (i) frequent seeding of PLs infected with different virus strains (Withyachumnarnkul, 1999) and (ii) pond drainage and cleaning regimens.

Why do the data suggest an effect only for ORF23/24, and not for the other loci? First, this is a preliminary study with a limited sample size, the power of the statistical test used is low. However, there appears to be an interesting trend for the ORF14/15 data. For samples from four out of five geographic locations in which the deletion size

changed over time, there was a shift from the 6031 bp deletion to the 5950 bp deletion. Moreover, the 5950 bp deletion was found only in samples from later years (2005-2008; see Table 3). This suggests that there was selection for a genotype carrying this slightly smaller deletion during that period of time. What could have caused the occurrence of selection at most of the sites? In this period of time, *Penaeus vannamei* was widely introduced in Vietnamese shrimp farms, replacing *P. monodon* (Raux *et al.*, 2006; Corsin, 2005). Others have shown that passaging in different host species can result in differential selection of WSSV genotypes (Waikhom *et al.*, 2006), lending credibility to this explanation. Moreover, the 5950 bp deletion appears to be selected for when WSSV samples obtained from *P. monodon* are passaged in *P. vannamei* (B.T.M. Dieu & J.M. Vlak, unpublished data).

We observed no trends in the VNTR data (Tables 2, 3 and 4). The data of Pradeep *et al.* (2008a, 2008b) suggest that VNTR loci (ORF75, ORF94, ORF125) are more variable than deletion loci (ORF14/15, ORF23/24). We have also found a similar trend for the spread of WSSV in Vietnam (Dieu *et al.*, 2004; Dieu et al., 2010). These observations may explain why we did not find an effect of farming practice on locus trait value for VNTRs: variation is generated too rapidly for VNTRs to be useful markers on larger spatial - and temporal - scales.

Our data suggest that extensive farming led to fewer changes in deletion size for the ORF23/24 variable region, as compared to intensive farming. This result has important ramifications. First, it suggests that virus populations in extensive farms will be more stable than in intensive farms, a result that we hypothesized based on the way ponds are managed under these farming practices. This will have consequences for WSSV evolutionary dynamics. For intensive farming systems, infection of a pond can be an evolutionary dead end as the pond will eventually be drained and cleaned, leading to the destruction of most virions. On the other hand, if ponds are not carefully cleaned and viruses reach into the surroundings by e.g. marine crabs, fresh water prawn *Macrobrachium rosenbergii* (Hossain *et al*., 2001) or polychaetes (Vijayan *et al*., 2005), a disease-free intensively-managed pond is then a resource which can be best exploited by highly virulent genotypes, as there is no cost of virulence (i.e. the pond will be drained irrespective of virus behavior). In extensive farming systems the costs of virulence may be maintained i.e. killing the host at any point in time means that host introduced into the pond at a later time cannot be directly infected. This line of thought may also extend to shrimp-rice farming, because the plots used are never completely drained and cleaned. WSSV is known to cause asymptomatic or avirulent infections (Withyachumnarnkul, 1999; Flegel *et al.*, 2004), which may be important for maintaining the virus in low-density host populations, for example by vertical transmission. Genetic composition of WSSV populations may be one factor which

determines virulence, as has been shown by Marks *et al.* (2005a) and suggested by Hoa *et al.* (2005). There will, however, be many other factors which will determine WSSV virulence, such as farm management (Corsin *et al.*, 2001), temperature (Rahman *et al.*, 2006), salinity (Liu *et al.*, 2006), ammonia-N (Jiang *et al.*, 2004) and adaptive shrimp responses (Flegel, 2007).

Finally, effects of shrimp farming practice on changes in locus trait value have implications for studying the spread of WSSV by means of molecular epidemiology (Dieu *et al.*, 2004; Pradeep *et al.*, 2008a, 2008b; Dieu et al., 2010). If WSSV populations in intensive farm systems are more variable, this means that the original genotype which colonized the farm - and the surrounding region - is less likely to be maintained in the virus population than in an extensive farm. Hence, if samples are taken retrospectively to determine virus spread (e.g. Dieu *et al.*, 2004, 2010), then it is best to sample from extensive farms, because the virus population sampled is more likely to be representative of the genotypes that were first introduced - or first became predominant - in that area. The effects of farming practice on the stability of WSSV populations should therefore be given consideration in the design of experiments to study the spread and epidemiology of WSSV.

Acknowledgements

We thank Lia Hemerik for critical comments and advice on the statistics and Hao Van Nguyen and Tuyen Xuan Ngo for providing WSSV samples. B.T.M.D. was supported by a WOTRO Ph.D. fellowship (grant WB 83-207).

Chapter 6

Adaptive trajectory of genome size evolution in an emerging shrimp virus

Abstract

White spot syndrome virus (WSSV) is the sole member of the novel Nimaviridae family, and the source of major economic problems in shrimp aquaculture. WSSV rapidly spread worldwide after the first reported outbreak in the early 1990s. Large genomic deletions occur at two loci in the WSSV genome, ORF14/15 and ORF23/24, and these variable loci have been used as molecular markers to study patterns of viral spread. Here we describe for the first time the dynamics underlying the process of genome size evolution using empirical data. We genotyped new WSSV isolates from five Asian countries, and analyzed this information together with published data. Genome size appears to stabilize over time, and deletion size at ORF23/24 was significantly related to when the first outbreak of WSSV occurred in a country. A simple mathematical model of genome size evolution was developed, and parameter estimates support a geometric model of genome size evolution: incrementally smaller genomic deletions are substituted over time. Based on an *in vivo* assay, we found that WSSV isolates with a smaller genome size had increased infectivity and decreased median host survival time, suggesting a link between genome size and fitness in an aquaculture environment.

Keywords: WSSV, aquaculture, genome, evolution, adaptive trajectory, genomic deletions, molecular epidemiology

This chapter has been submitted as: Dieu, B. T. M., Zwart, M. P., Hemerik, L. & Vlak, J. M. (2010). Adaptive trajectory of genome size evolution in an emerging shrimp virus.

Introduction

White spot syndrome virus (WSSV) was first reported in shrimp aquaculture in Taiwan in the early 1990s (Lotz, 1997). The virus has since spread worldwide, and has had a major negative impact on shrimp aquaculture (Escobedo-Bonilla *et al*., 2008; Walker & Mohan, 2009). WSSV is a dsDNA virus with a 300 kilo base pair (kbp) genome (van Hulten *et al*., 2001a; Yang *et al*., 2001), having one of the largest genomes of the animal viruses (Filee & Chandler, 2008). The virus has been recently assigned as the sole member of a new virus family, the Nimaviridae (Vlak *et al*., 2005).

During WSSV spread in Asia the virus has shown striking changes in biological characteristics (Marks *et al*., 2005a) and genotype (Dieu *et al*., 2004; Marks *et al*., 2004; Pradeep *et al*., 2008a,b). Relative to the putative ancestral virus, a WSSV variant that evolved in shrimp aquaculture is characterized by (i) causing higher host mortality, (ii) having a shorter host survival time, and (iii) having a higher within-host competitive fitness (Marks *et al*., 2005a). The most important genotypic changes during WSSV spread in Asia appear to be two genomic deletions, in the ORF14/15 and ORF23/24 variable regions (Dieu *et al*., 2004; Marks *et al*., 2004; Marks *et al*., 2005a). The total size of the genomic deletions which have occurred between the oldest and the most recent WSSV isolates analyzed to date is almost 15 kbp (Marks *et al*., 2004; Pradeep *et al*., 2008a,b), accounting for approximately 5% of the genome.

Deletions in the ORF14/15 and ORF23/24 variable regions have become progressively larger as the virus has spread and evolved, to the extent that these deletions can be used as molecular markers to study the patterns of virus spread on intermediate to large spatiotemporal scales. This approach has been used to study WSSV spread to Vietnam (Dieu *et al*., 2004; Dieu *et al*., 2010) and India (Pradeep *et al*., 2008). However, beyond the patterns of virus spread from Taiwan to Thailand (Marks *et al*., 2004; Marks *et al*., 2005a), Vietnam and India, little is known about the dynamics in space and time of the change in these genomic deletions and the consequences for WSSV evolution and virulence.

A number of relevant questions on the spread and evolution of WSSV therefore remain unanswered. First, it remains to be seen whether genome size has stabilized during the spatiotemporal spread of the virus. Genome size can only decrease until non-redundant or essential genes and regulatory sequences are disrupted or lost. What is the limit to deletions at the ORF14/15 and ORF23/24 variable loci, and has this deletion size been reached? Second, if one considers when WSSV outbreaks occurred in different countries (see Fig. 1, Table 1; see also pg. 2 in (Escobedo-Bonilla

et al., 2008), the temporal pattern does not suggest that the virus spread through a constant geographic radiation. Rather, the virus seems to rapidly traverse some large geographic distances (e.g., from Taiwan in 1992 to Thailand and India in 1994), whereas other, shorter distances are slowly traversed (i.e., the first WSSV outbreak in the Philippines occurred in 1999). Does molecular evolution of WSSV recapitulate the temporal pattern of spread?

These genomic deletions in the ORF14/15 and ORF23/24 variable regions have supposedly arisen because (i) they allow for faster replication due to a smaller genome size, and (ii) these sequences have become largely redundant in the novel shrimp aquaculture environment (Marks *et al*., 2004). The striking patterns in the evolution of these deletions over space and time (Dieu *et al*., 2004; Dieu *et al*., 2010) do not only have utility as molecular markers of virus spread; they are in effect a record of virus adaptation to shrimp aquaculture – spanning 10 years since the first known WSSV outbreak – and virus spread covering distances of thousands of kilometers, during which the virus continually encountered naive shrimp populations. The spread of WSSV in aquaculture therefore provides a unique opportunity to address questions about the dynamics of genome size evolution. If an organism has redundant genome sequences present, and if the population genetics of this organism allow for rapid evolution of genome size (Lynch, 2006), then we need to ask two questions: (i) what does the adaptive trajectory look like, and (ii) what mechanism accounts for this observed trajectory? To explore these issues we have characterized novel WSSV samples from five countries in Asia, and used these together with previously reported information to test a simple model of genome size adaptation.

Materials and methods

Collection of WSSV isolates and analysis of variable loci

The origins of the WSSV isolates analyzed in this study are shown in Table 1 and Fig. 1. Shrimp were cleaned with 70% ethanol and kept frozen during transportation to Wageningen University, The Netherlands, where samples were stored at -20° C until further processing. DNA extracts of collected shrimp were screened for the presence of WSSV with specific primers for *VP26* (Dieu *et al*., 2004). The five WSSV variable loci previously identified (Marks *et al*., 2004), variable number tandem repeat (VNTR) loci ORF75, ORF94 and ORF125, and variable regions ORF14/15 and ORF23/24, were characterized up to the nucleotide level as described (Dieu *et al*., 2004). PCR on WSSV variable loci was performed with 250ng viral DNA, using Taq DNA polymerase (Promega). The specific primer sets, PCR conditions used and sizes of the PCR products are shown in Table 2. PCR products were analyzed and sequenced according to published procedures (Dieu *et al*., 2004).

Table 1. Origins of the Asian WSSV isolates used in this study. "First WSSV outbreak" is the year in which a WSSV outbreak was first reported in the country where the sample was collected. Abbreviations in bold indicate the sample was genotyped in the present study.

Country	Host	First WSSV Outbreak	Place (Province)	Origin of post larvae	Date of Collection	Abbreviation
Taiwan	*P. monodon*	1992	Southern	ND	1994	*WSSV-TW*
China	*P. japonicus*	1993	Xiamen	ND	1996	*WSSV-CN*
Vietnam	*P. monodon*	1993	Quang Ngai	CentralVN	3/2003	*WSSV-VN-T*
Vietnam	*P. vannamei*	1993	Long An	CentralVN	2003	**WSSV-VN-LA**
Thailand	*P. monodon*	1994	Suratthan	ND	1996	*WSSV-TH-96-I*
Thailand	*P. vannamei*	1994	ND	ND	1996	**WSSV-TH-S**
India	*P. monodon*	1994	East coast	ND	2005	*WSSV-In-05*
India	*P. monodon*	1994	East coast	ND	2006	*WSSV-In-06*
Philipp.	*P. monodon*	1999	Iloilo	Local	04/2008	**WSSV-Phi**
Indonesia	Polychaete	1996	Jambak, Java	ND	02//2008	**WSSV-Indo**
Cambodia	*P. monodon*	1996	Shihanuk Ville	Thailand	02/2006	**WSSV-Cb**
Japan	*P. japonicus*	1993	ND	ND	1995	**WSSV-Ja**
Iran	*P. indicus*	2002	Abadan	ND	6/2002	**WSSV-Ir**

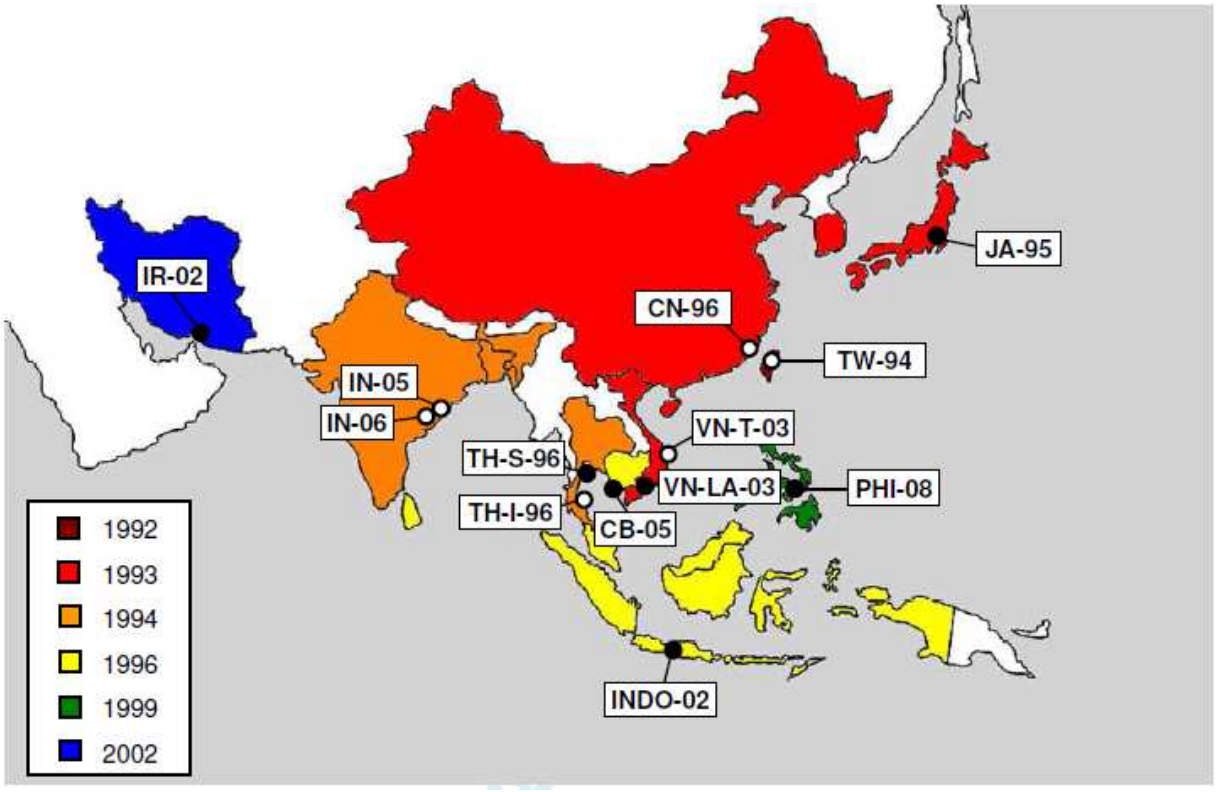


Figure 1: Map of Asia, showing the geographical origins of WSSV isolates used for this study. New isolates characterized in this study are marked with black circles, whereas isolates previously reported in the literature are marked with white circles. Further information on the isolates is given in Table 1. Colors denote the year of first outbreak for Asian countries for which this information is known (see Materials and Methods).

Table 2. Primers used in PCR analysis for the variable loci of WSSV.

Primer pair name	Primer orient.	Sequence (5'-3')	Anneal. temp. (^{0}C) / elong-ation time (s)	WSSV-CN sequence coordinates	Size (bp) of PCR product
VR23/24 -Ja	Forward	CAGATAATGCAAACACGAGACAC	49 / 100	275794-275816	~700
	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 – Asia screen	Forward	GAGTAGTCTTCAATGGCAATGT	55 / 80	275008-275029	~400
	Reverse	GATGACTCGGTACGCTTTAG		287376-287357	
VR14/15-screen	Forward	GAGATGCGAACCACTAAAAG	49 / 75	22904-22923†	~500/600
	Reverse	ATGGAGGCGAGACTTGC		24157-24141†	
VR14/15-TH	Forward	GAGATGCGAACCACTAAAAG	49 / 80	22904-22923†	~750
	Reverse	GAAAAATAAATCACGGGCTAATC		23646-23624†	

† According to WSSV-TH-I-96 sequence

Statistical analysis of ORF14/15 and ORF23/24 variable region data

For statistical analysis and modeling of WSSV genome size evolution, we combined the samples characterized here with other published reports containing information on the ORF14/15 and ORF23/24 variable regions (see Table 1). A maximum of 2 samples per country were included, so that none of the countries would heavily affect the outcome. If more samples were available (i.e. for India and Vietnam), we chose samples that were collected earliest. WSSV TH-96-II was not included in this analysis because this sample is the putative common ancestor (archetype) for virus strains in shrimp aquaculture (Marks *et al*., 2005a).

For the ORF14/15 and ORF23/24 variable regions, the non-parametric Jonckheere-Terpstra test (Bewick *et al*., 2004) (SPSS 15.0, SPSS Inc., Chicago, IL) was used to determine if median deletion size significantly increased or decreased when the samples were ordered according to: (i) the year of first outbreak in the country where the sample was collected ("first outbreak year"), or (ii) the ranked geographic distance of the sample collection site to Taiwan, the location of the first WSSV outbreak ("distance"). We are performing four comparisons (two independent variables and two loci), and a Bonferroni correction (Sokal & Rohlf, 1995) was therefore made to the significance threshold α so that the corrected threshold *P*-value (α') is 0.013.

In order to determine 'year of first outbreak', we used a published list of outbreak years (see Table 1 on pg. 2 (Escobedo-Bonilla *et al*., 2008). The presence of WSSV in Vietnam was confirmed by PCR in 1997 (Corsin *et al*., 2001), but the first outbreaks occurred in 1993 (Bondad-Reantaso *et al*., 2005; Khoa *et al*., 2000) and we used this date for our analysis. Cambodia is not included in the list of outbreak data we used (Escobedo-Bonilla *et al*., 2008). Viral disease was implicated

in the collapse of Cambodian shrimp aquaculture in the late 1990s, and we therefore took the year 1996, when shrimp production first declined (Sour & Viseth, 2004; Viseth & Pengbun, 2005) as an estimate for the first outbreak.

Model of WSSV genome size evolution

Fisher proposed that an organism adapts to its environment by the substitution of mutations that slightly enhance fitness, because mutations resulting in small fitness changes are more likely to be beneficial than mutations causing large fitness changes (Fisher, 1999). Kimura then showed that mutations leading to larger fitness enhancement had a larger probability of becoming established, suggesting that mutations leading to intermediate fitness enhancement are most likely to be substituted (Kimura, 1983). More recently theoretical work by Orr has shown that mean effects on fitness of the substituted mutations are similar to a geometric distribution, with each new mutation substituted in the population having a proportionally smaller effect on fitness than the previous mutation (Orr, 1998; Orr, 2002; Orr, 2005). In other words, whereas mutations with large effects on fitness are initially substituted, as the population approaches a fitness peak, mutations with smaller effects on fitness will be substituted. This pattern has been empirically observed for the evolution of fitness and morphological traits by experimental evolution (Bull *et al*., 1997; Holder & Bull, 2001; Lenski & Travisano, 1994; Schoustra *et al*., 2009).

We can apply this perspective to genome size evolution for WSSV, given that: (i) there are redundant genomic sequences present in the ancestral WSSV (Marks *et al*., 2004), (ii) replicative fitness is inversely proportional to genome size (Marks *et al*., 2005a), and (iii) if deletions occur at multiple loci there is no epistasis. The assumption that no epistasis occurs is plausible because the deleted sequences are thought to be redundant sequences. We then expect that the first substituted genomic deletion will be very large, and that subsequent genomic deletions substituted will be progressively smaller, until the optimum genome size is reached. In order to describe WSSV genomic size (S) evolution with a mathematical model, we assume the genome size of ancestral virus introduced into aquaculture (S_0) is a constant. We can then describe the evolution of genome size as follows:

$$S_{t+1} = S_t - b_t = S_t - ck^t \quad (1)$$

where t is time (measured in years), b_t is the deletion size at time t, c is the size (in base pairs) of the first deletion b_0 and k is the multiplication factor for this initial deletion size. We are interested in the multiplication factor k, because its value indicates whether the geometric model of adaptation applies to the evolution of

genome size. If $k = 1$ genome size evolution is linear: each new deletion substituted is of the same size as the previous deletion. If $k > 1$ genome size evolution is a process involving geometric growth: each new deletion substituted is larger than the previous deletion. If $k < 1$ genome size evolution is a process involving geometric decay: each new deletion substituted is smaller than the previous deletion. The last situation ($k < 1$) is predicted by the geometric model of adaptation to novel environments proposed by Orr (Orr, 1998; Orr, 2002; Orr, 2005).

We can then calculate genome size at t by subtracting the summation of the geometric sequence of all deletions which have occurred from genome size S_0:

(2)
$$S_t = S_0 - \sum_{i=0}^{t-1} b_i = S_0 - \sum_{i=0}^{t-1} ck^i = S_0 - \frac{c\left(1-k^t\right)}{1-k} \quad \text{for} \quad k \neq 1$$
$$S_t = S_0 - \sum_{i=0}^{t-1} b_i = S_0 - \sum_{i=0}^{t-1} c = S_0 - ct \quad \text{for} \quad k = 1$$

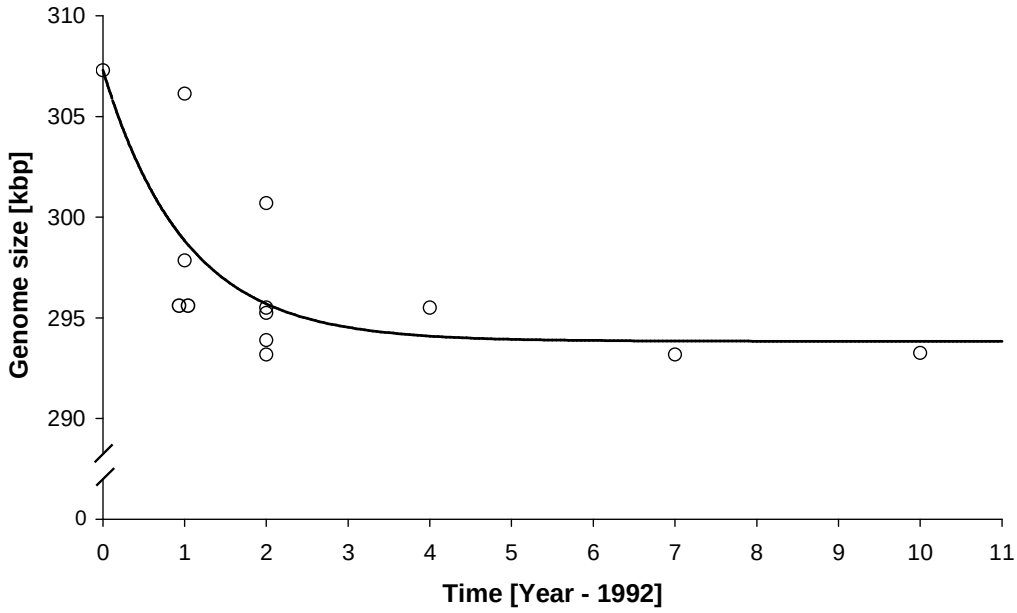
To test whether our data support this model, we fitted the upper part of equation 2 to the observed genome size ($S_{obs,i}$), which was calculated as:

(3)
$$S_{obs,i} = S_0 - D_{1,i} - D_{2,j}$$

where S_O is the genome size of the earliest WSSV isolate, WSSV-TW (S_O = 307,287 bp (Marks et al. 2004; Wang et al. 1995)), and $D_{1,i}$ and $D_{2,i}$ are the deletion sizes at ORF14/15 and ORF23/24 relative to the WSSV-TW sequence, for a virus isolate from a country in which the outbreak originated at time i, (number of years after 1992, thus $i = 0$ represents 1992). We only need to consider these two regions because the WSSV genome is surprisingly stable, with the exception of the ORF14/15 and ORF23/24 regions and VNTR loci (Marks *et al.*, 2004). Non-linear regression (SPSS 15.0) was used to fit the upper part of equation 2 to the data and obtain 95% confidence intervals for the fitted constants c and k. Time of first outbreak was used as the independent variable in non-linear regression, performed first for all the available data. However, the data points from later outbreaks ('first outbreak year' >1996) might strongly influence the parameter estimates and introduce a bias towards rejecting the null-hypothesis (k-values ~ 1).

Non-linear regression was therefore repeated for the trajectory where total attenuation was not yet observed, i.e. the samples of the Philippines and Iran were removed (see Fig. 4A). We refer to this subset as only 'early outbreak' data.

A



B

Parameter	All Data		Early Outbreak Samples (≤ 1996)	
	Estimate ± SE	95% C.I.	Estimate ± SE	95% C.I.
c	8,457 ±1312	5,568 – 11,345	8,699 ±1638	4,994 – 12,404
k	0.371 ±0.150	0.040 – 0.702	0.303 ±0.246	-0.251 – 0.860

Figure 4: The evolution of WSSV genome size. Panel A: the time of first outbreak for the country a WSSV isolate was collected from is plotted on the x-axis, and the genome size in kpb is plotted on the y-axis. The mean genome size is given by the squares, with standard errors were applicable, and the line indicates the model fitted to all data (equation 2). Panel B: results of model fitting, for all data or only the early outbreak data. The standard errors (SE) and confidence intervals (C.I.) of parameter estimates are given. In both analyses, *k* is significantly smaller than 1 as predicted by a geometric model of adaptation, where each new deletion occurring is smaller than the previous deletion.

In vivo assay for host survival and median host survival time

In our model development we assumed that replicative fitness is inversely proportional to genome size, based on the observation that a putative ancestral WSSV isolate with the largest known genome caused lower host survival, had a longer median host survival time and had a lower within-host competitive fitness than a variant with a smaller genome (Marks *et al*., 2005a).

Others have made similar observations when comparing virulence of early outbreak and late outbreak WSSV isolates (Laramore *et al*., 2009), and when relating genome size to competitive fitness (Pradeep *et al*., 2009). To confirm and further detail these results, we used an *in vivo* assay to determine the virulence and median survival time of the following WSSV isolates: (i) TH-96- II: putative ancestral

WSSV variant with a 312 kbp genome, the largest known WSSV genome (Marks *et al*., 2005a), (ii) VT-T, VT-X and VT-S: Vietnamese isolates with an intermediate genome size of 298 kbp (Dieu *et al*., 2004), and (iii) TH-96-I: a WSSV isolate collected early in the epizootic (1996) with small genome size of 293 kbp (van Hulten *et al*., 2001a). These five isolates were amplified in crayfish and virions purified as described elsewhere (Xie *et al*., 2005). Subsequently, we determined the virion concentration for each purified virus stock by ELISA using IgY against WSSV-VP28 produced in bacteria, competitive PCR (Tang & Lightner, 2000), and by counting intact virions with transmission electron microscopy. All methods gave similar results, and the ELISA data were then used to dilute all virus stocks to the same concentration (an absorbance of 0.445) using 330 mM NaCl buffer. 10^5, 10^6, and 10^7 dilutions (also in 330 mM NaCl) of these stocks were then used for the injection of shrimp.

The shrimp used for the experiment were SPF *Penaeus monodon* post larvae obtained from a commercial hatchery in Thailand, which we also PCR screened for viral diseases (Witteveldt *et al*., 2004). The shrimp were communally kept at 28 °C and fed commercial food pellets (Coppens International; Helmond, The Netherlands) prior to and during the experiment. Shrimp with a weight of 5-10 grams were intramuscularly injected with 10 µl dilutions of the WSSV stock using a 1.5 ml volume B-D Pen (Becton Dickinson) and 28Gx1/2" NovoFine needles (Novo Nordisk). For each virus (5 isolates) and each dose (3 doses), 14 shrimp were injected. Shrimps were subsequently housed individually, and mortality was recorded daily. Non-injected control shrimp were kept, and no mortality was observed in these shrimp. Shrimp were maintained until 29 days post-injection. PCR with specific primers for *VP26* (Witteveldt *et al*., 2004) was performed to confirm deaths were due to WSSV infection, and randomly selected surviving shrimp were also screened for WSSV infection.

For analysis of host survival and median host survival times, we pooled data from all three doses to increase statistical power. Differences in the proportion of hosts surviving until the end of the experiment were made using a χ^2 test for trend in proportions ('prop.trend.test', R2.7.0, The R Foundation; Vienna, Austria). For this test the samples were ranked in order of increasing genome size, and VT-T, VT-X, and VT-S were assigned the same score given the small differences in genome size (< 100 bp or 0.03% difference in genome size). Pairwise comparisons – with a Holm-Bonferroni correction – were also made between all treatments ('pairwise.prop.test', R2.7.0). Median survival times were calculated from the estimated Kaplan- Meier survival curve, and the log-rank test was used to look for significant differences between treatments (SPSS 15.0).

Results

Description of WSSV isolates

WSSV isolates originating from a single host were collected from ponds distributed over five different Asian countries (Fig. 1, Table 1). Among these, four isolates were derived from *Penaeus* shrimp culture ponds, and one was derived from a polychaete (Indonesian isolate; see Table 1), which are known to be WSSV carriers (Vijayan et al. 2005). All chosen isolates tested positive for the presence of WSSV using a single-step PCR, according to published procedures (Dieu *et al*., 2004).

Variable region ORF23/24

In order to map the ORF23/24 locus, PCR with the "VR23/24-Asian screen" primers (Table S1) was performed on all chosen samples (Fig. 2). The primer annealing sites flank the ORF23/24 variable region, based on WSSV-TH sequence (AF 369029, van Hulten *et al*., 2001a). The WSSV isolates from the Philippines, Indonesia and Iran rendered a 400-bp amplicon. Cloning and sequencing of this PCR fragment indicated that it was 100% identical to WSSV-TH isolate, with a deletion of 13,210 bp compared to WSSV-TW. The WSSV isolates from Japan and Cambodia gave a product of approximately 700 bp with primer set VR23/24-Ja (Table 2). Cloning and sequencing of these PCR fragments indicated that they were 100% identical to Indian isolate (ACC. No. EU 327499; (Pradeep *et al*., 2008a,b)), with a deletion of about 10,970 bp compared to WSSV-TW. All the characterized isolates therefore had relatively large deletions in the ORF23/24 region (Fig. 2). A Jonckheere-Terpstra test demonstrated that the ORF23/24 variable- region deletion size increased significantly with 'first outbreak year', but was not significantly related to 'distance from Taiwan' (Table 3).

Variable region ORF14/15

The location and size of the genomic deletion in ORF14/15 was determined for the WSSV-Asian isolates using a similar approach as for the ORF23/24 variable region. The archetype WSSV isolate TH-96-II, which has a 6,436 bp insertion compared to WSSV-TW (Marks *et al*., 2005a), was used as a reference sequence for determining the size of the deletion. A PCR reaction with the "VR14/15-screen" primers (Table 2) was first performed. The WSSV isolates from the Philippines and Indonesia showed an approximately 500 bp amplicon, similar in length to that previously reported for isolate K from Vietnam. Restriction enzyme analysis of the PCR products confirmed that these isolates have the same 6,031 bp deletion present in most WSSV-VN isolates previously analyzed. The isolates from Japan,

Iran and Cambodia gave an approximately 600 bp amplicon. Restriction enzyme analysis of the PCR products confirmed that these isolates have the same 5,950 bp deletion present in VN-X and VN-S (Fig. 3; see also (Dieu *et al*., 2004). A Jonckheere- Terpstra test demonstrated that the ORF14/15 variable-region deletion size was not significantly related to 'first outbreak year' or 'distance from Taiwan' (Table 3).

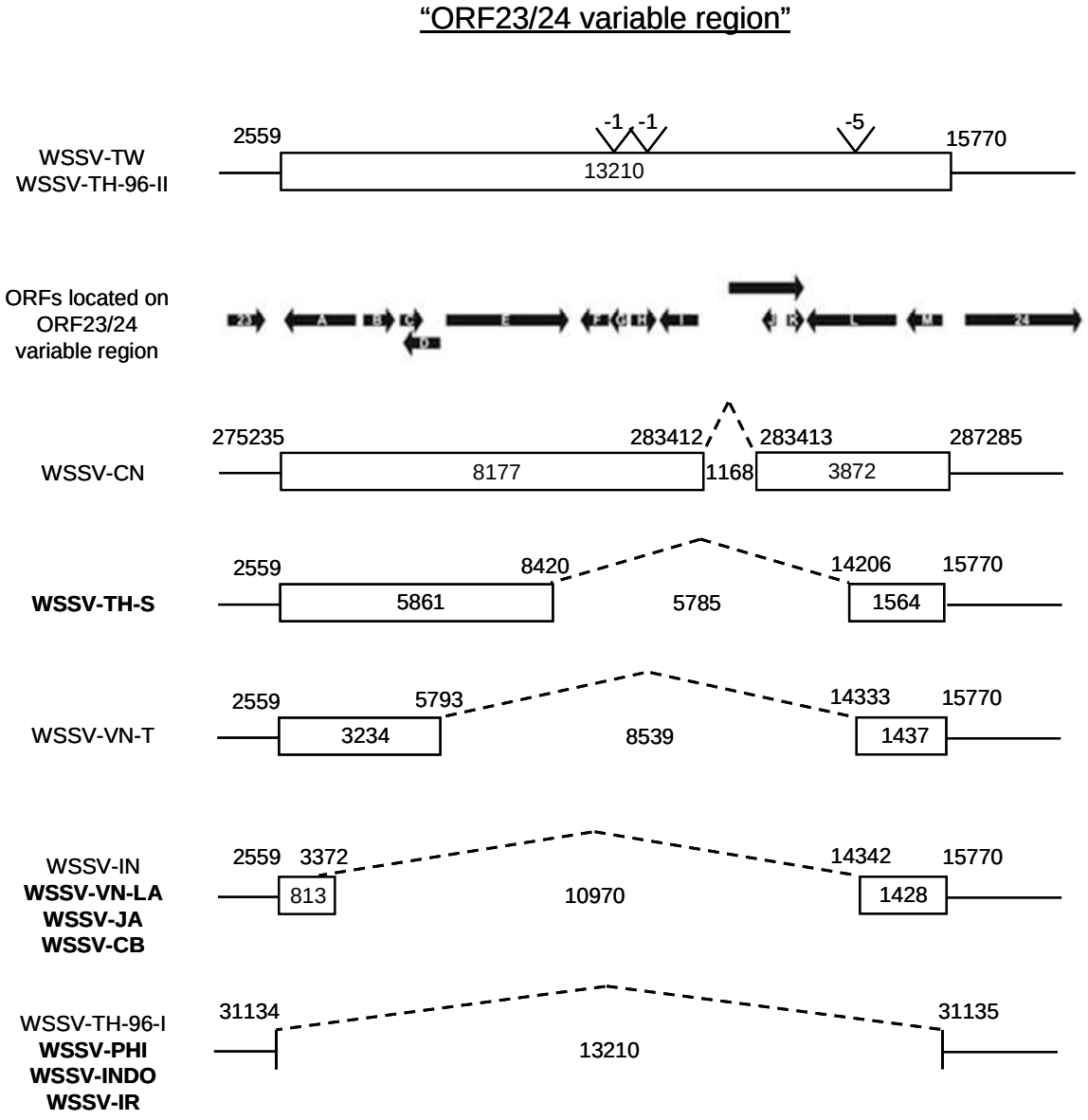


Figure 2: Schematic representation of the ORF23/24 variable region of the Asian WSSV isolates. The coordinates of the WSSV-Asian isolates are according to the WSSV-CN or WSSV- TH annotation. Names of WSSV isolates characterized in this study are in bold. The length of the fragments is indicated within boxes or sequences. The positions of the ORFs located in this region are indicated by closed arrows, which also represent the direction of transcription. ORFs are numbered in accordance to previous studies (Dieu et al. 2004; Marks et al. 2004).

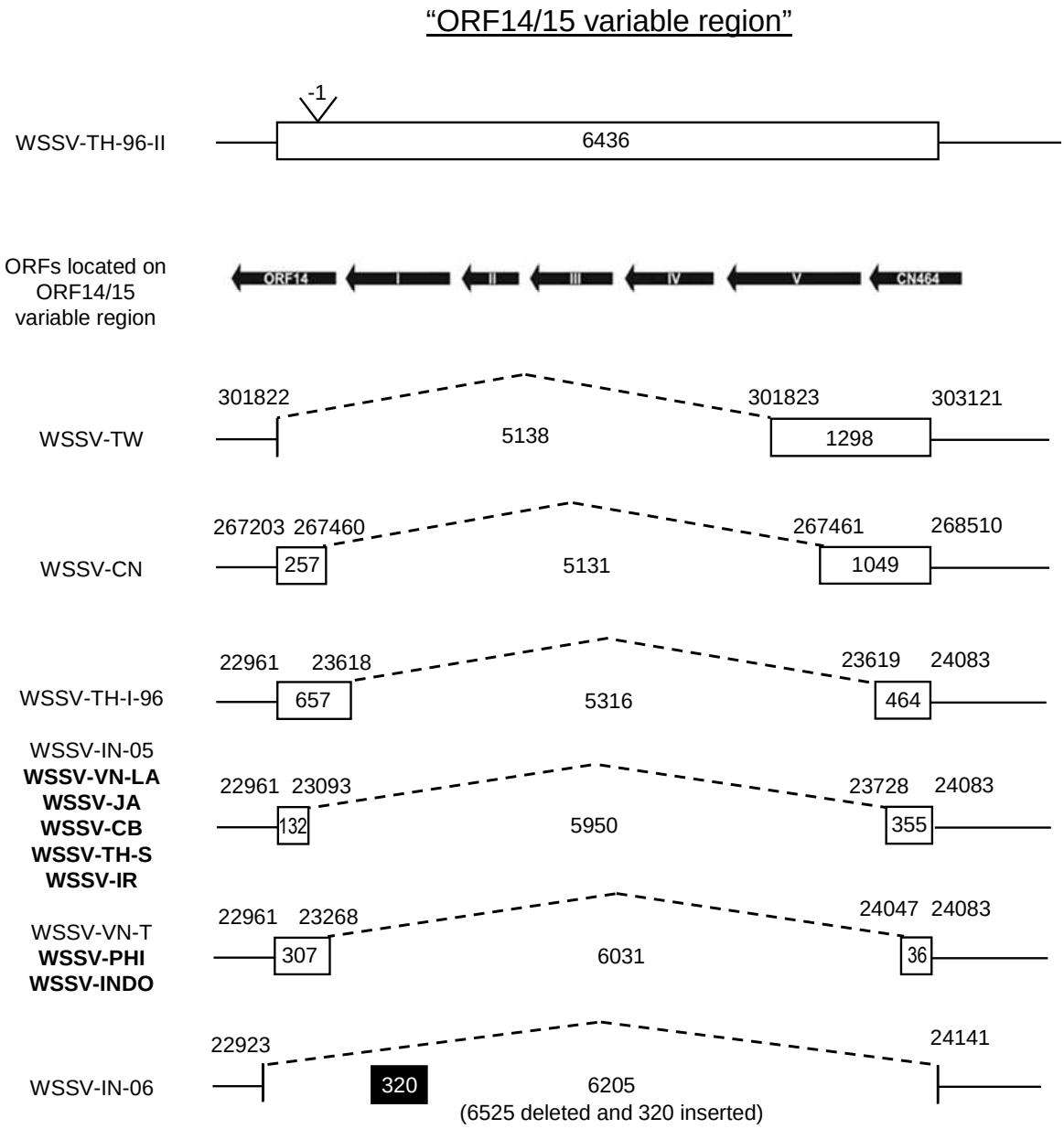


Figure 3: Schematic representation of the ORF14/15 variable region of the WSSV putative common ancestor (WSSV Th-96-II), and the WSSV-Asian isolates. Names of WSSV isolates characterized in this study are in bold. The coordinates of the WSSV-Asian isolates are given according to the WSSV TH-96-II annotation (Marks *et al*., 2005a). The length of the fragments is indicated within boxes or sequences. WSSV IN-06 has an inserted sequence, which is marked with a black box.

Table 3. Statistical analysis of ORF14/15 and ORF23/24 deletion sizes of Asian WSSV isolates**.**

Locus	Independent variable	N	Standardized *JT*	*P* value
ORF14/15	First outbreak year	13	1.410	0.158
	Distance from Taiwan	13	1.040	0.298
ORF23/24	First outbreak year	13	2.848	0.004*
	Distance from Taiwan	13	2.051	0.040

Jonckheere-Terpstra tests were performed on the deletion size of the two variable loci. N is the number of samples, Standardized *JT* is the test statistic, and *P* value denotes the significance, which is marked with an asterisk if below the threshold *P* value of 0.013 (see Materials and Methods). First outbreak year and distance from Taiwan were not significantly correlated (Pearson correlation coefficient = 0.432, $P = 0.141$).

WSSV genome size evolution

To test whether a geometric model of adaptation (Orr, 1998; Orr, 2002; Orr, 2005) applies to the evolution of WSSV genome size, the upper part of equation 2 (see Materials and Methods) was fitted by non-linear regression to the genome size estimated from the data (Fig. 4A). The value for the multiplication factor *k* was significantly less than 1 (Fig. 4B), both when all data or only the "early outbreak" data were analyzed. The "early outbreak" data (samples for which "first outbreak year" $\leq$ 1996) were also analyzed to avoid rejection of the null-hypothesis due to attenuation. Both *k*-value estimates indicate that the data support a geometric model of adaptation, i.e. each new genomic deletion substituted is on average smaller than the previous deletion (Fig. 4B).

Host survival and median host survival time of WSSV isolates

To test the previously reported relationship between genome size and virus fitness (Marks *et al*., 2005a), we performed an assay to determine host survival (at the end of the experiment) and median host survival time for five WSSV isolates with varying genome sizes (Fig. 5). We consider low host survival and short median host survival time as indicators of high fitness in an aquaculture environment. The proportion of host survival increased significantly with genome size ($\chi^2 = 21.378$, $P < 0.001$), supporting our hypothesis that fitness in an aquaculture environment increases as genome size decreases. However, only WSSV TH-96-II had a significantly higher host survival than the other isolates when pairwise comparisons were made ($P < 0.01$ for all comparisons to TH-96-II; $P > 0.05$ for all other comparisons). Significant differences between isolates in median host survival time were also found when making pairwise comparisons with a log-rank test. Isolate TH-96-II had a

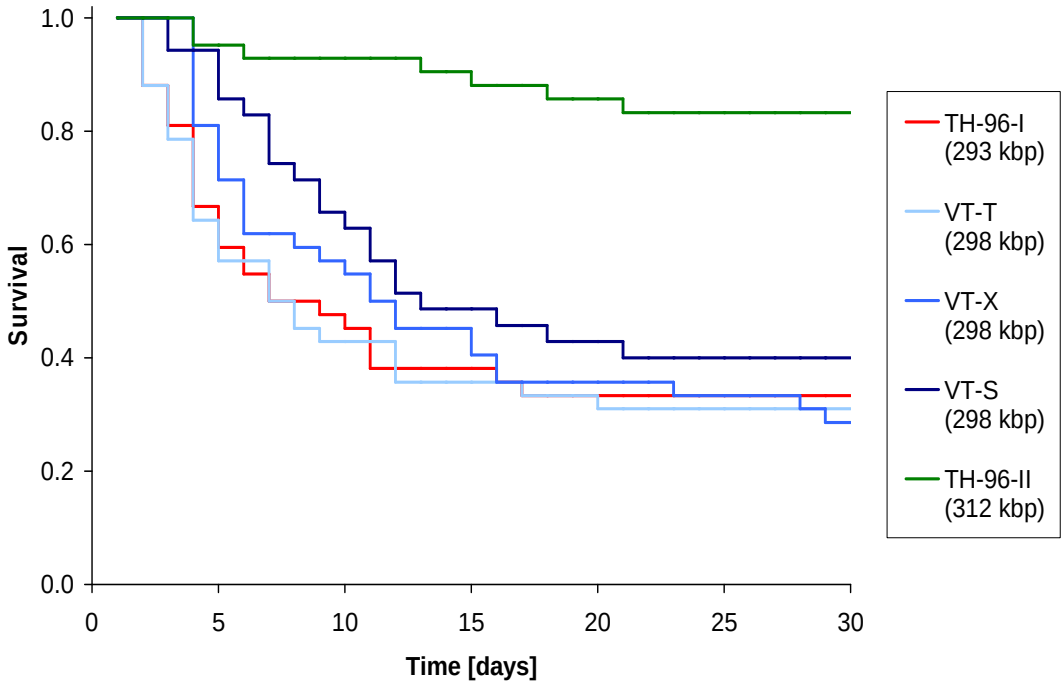


Figure 5: Kaplan-Meier survival curves for WSSV isolates with different genome sizes. Time in days is on the x-axis, and survival on the y-axis. The virus isolates and their genome sizes are given. Isolate TH-96-II has a significantly higher final survival, and a significantly longer median survival time. The data are pooled from three different virus doses.

significantly longer host survival time than the other four isolates ($P < 0.001$ for all comparisons), but there were no other significant differences between isolates ($P > 0.05$ for all other comparisons). The long host survival time for TH-96-II supports the hypothesis that genome size is linked to virus fitness. On the other hand, the median host survival time for intermediate genome size variants (WSSV VN-X, VN-S and VN–T) were not significantly different from the small genome size variant (TH-96-I). This observation is not contradictory to our hypothesis, however, because the genome size of the intermediates (298 kbp) is much closer to TH-96-I (293 kbp) than TH-96-II (312 kbp).

Discussion

We genetically characterized WSSV isolates from five new Asian countries. For the two variable regions in which large genomic deletions occur – ORF14/15 and ORF23/24 – we found that the size of the deletions appears to stabilize over time. This suggests that there is a limit to deletion size at these loci, and those genes or regulatory sequences up or downstream of the deletion are critical for virus replication. ORF25 is an immediate early gene, whereas ORF13 and ORF16 are late

genes, but no functions have been assigned (Marks *et al*., 2005b). We then tested whether there is a relationship between the size of the deletion at ORF14/15 and ORF23/24, and (1) 'first outbreak year' (the year of first outbreak in the country where the sample was collected), and (2) the 'distance from Taiwan' (the ranked distance of the sample collection site from Taiwan). The ORF14/15 variable region was not significantly related to either independent variable (Table 2). The ORF23/24 variable region was only significantly related to 'first outbreak year', and not to 'distance from Taiwan' (Table 2). These results are similar to a recent study on WSSV spread within Vietnam (Dieu *et al*., 2010), although the spatiotemporal scale we consider is much larger.

Our results show that WSSV molecular evolution at this ORF23/24 locus recapitulates the pattern of temporal spread, rather than being indicative of a smooth geographic radiation. This observation strongly suggests that commercial activities – such as the long-range transport of broodstock and post larvae – have been instrumental in the spread of WSSV. This conclusion will be moot for preventing the spread of WSSV in shrimp farming, as the virus has already spread worldwide. In the event of future disease outbreaks in shrimp or other aquaculture species, however, intervention strategies should sufficiently focus the effects of long-range transport of shrimp. For example, strict measures were in place in the Philippines to prevent the entrance of WSSV. These measures included: (i) prohibition on the import of all exotic shrimp species, (ii) an active surveillance and reporting system, (iii) regulation of the within-country movement of shrimp fry, and (iv) hatchery accreditation schemes. Although the virus was eventually established in 1999, these measures did provide protection to national shrimp aquaculture for a number of years (Regidor *et al*., 2005). Similar measures have probably contributed to Australia's disease-free status to the present day.

The WSSV isolates genetically characterized here allow us to investigate the trajectory of adaptive evolution of a virus in a novel environment. WSSV evolution in cultured shrimp has been marked by large genomic deletions (Dieu *et al*., 2004; Marks *et al*., 2004; Marks *et al*., 2005a). The deleted regions appear redundant in this new environment and their absence has been linked to higher host mortality, a shorter median host survival time and higher competitive fitness (Marks *et al*., 2005a). It was postulated that these effects were brought about due to a decreased genome size, which allowed for faster replication. We therefore postulated that genome size evolution proceeds according to a geometric model of adaptation (Orr, 1998; Orr, 2002; Orr, 2005), and formulated a simple mathematical model of this process. A biological interpretation of this model is that those viruses with the smallest genome – but still retaining sufficient genomic sequences to replicate – are

selected, at every time point in the evolutionary pathway. After initial selection for large deletions, there is fine-tuning of the genome size by 'trimming away' remaining redundant sequences flanking these large deletions (Fig. 6). Our data provide support for this model (Fig. 4A and B), which also provides further confirmation that the genomic deletions in the ORF14/15 and ORF23/24 variable regions have stabilized over time.

Our data also suggest a rugged fitness landscape in the vicinity of optimum deletion and genome size (Fig. 6). Deletion of sequences beyond the limits of the proposed optimum deletion size was not observed for the ORF23/24 variable region. For the ORF14/15 variable region, limits to deletion size appear to be less strict; an approximately 300 bp region in the 5' end is deleted in early samples but then re-appears, and WSSV-IN-06 has a larger deletion than other isolates, while also carrying an insertion (Fig. 3). These observations suggest that WSSV variants carrying larger genomic deletions pay a high fitness cost due to the deletion of important, non-redundant sequences, or lose viability all together.

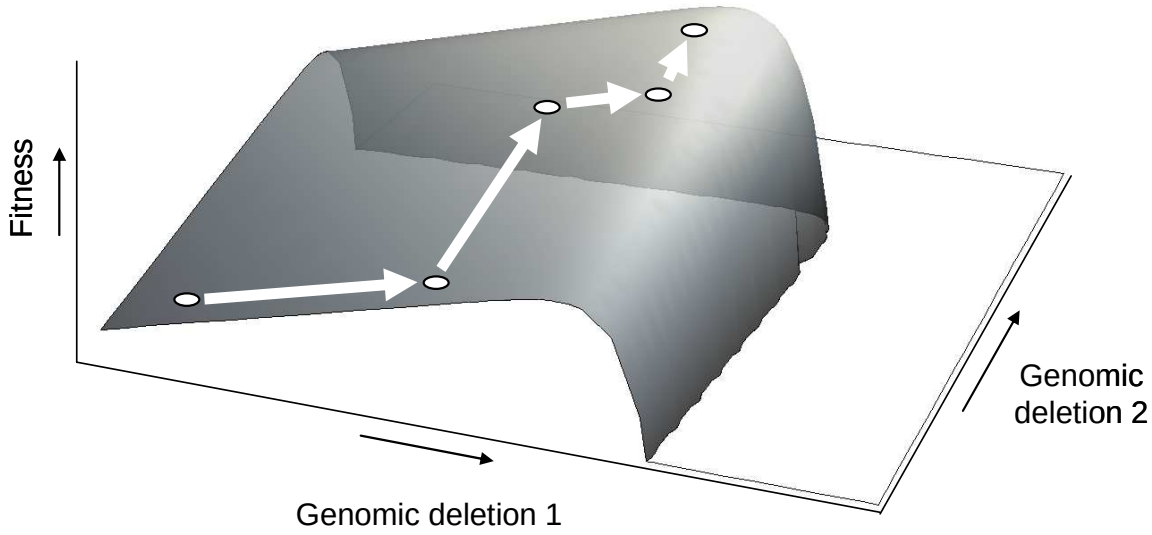


Figure 6: Conceptual fitness landscape for an organism evolving a smaller genome size. Arrows near the axes indicate the direction along which deletion size or fitness increases. Genomic deletions in two loci result in increased fitness due to a smaller genome size and faster replication. However, if the genomic deletions become too large non-redundant sequences are deleted and the organism may no longer be viable. The white arrows show a hypothetical example of our proposed model of evolution of genome size: initially large deletions occur, followed by 'fine-tuning' of genome size. Note that until both deletions approach the optimal size, there is no epistasis and the fitness landscape is a flat, inclined plane.

The compact organization of the genome of many micro-organisms suggests a link between genome size and replicative fitness (Lynch, 2006), although there are clearly other determinants of replicative fitness (Bull *et al*., 2004). The putative ancestral WSSV with a large genome caused higher host survival, a longer host survival time and had reduced within-host competitive fitness than an evolved WSSV strain with a smaller genome (Marks *et al*., 2005a). This observation suggests that genome size is linked to virulence and competitive fitness. Other recent studies also suggest that WSSV genome size is linked to competitive fitness (Pradeep *et al*., 2009), and that WSSV adaptation to aquaculture conditions may lead to higher virulence (Laramore *et al*., 2009). To confirm and further detail these results, we determined host survival and median host survival time of five WSSV isolates with varying genome sizes. Host survival significantly increased with genome size, and the WSSV isolate with the largest genome size (Th-96-II) had a significantly longer survival time (Fig. 5). These observations provide support for our hypothesis that the evolution of a smaller genome has in this instance led to increased fitness in an aquaculture environment. The ability of transmission stages to cause infection and host mortality – and therefore low host survival - is considered an important component of micro-parasite fitness, also in the case of WSSV (Marks *et al*., 2005a). In aquaculture conditions, the main route of WSSV transmission is via ingestion of infected cadavers (Soto & Lotz, 2001; Wu *et al*., 2001), implying it is advantageous to kill the host quickly and hereby achieve earlier transmission than competitor strains. We therefore also consider median survival time an indicator for fitness in aquaculture conditions. One important limitation to this experiment is that natural virus isolates were used, and therefore other genomic variations within the isolate could in principle be responsible for the observed differences. However, variation in the WSSV genome seems to be concentrated in the ORF14/15 and ORF23/24 variable regions (Dieu *et al*., 2004; Marks *et al*., 2004; Marks *et al*., 2005a).

To our knowledge, we are the first to report the adaptive trajectory during genome size evolution, although others have suggested a pattern of incrementally smaller deletions may apply to genome shrinkage in bacteria (Gomez-Valero *et al*., 2007; Nilsson *et al*., 2005; Ochman, 2005).

This topic should receive further consideration, as there are many conceivable scenarios in which genome size will be under strong selection (Lynch, 2006) and show rapid evolution. Three obvious cases are (i) emerging infectious disease outbreaks, as discussed here, and (ii) the evolution of defective interfering particles (Huang, 1973; Von Magnus, 1954; Zwart *et al*., 2008), and (iii) evolution of genome size following genome duplication (van Hoek & Hogeweg, 2007). Experimental evolution (Buckling *et al*., 2009; Nilsson *et al*., 2005) would be an excellent tool to

study the evolution of genome size and complexity, and further test whether a geometric model of adaptation is relevant to this process.

An important assumption when inferring patterns of spread from molecular epidemiological data is that WSSV populations are stable over time; obtaining data from different geographic locations at one point in time can be difficult, if not impossible. The striking patterns in WSSV molecular epidemiology (Dieu *et al*., 2004; Pradeep *et al*., 2008a,b) however, suggest that this assumption is largely met. Moreover, in a preliminary study it has been observed that intermediate-sized deletions in the ORF23/24 variable region can be stably maintained in WSSV populations in extensive farms in Vietnam over many years (B.T.M. Dieu, J.M. Vlak and M.P. Zwart, unpublished manuscript). Others recently reported that intermediate ORF23/24 variants were still present in Southern China in 2007 (Tan *et al*., 2009). This observation is also congruent with the idea that WSSV populations with intermediate-sized deletions can be stable over many years, because China was affected by WSSV very early (Fig. 1). However, the stability of WSSV populations – at a one location over time – deserves further consideration. Our data imply that the spatial spread of WSSV is paired with rapid molecular evolution (i.e. the occurrence of progressively larger genomic deletions), whereas persistence of established WSSV population at a one location is marked by stasis. This pattern could be described by the importance of within-host competitive fitness – and therefore strong selection for faster replication – during invasion of populations of naïve hosts. Investigating these issues will be facilitated by continued characterization of WSSV variable loci for virus samples from new geographic locations and different times of collection (Tan *et al*., 2009).

Acknowledgements

We thank Desrina Haryadi for the Indonesian WSSV samples, Eleonor Tendencia for Philippine WSSV samples, Dr. M. Afsharnasab for Iranian WSSV samples, Prof. Toshiaki Itami for Japanese WSSV samples, and Angela Vermeesch, Jeroen Witteveldt and Hendrik Marks for technical assistance with the *in vivo* assay. B.T.M.D. was supported by a WOTRO Ph.D. fellowhip (grant WB 83-207).

Chapter 7

General Discussion

Synopsis

White spot syndrome virus (WSSV) causes a devastating disease for shrimp and quickly spread world-wide in shrimp aquaculture since its discovery in the early 1990s. Many methods of disease prevention and control have been tried. Some of these methods have resulted in significant improvements in shrimp health, in particular PCR screening of post-larvae (Thakur *et al.*, 2002), and the introduction of more resistant shrimp species, such as *P. vannamei* (Briggs *et al.*, 2005). However, despite these improvements, WSSV remains a serious problem and most major shrimp operations are at risk of disease outbreaks (OIE, 2003a). Understanding epidemiology and evolution of the virus could give information for designing effective prevention strategies. This raises the question whether the combination of a suitable method and sufficiently variable WSSV loci can be found. This combination would be used as a marker for inferring the relationship between WSSV isolates, and tracking the evolution of this virus through space and time. This question links together the work presented in this thesis: understanding the origin of WSSV isolates from different geographical locations and inferring patterns of virus spread and evolution over space and time using molecular markers (phylogeography). This thesis goal necessitates addressing four separate issues: (i) Valuation of WSSV variable loci, more specifically which loci are suitable markers for studying WSSV spread on different spatiotemporal scales, (ii) Exploration as to whether VNTRs could be used to study the heterogeneity within WSSV isolates, (iii) Evaluation of the longitudinal effects of shrimp production practices on the genetic structure of WSSV populations and (iv) Investigation of the dynamics of change of WSSV variable loci and genome size: does the trajectory of adaptation render information about the underlying process?

The ORF23/24 and ORF14/15 variable loci with large genomic deletions, and variable number tandem repeat (VNTR) sequences in ORF75, ORF94 and ORF125 were demonstrated as the major variable loci in the WSSV genome (Marks *et al.*, 2004). The clear patterns of variation in these loci between WSSV isolates at various spatiotemporal scales, which originated from the key shrimp production areas in Vietnam and Asian countries, indicate that they can be suitable markers for studying WSSV epidemiology (Chapter 2, 3 and 6). However, care must be taken to choose an appropriate marker for a given spatiotemporal scale. These results, supported by statistical analysis and a mathematical model, indicate the usefulness of integrated approaches in epidemiology (Chapters 3 and 6). Molecular tools such as the

polymerase chain reaction (PCR) and sequencing allow for the characterization of large numbers of virus isolates down to the nucleotide level, but a quantitative framework for the interpretation of data and rigorous testing of hypotheses are indispensable. The factors that affect the observed genetic patterns were further investigated. Methods for exploring genetic heterogeneity within WSSV isolates were explored in Chapter 4. We found that molecular cloning induced variation in VNTR sequences and therefore explored the possibility of *in vivo* cloning (Smith & Crook, 1988) of WSSV genotypes. Finally it was found that WSSV populations in extensive shrimp farming systems were genetically stable over time, as opposed to those in intensive systems. This observation suggests that the earlier system is a good source of WSSV isolates that are likely to be more representative of the ancestral viruses that first 'colonized' a geographic region, when retrospectively sampling WSSV isolates to study virus spread (Chapter 5).

Patterns of WSSV spread and their implications for the evolutionary history of this virus are discussed in many chapters (Chapters 2, 3 and 4). One simple but important finding is consistently supported: all WSSV strains so far studied share a very recent common ancestor – probably close to the start of the world-wide outbreak in the early 1990s. An evolutionary framework was used to explore the patterns of WSSV molecular evolution (Chapter 6), in which it was found that the adaptive trajectory of WSSV genome size evolution conformed to a geometric model of adaptive evolution proposed by Orr (1998). It is very well possible that the isolate, found in Thailand in 1996 (TH-2) and supposedly containing the largest WSSV genome to date, is closer to the ancestral virus (Marks *et al.*, 2005a).

Why are the gradients in ORF23/24 conserved?

Overall, the ORF23/24 locus showed a gradually increasing deletion size at various spatiotemporal scales (Chapters 2, 3 and 6). A number of underlying processes could potentially explain this observed pattern, raising a number of questions. Three hypotheses on molecular evolution at the ORF23/24 locus during WSSV spread are illustrated in Figure 1. When the virus spreads to a new box (i.e. an area in which the virus was previously not present), this is referred to as an 'epidemic situation'. When the virus stays in the same box, this is referred to as the 'endemic situation'.

Hypothesis 1 (Figure 1A) is that WSSV molecular evolution at the ORF23/24 locus occurs at a constant rate in both epidemic and endemic situations. In other words, whether the virus spreads or not does not affect the rate of molecular evolution, and the environment is similar at the different geographic locations (i.e. all boxes). It is then expected that the virus genotypes found at all locations and collected at one time

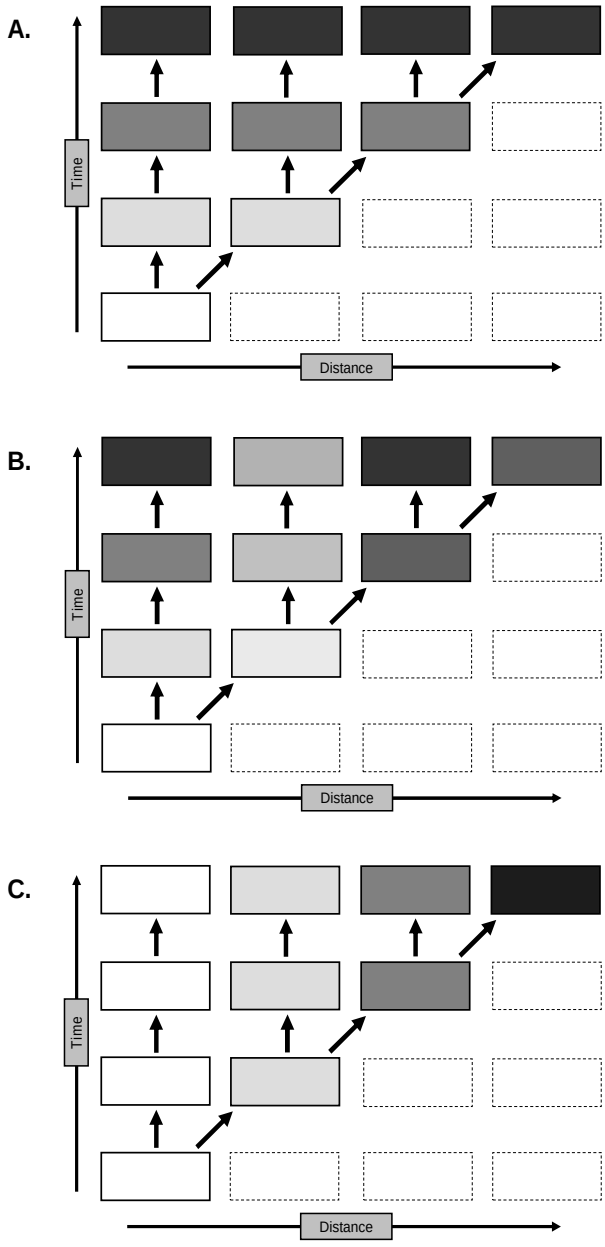


Figure 1. Three hypotheses of molecular evolution during geographic spread of WSSV. Geographic distance is on the horizontal axis and time on the vertical axis. Boxes with solid boundaries are invaded geographic locations, while boxes with dotted boundaries have not yet been invaded. Shading of the boxes indicates a molecular trait that is evolving (e.g. deletion size at ORF23/24). In A the rate of molecular evolution is the same in epidemic (invading a new box) and endemic (staying at the same box) situations and independent of the geographic location. In B the rate of molecular evolution, or genotype under selection, is dependent on geographic location. In C the rate of molecular evolution is faster in epidemic situations than in endemic situations. The patterns of genome size evolution of WSSV, based on the variable regions at ORF14/15 and ORF23/24, are most consistent with the third hypothesis.

point will be similar. Hypothesis 2 (Figure 1B) is that the virus encounters different environments as it spreads and that these environments (i) select for different virus genotypes, (ii) lead to different rates of molecular evolution or (iii) both. As a consequence it is expected that the genotypes at each location are different, but that there is no geographic 'gradient' for ORF23/24. A gradient in ORF23/24 would only occur if there is also a gradient in the environment (i.e. an environmental condition leads to progressively faster molecular evolution – i.e. increasing temperature along the 'route' the virus has spread). Hypothesis 3 (Figure 1C) is that different rates of evolution occur, depending on whether the virus is in an endemic or epidemic situation. During epidemic spread, the rate of molecular evolution is higher than during an endemic situation. This hypothesis would lead to a gradient, as the more time a virus is in the epidemic situation (i.e. those individuals in the 'leading edge' of virus spread), the farther it will have spread and the more molecular evolution it will have undergone.

Which hypothesis fits the thesis data? Hypothesis 1 does not fit because there is a gradient of the genotypes for ORF23/24 (Chapters 2, 3 and 6). Genetic drift, which could cause genetic changes, is excluded as it would lead to a random pattern but not a gradient. Hypothesis 2 probably does not fit because it is highly unlikely that any such gradient exists. Firstly, the pattern observed (increasing deletion size) occurred on two different scales: within Vietnam (spread north and south from central Vietnam, Chapter 3) and during spread throughout Asia. Since the magnitude of change in deletion size was approximately the same, this suggests that WSSV was not adapting to gradients at these different scales. Secondly, the patterns observed in Vietnam and Asia occur as the virus radiates out from the original outbreak site (central Vietnam or Taiwan, respectively). This means the gradient must be the same in all directions the virus radiates out, again making this a highly unlikely situation. Hypothesis 3 is most congruent with the data, because it is congruent with the observation of (i) a gradient at both intermediate (Chapter 3) and large scales (Chapters 2 and 6), and (ii) stability of genotype at a given geographic location (Chapter 5).

Overall, the thesis data therefore suggest that WSSV molecular evolution at the ORF23/24 variable region occurs at different rates in endemic and epidemic situations. But why would these different rates of evolution occur? One possible explanation is that during epidemic situations and at high shrimp density, there is very strong selection for fast replication. The infection in a host must proceed quickly so that it can be spread to other naive shrimp. During endemic situations fast replication is no longer important, because most shrimp are infected and thus there is no benefit to replicate quickly. Rather each shrimp is a resource that must be prudently exploited by the virus. This proposed explanation has parallels with *K* and *r* strategies, as used by

ecologists (Parry, 1981). The *r* strategy applies to the epidemic situation, when the virus should spread quickly to new hosts, and the *K* strategy applies to the endemic situation, when the virus must prudently exploit the host.

Is there evidence of adaptation to unique, local environments for WSSV?

The genotype in ORF23/24 with 8,539 bp deletion was only detected in the seven provinces located in the central region of Vietnam (isolates H, K, T, L, X, S, A; Chapter 2 and 3). The weather in this entire region is the hottest and driest in the entire country. This observation suggests that there may be selection for a specific WSSV genotype that thrives in this unique local environment. On the other hand, this is also the site in which WSSV was probably first introduced in Vietnam (Chapter 2 and 3). Our interpretation of the data suggests that this unique genotype is simply a surviving intermediate, reflecting WSSV's evolutionary history rather than local adaptation.

ORF14/15 was not a suitable marker locus for WSSV spread on an intermediate scale (Chapters 3 and 4), although it is perhaps suitable on very large spatial scales (Chapter 6). Two other ORF14/15 variants are present as unique subgroups for Vietnam, represented by: (i) isolates X and S, and (ii) isolate A. A variant with a 6,030 bp deletion was found for almost all other Vietnamese isolates (twelve out of seventeen isolates) (Chapter 3). The genotype of isolates X and S was found later in other late WSSV-VN isolates (from 2005 till 2008), which were derived from *P. vannamei,* a shrimp species introduced for aquaculture purposes only recently in Vietnam (Corsin, 2005; Chapter 3). The genotype found in isolate A has not been found in any other WSSV isolate to date. Therefore these subgroups may have originated not only because of the geographic spread of WSSV, but also because of environmental conditions, such as different hosts or habitats. However, this aspect has not been given much consideration and further studies on the effects of environment and host factors on WSSV genetic variation are needed.

Implications of the thesis results

The results from this work confirm that long range transport of post-larvae, broodstock and infected shrimp food plays an important role in WSSV spread (Chapter 3, 5 and 6). Other studies have reached this conclusion (Lightner *et al*., 1997; Durand *et al*., 2000), although this work provides confirmation from a molecular epidemiological perspective. Moreover, trans-boundary policy has been demonstrated to prevent WSSV spread to virus-free areas, such as the Philippines and Australia (Flegel and Fegan, 2002). Therefore, despite the fact that WSSV has already been very widely disseminated, blocking the transport of infected material is indispensable to prevent spread to remaining virus-free areas, such as Australia. Trans-boundary policies should

also be widely applied in intervention strategies for other disease outbreaks in aquaculture.

There are good indications that WSSV genotypes with larger genomes are less virulent than those with smaller genomes (Chapter 6, Marks *et al*., 2005a). Moreover, the current work suggests that in an endemic situation WSSV variants are relatively stable (Chapter 5) and a suggestion has been made that genome size evolves slower during an endemic than an epidemic situation (see above and Chapter 6). A logical prediction which follows from this interpretation is that less virulent WSSV variants – those with a relatively large genome size – were present for a long time in those regions which first experienced WSSV outbreaks (i.e. Taiwan, China, Japan and to a lesser extent perhaps Vietnam). Whether this prediction is correct remains to be seen, although Tan *et al.* (2009) reported finding intermediate WSSV ORF23/24 variants in Southern China in samples collected in 2007. Moreover, even if these large genome variants with lower virulence have been preserved for a considerable period of time, it remains to be tested whether the biological differences (e.g. Marks *et al.*, 2005) actually result in differences that significantly impact shrimp production on a commercial scale. In other words, less virulent genotypes may have been conserved due to evolutionary dynamics, but did this impact shrimp production?

What next for the future study in this field?

It is necessary to have further studies to confirm these results and to answer many remaining questions. Firstly, a complete framework for selecting suitable molecular markers and interpreting the data at different spatiotemporal scales is required. Although we have considered large (Chapters 2 and 6) and intermediate (Chapter 3) scales, there have been no systematic tests on small or very small spatiotemporal scales (e.g. provincial to pond level). However, our data (Chapter 3) and studies published by others (e.g. Hoa *et al.*, 2005; Pradeep *et al.*, 2008a,b) suggest that VNTRs are probably suitable markers at these small scales. Suitable data sets with a sufficient number of samples and suitable sample sources (as suggested by Chapters 3 and 5) that are representative for a small spatiotemporal scale will be required. Data published by Pradeep *et al.* (2008a,b) may very well be suitable for such a test. Study design will require careful attention to the sampling regimen and obtaining detailed histories of the samples (i.e. post larvae and host sources, environment conditions). These data may then be suitable for inferring virus origin and other factors that could be involved in the virus spread.

Secondly, longitudinal studies in which WSSV samples are collected in and around a large number of ponds should be performed. The samples should then be genotyped to valuate the different WSSV variable loci as markers. It is expected that

the VNTR loci will be suitable as molecular markers at small to very small scales, also when a temporal component is explicitly analyzed. Host species, including intermediate hosts such as mud crabs, are thought to have an effect on genotypic composition and virulence of WSSV populations (B.T.M. Dieu & J.M. Vlak, unpublished data). Virulence may be linked to WSSV genome size (Chapter 6) and it may therefore also be interesting to genotype ORF14/15 and ORF23/24 in such a study.

The extent to which genetic heterogeneity occurs within single-host WSSV isolates is an issue that remains to be clarified. This is important both from fundamental and applied perspectives, as genetic heterogeneity could be determinant of both the fitness and virulence of WSSV populations. Not only should VNTRs be further studied with the *in vivo* cloning methods as suggested in chapter 4; ORF23/24 and ORF14/15 could also be used as markers for such a study. These variable sequences will probably not be prone to experimental artifacts reported for VNTR loci in Chapter 4.

In addition, infected but surviving shrimp, and virus-induced dead shrimp have been found in the same pond (Luo *et al.*, 2003). However, a genetic characterization of the viruses found in surviving and dead shrimp is still missing. The variable loci explored in this thesis could be used as markers for this analysis to see whether there is a relationship between these loci and virulence. The results of Chapter 6 and Marks *et al.* (2005) strongly suggest that such a relationship may be found.

Many important questions about the WSSV biology and epidemiology remain unanswered. However, our results shed light on simple methods that can be applied in ecological studies of emerging viruses with only limited genomic variation.

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Summary

White spot syndrome virus (WSSV) is a large double-stranded DNA virus and the sole member of the Nimaviridae virus family. This virus causes devastating damage to shrimp farming by rapidly inflicting high levels of mortality. WSSV quickly spread worldwide since it was first described for Taiwan and China in the early 1990s. No adequate methods for fully controlling this disease have been found so far. Understanding the origin of the different WSSV isolates and making inferences on geographical spread of the virus and evolution over space and time would be an important step to developing methods to mitigate the effects of WSSV on shrimp farming. This thesis develops genetic markers for identification and discrimination of WSSV isolates at different spatiotemporal scales. These markers have important applications in determining patterns of WSSV spread and further understanding WSSV evolution.

The variable regions with genomic deletions and variable number tandem repeat (VNTR) loci were indicated as the major variable loci in the WSSV genome by alignment of the three completely sequenced virus isolates from Taiwan (WSSV-TW), China (WSSV-CN) and Thailand (WSSV-TH). The pattern of variation between these isolates suggests that all three WSSV isolates originate from a common ancestor, perhaps in or around the Strait of Taiwan. The virus then spread to Thailand, but intermediate isolates – both in terms of geographic location and genotype – were missing. Vietnamese WSSV isolates fit this requirement and were used to evaluate whether these loci are suitable as genetic markers for identification and discrimination of WSSV isolates. Restriction fragment length polymorphism (RFLP) analysis and further genotyping by means of PCR amplification and sequencing were used to reveal genetic variation between these isolates. The gradually increasing deletions of both the variable regions with genomic deletions further support the supposition that multiple introductions of WSSV occurred in central Vietnam, a geographic location which was probably passed during spread from the straits of Taiwan to Thailand (Chapter 2).

The coverage within Vietnam afforded by the low number of samples analyzed was not sufficient, however, to allow for a test of the markers at an intermediate geographical scale and meaningful statistical analysis of the data. Therefore, more isolates from the key shrimp production regions of Vietnam were analyzed to achieve a quantitative framework for a more rigorous interpretation. This larger data set allows for statistical analysis and more detailed inferences on WSSV spread in Vietnam – an intermediate spatiotemporal scale. Progressive increases of the genomic deletion size

were shown for the variable region with larger genomic deletion. The detailed molecular data and the history of shrimp aquaculture suggest that WSSV was introduced in central Vietnam and then radiated out to the North and the South. The data also indicated that these genetic loci are suitable markers for determining WSSV spread on different spatiotemporal scales. Specifically, the variable region with larger genomic deletion is the most appropriate marker at intermediate scale. VNTR loci were indicated as the suitable markers for smaller scales, whereas the variable region with smaller genomic deletion is more stable and may be suitable for studying larger spatiotemporal scales (Chapter 3).

An evaluation of whether VNTR can be used to study genetic heterogeneity within WSSV isolates from a single shrimp was performed. In a control experiment variation in molecularly cloned VNTR sequences was observed when using clonal VNTR sequences as source material. This indicates that the cloning procedure introduces spurious variation, making it problematic to study VNTRs with approaches requiring molecular cloning. However, no variation was observed in multiple replicates of PCR amplification of a single clone, indicating that VNTR PCR-based genotyping alone is suitable. Moreover, after 20 replicates of two passages of WSSV in *Penaeus vannamei* shrimp no variation was detected, indicating that WSSV VNTR loci are sufficiently stable to be used as molecular markers on short time scales. *In vivo* cloning was proposed to study polymorphism within WSSV populations as the WSSV dose-response relationship corresponds to theoretical predictions (Chapter 4).

Effects of extensive and intensive farming practices on the genotypic composition of WSSV populations were evaluated by genotyping the variable genomic deletion loci of WSSV samples collected from Vietnamese farms at various sites over a period of several years. Only the ORF23/24 variable region was stable over time in extensive farming systems, whereas none of the variable loci were stable in intensive systems. This observation implies that extensive farms are a good source of samples when the spread of WSSV is to be studied from retrospectively collected virus isolates (Chapter 5).

Deletion sizes in the deleted variable region of WSSV isolates from Japan, Iran, the Philippines, Indonesia and Cambodia were significantly related to the ‘time of first WSSV outbreak in a country’ when analyzed together with genotyping data from other studies. This relationship also confirmed that the genomic deletion loci – in particular the larger deleted variable region – are useful markers on large spatiotemporal scales. A simple model of genome size evolution was formulated based on a geometric model

of adaptation. Fitting of the model to the data provided support for the hypothesis that the WSSV genome adapted to shrimp aquaculture by the substitution of incrementally smaller genomic deletions over time. The data also suggests that there is a maximum deletion size for both loci because the deletion size stabilizes over time. Moreover, the congruence between the temporal spread pattern and molecular epidemiological data provide further support for the notion that WSSV spread on large scales is heavily dependent on long range transport of broodstock and post larvae (PL) (Chapter 6).

In conclusion, different variable loci in WSSV genome were evaluated as the suitable makers for molecular epidemiology at various spatiotemporal scales. A combination of molecular data and simple quantitative analyses lead to a detailed spread model of WSSV within Vietnam and ideas about the evolutionary trajectory of viral genome size evolution. The results have implications for the design of molecular epidemiology studies and intervention strategies for WSSV, and also other pathogens in aquaculture.

Samenvatting

White spot syndrome virus (WSSV) is een groot dubbelstrengig DNA-virus en de enige vertegenwoordiger van de virusfamilie Nimaviridae. Dit virus veroorzaakt grote schade in de garnalenteelt door het snelle optreden van sterfte als gevolg van WSSV-infectie. WSSV heeft zich vanuit China en Taiwan, waar het voor het eerst in de jaren 1990 is ontdekt, snel over de hele wereld verspreid. Er zijn tot op dit moment geen afdoende methoden beschikbaar om de ziekte te bestrijden of te behandelen. Kennis over de oorsprong van de diverse WSSV-isolaten en hun geografische verspreiding in ruimte en tijd zouden een grote stap voorwaarts zijn bij het ontwikkelen van methoden ter voorkoming van ziekt door WSSV in de garnalenteelt. In dit proefschrift worden genetische merkers beschreven voor de identificatie van en het maken van onderscheid tussen WSSV-isolaten Deze merkers moeten bruikbaar zijn om WSSV in geografische ruimte en in tijd te vervolgen. Met behulp van deze merkers kunnen verspreidingspatronen van WSSV en de evolutie van WSSV beter worden gereconstrueerd.

De variabele gebieden in het WSSV-genoom, gekenmerkt door deleties (ORF14/15 en ORF23/24) en een variërend aantal ‘repeats’ (sequentieherhalingen), werden in kaart gebracht door een vergelijking te maken van de genomen van een drietal WSSV-isolaten, waarvan de gehele basenvolgorde is bepaald, te weten isolaten uit Taiwan (WSSV-TW), China (WSSV-CN) en Thailand (WSSV-TH). Deze vergelijking maakte duidelijk dat deze drie WSSV-isolaten een gemeenschappelijke voorouder moet hebben gehad, die rond de Straat van Taiwan moet zijn ontstaan. Vermoedelijk is het virus vervolgens in Thailand beland, maar tussenvormen van WSSV zijn nog niet gevonden. Vietnam vormt de geografische verbinding tussen China en Thailand en daarom is dit land uitgekozen voor nader onderzoek naar het vóórkomen en de verspreiding van WSSV en om te bezien of de geïdentificeerde genetische merkers geschikt zijn om de diverse Vietnamese virusisolaten te onderscheiden. Polymorfismen in het virale DNA, via verschil in lengte van restrictie-enzymfragmenten (RFLP) en via polymerasekettingreactie (PCR) gevolgd door basenvolgordebepaling, werden gebruikt om de genetische variatie tussen de diverse WSSV-isolaten te bepalen. De in lengte toenemende deleties in de diverse variabele gebieden van het virusgenoom vormden een ondersteuning voor de hypothese dat WSSV via meerdere, onafhankelijke introducties in Vietnam is terechtgekomen. Dit is niet onwaarschijnlijk, gezien de geografische positie van Vietnam tussen enerzijds China en Taiwan en anderzijds Thailand. (Hoofdstuk 2).

De dekking met monsters en de aantallen uit Vietnam waren onvoldoende om te bezien in hoeverre de genetische merkers konden worden gebruikt op regionale schaal om statistisch betrouwbare uitspraken te kunnen doen. Daarom werden meer monsters verzameld uit vooral gebieden met garnalenproductie in Vietnam om een kwantitatief model te maken dat robuuster is. Deze grotere dataset laat een betere statistische analyse toe, die meer gedetailleerde uitspraken kan doen over de wijze van verspreiding van WSSV over Vietnam, in ruimte en tijd op regionale schaal. Isolaten met toenemende deleties werden gevonden in de variabele gebieden op het WSSV-genoom. Deze gedetailleerde genetische data en de geschiedenis van de garnalenkweek in Vietnam suggereren dat WSSV aan land is gekomen in Centraal Vietnam en vandaaruit naar het noorden en zuiden is verspreid. De gegevens gaven ook aan dat deze variabele gebieden bruikbare markers zijn om de verspreiding van WSSV in ruimte en tijd op regionale schaal te verklaren. Vooral het variabele gebied met de grootste deletie (ORF23/34) is de meest geschikte merker voor WSSV op regionale schaal. De ‘repeat’-gebieden zijn geschikter voor het karakteriseren van WSSV op kleine schaal, terwijl het variabele gebied met de kleinste deletie (ORF14/15) goed bruikbaar is op iets grotere subregionale schaal. (Hoofdstuk 3).

Om de genetische heterogeniteit van WSSV binnen een isolaat, afkomstig uit een enkele garnaal, te bestuderen werd bekeken in hoeverre de ‘repeat’-gebieden hiervoor konden worden gebruikt. In een controle-experiment werd gekeken naar de variatie in via PCR verkregen en gecloneerde ‘repeats’ met een ‘repeat’-kloon als uitgangsmateriaal. Daarbij bleek dat hier ook variatie optrad en dat deze procedure op zichzelf variatie oproept door fouten in het kopiëren van gekloneerd DNA tijdens de PCR. Echter, wanneer alleen werd gekeken naar het primaire PCR-product van een ‘repeat’-kloon, trad deze variatie niet op en daarom werd de ‘PCR’-methode gebruikt om verschillende WSSV-isolaten te genotyperen. De repeats bleken ook zeer stabiel te zijn. Na twee passages, met twintig herhalingen, van WSSV in garnaal (*Penaeus monodon*) trad geen variatie in het ‘repeat’-gebied op. Dit betekent dat deze ‘repeat’-gebieden bij beperkte passages genetisch stabiel zijn en geschikt om WSSV te karakteriseren op kleine schaal in ruimte en tijd. Voorgesteld wordt om het polymorfisme binnen een WSSV-isolaat verder te bestuderen door het virus in vivo te kloneren op basis van gegevens over dosis-afhankelijke mortaliteit, bij een 5% overleving. (Hoofdstuk 4).

Het effect van extensieve en intensieve garnalenteelt op de samenstelling van genotypes binnen WSSV populaties werd onderzocht door de variabele gebieden (ORF14/15 en ORF24/25) te genotyperen via monsters die op dezelfde plaats in een reeks van jaren werden genomen. Alleen het variabele gebied ORF23/ORF24 was stabiel in externe garnalenteelt, terwijl geen enkel genotype stabiel was in intensieve systemen. Deze waarneming geeft aan dat extensieve garnalenteeltsystemen een goede bron zijn voor bemonstering, in het geval men geïnteresseerd is in 'historie' van de ziekteontwikkeling. (Hoofdstuk 5)

De omvang van de deleties in de variabele gebieden, die in monsters uit diverse landen zoals Japan, Iran, de Filippijnen, Indonesië en Cambodia werden aangetroffen en werden geanalyseerd via genotypering, zegt iets over het moment van de eerste WSSV-uitbraak in een bepaald land. Deze relatie bevestigde ook dat een van de variabele gebieden, ORF23/24, een heel bruikbare merker is voor oorspronkelijke uitbraken in ruimte en tijd. Er werd een eenvoudig model geformuleerd voor de ontwikkeling van genoomgrootte op basis van een geometrisch model van adaptatie. Invoer van de experimenteel verkregen data in het model leidde tot bevestiging van het model en dit resultaat bevestigt de hypothese dat WSSV zich in de tijd via snelle evolutie aangepast heeft aan de garnalenteelt door vervanging kleinere deleties door grotere. Het model bevestigde ook dat er een maximale deletie is, die kan worden bereikt voor beide variabele loci (ORF14/15 en ORF24/25). Bovendien ondersteunt de overeenkomst tussen de verspreiding van het virus in de tijd en de moleculair-epidemiologische gegevens ook de zienswijze dat WSSV zich over grote gebieden kon verspreiden dank zij lange afstandstransport van uitgangsmateriaal en postlarvae, en niet dankzij natuurlijke verspreiding in ruimte en tijd. (Hoofdstuk 6).

Samenvattend kan worden gesteld dat de diverse variabele gebieden in het WSSV-genoom werden geëvalueerd voor hun bruikbaarheid als moleculaire merker om de epidemiologie van dit virus in ruimte en tijd te bestuderen. Een combinatie van moleculaire gegevens en eenvoudige kwantitatieve analyses leidde tot een gedetailleerd verspreidingsmodel van het virus voor Vietnam en gaf nieuwe ideeën over het ontstaan en de evolutiegang via moleculaire veranderingen in het virale genoom. De resultaten van dit onderzoek zijn bruikbaar bij het ontwerpen van (moleculair) epidemiologische studies en interventie- / mitigatiestrategieën voor WSSV, en wellicht ook van andere pathogenen in de aquacultuur. (Hoofdstuk 7).

Tóm lược

Virut gây bệnh đốm trắng trên tôm (White spot syndrome virus - WSSV) là virut có bộ gen lớn với ADN sợi đôi và là thành viên duy nhất của họ virut Nimaviridae. Virut nầy là yếu tố hủy diệt đối với nghề nuôi tôm do việc gây nên tỉ lệ chết rất lớn và nhanh. WSSV lan truyền nhanh chóng khắp thế giới sau khi được mô tả lần đầu tiên ở Đài Loan và Trung Quốc những năm đầu thập niên 90. Cho đến nay vẫn chưa tìm ra phương pháp phù hợp nào có thể kiểm soát hoàn toàn bệnh nầy. Vì vậy, hiểu được nguồn gốc của các chủng WSSV khác nhau và suy luận ra mô hình lan truyền địa lý và sự tiến hóa của virut qua không gian và thời gian sẽ là một bước quan trọng để phát triển các phương pháp làm giảm nhẹ ảnh hưởng của virut đối với nghề nuôi tôm. Luận án nầy phát triển các yếu tố đánh dấu di truyền để xác định và phân biệt các chủng WSSV ở các qui mô không gian và thời gian khác nhau. Các yếu tố đánh dấu nầy có ứng dụng quan trọng trong việc xác định các mô hình lan truyền và hiểu được sự tiến hóa của WSSV.

So sánh trình tự của ba chủng virut có nguồn gốc từ Đài Loan (WSSV-TW), Trung Quốc (WSSV-CN) và Thái Lan (WSSV-TH) đã xác định các vùng gen biến đổi có tiềm năng bị mất đoạn hoặc chứa các số lượng biến đổi của các đơn vị lập lại liên kề (VNTR) là các vùng biến đổi chính trong bộ gen WSSV. Kiểu biến đổi gen giữa ba chủng nầy đề nghị chúng có tổ tiên chung có lẽ ở quanh eo biển Đài Loan. Từ đó virut lan truyền đến Thái lan, tuy nhiên dữ liệu về các chủng trung gian – cả về vị trí địa lý và kiểu gen – đều thiếu. Các chủng WSSV Việt Nam đáp ứng với yêu cầu nầy và được sử dụng để đánh giá vai trò yếu tố đánh dấu di truyền của các vùng gen biến đổi trong việc xác định và phân biệt các chủng WSSV. Biến đổi di truyền giữa các chủng nầy được xác định thông qua phân tích đa hình về chiều dài của các đoạn cắt giới hạn (RFLP) và xác định kiểu biến đổi gen thông qua giải trình tự các đoạn ADN thu được bằng phản ứng PCR. Chiều dài tăng dần của các đoạn gen bị mất ở cả hai vùng biến đổi mang đặc tính mất đoạn đã hỗ trợ cho giả thiết là nhiều nguồn WSSV xâm nhập trước tiên vào miền trung Việt Nam, một vùng địa lý mà có lẽ là trung gian của sự lan truyền WSSV từ eo biển Đài Loan đến Thái Lan (Chương 2).

Tuy nhiên, các mẫu đại diện cho Việt Nam được cung cấp bởi số lượng mẫu nhỏ không đủ cho kiểm định yếu tố đánh dấu di truyền ở qui mô địa lý trung bình và phân tích thống kê của số liệu. Vì vậy, nhiều mẫu từ các vùng sản xuất tôm chủ yếu của Việt Nam được phân tích để đạt yêu cầu khung định lượng cho một lý giải kết quả chính xác hơn. Bộ số liệu lớn hơn nầy cho phép phân tích thống kê và suy luận chi tiết hơn về sự lan truyền của WSSV ở Việt Nam – một phạm vi không gian và thời gian

trung bình. Trong vùng biến đổi có khuynh hướng mất những đoạn gen lớn cho thấy đoạn gen bị xóa bỏ có kích thước tăng dần. Sự kết hợp giữa số liệu phân tử chi tiết và lịch sử phát triển của ngành nuôi tôm ở Việt Nam đưa ra giả thiết là WSSV đã xâm nhập trước tiên vào miền trung Việt Nam và sau đó lan truyền vào miền nam và ra miền bắc. Số liệu cũng cũng chỉ thị rằng các vùng gen biến đổi nầy là các yếu tố đánh dấu di tuyền thích hợp cho việc xác định sự lan truyền của WSSV ở những qui mô không gian và thời gian khác nhau. Đặc biệt là vùng biến đổi thường mang những đoạn gen bị xóa bỏ lớn là yếu tố đánh dấu di truyền thích hợp nhất ở qui mô trung bình. Vùng gen mang các đoạn lập lại liên kề được xác định là những yếu tố đánh dấu di truyền ở các qui mô nhỏ hơn, trong khi vùng gen biến đổi thường bị mất các đoạn gen ngắn thì ổn định hơn và có lẽ thích hợp cho qui mô không gian và thời gian lớn hơn (Chương 3).

Thí nghiệm để đánh giá xem VNTR có thể được sử dụng để nghiên cứu sự đa dạng di truyền trong mỗi chủng WSSV từ một cá thể tôm cũng được thành lập. Trong thí nghiệm đối chứng, số lượng các đơn vị lập lại trong VNTR thể hiện sự biến đổi qua quá trình phân tử khi sử dụng một dòng đơn làm nguồn vật liệu tạo dòng. Điều nầy cho thấy rằng qui trình tạo dòng phân tử đã tạo nên sự biến đổi giả khiến cho qui trình nghiên cứu về các đoạn gen mang các đơn vị lập lại liên kề với kỹ thuật có yêu cầu công đoạn tạo dòng phân tử gặp vấn đề. Tuy nhiên, không quan sát thấy có sự biến đổi gen qua kết quả nhiều phản ứng lập lại của PCR, cho thấy rằng kỹ thuật xác định kiểu di truyền của VNTR dựa trên PCR là hữu dụng. Hơn nữa, không phát hiện sự biến đổi di truyền nào trên hai mươi mẫu nhiễm WSSV qua hai thế hệ trên tôm chân trắng (*P. Vannamei*) cho thấy rằng các vùng biến đổi chứa các đơn vị lập lại liên kề của WSSV đủ ổn định để được sử dụng như yếu tố đánh dấu phân tử trên qui mô thời gian ngắn. Tạo dòng trên cơ thể sống được đề nghị để nghiên cứu sự đa hình trong quần thể WSSV khi mối liên hệ đáp ứng với nồng độ WSSV phù hợp với dự đoán lý thuyết (Chương 4).

Ảnh hưởng của mô hình nuôi tôm quảng canh và thâm canh lên thành phần di truyền của quần thể WSSV được đánh giá bằng việc xác định kiểu di truyền các vùng biến đổi mang các đoạn gen bị xóa của các mẫu WSSV thu được từ các ao nuôi tôm qua nhiều năm. Chỉ có vùng biến đổi ORF23/24 thể hiện sự ổn định qua thời gian ở hệ thống nuôi quảng canh, trong khi không có vùng biến đổi nào ổn định trong hệ thống nuôi thâm canh. Quan sát nầy cho thấy rằng các ao với mô hình nuôi quảng canh là nguồn mẫu thích hợp để nghiên cứu sự lan truyền của WSSV dựa trên các mẫu virut thu thập theo kiểu truy ngược lại quá khứ (Chương 5).

Kích thước các đoạn gen bị mất trong vùng gen biến đổi của các chủng WSSV từ Nhật, Iran, Philippines, Indonesia và Cambodia có mối liên hệ dáng kể đến "thời gian xảy ra dịch đầu tiên trên lãnh thổ" khi được phân tích kết hợp với dữ liệu di truyền của những nghiên cứu khác. Mối liên hệ nầy cũng xác định rằng vùng gen biến đổi chứa các đoạn có khả năng bị mất – cụ thể là ORF23/24 – là các yếu tố đánh dấu di truyền hữu dụng cho qui mô không gian và thời gian lớn. Một mô hình đơn giản về sự tiến hóa của kích thước bộ gen được trình bày dựa trên mô hình hình học của sự thích nghi. Sự tương thích giữa mô hình nầy và dữ liệu di truyền đã hổ trợ cho giả thiết rằng bộ gen WSSV đã thích nghi với nghề nuôi tôm bằng sự thay thế các đoạn gen bị mất với kích thước tăng dần theo thời gian. Số liệu nghiên cứu cũng đề nghị rằng có một mức cực đại của kích thước đoạn gen bị xóa cho cả hai vùng biến đổi thể hiện qua sự ổn định của kích thước mất đoạn qua thời gian. Hơn nữa, sự tương thích giữa các kiểu lan truyền của virut qua thời gian và số liệu về dịch tễ học phân tử hổ trợ cho ý kiến cho rằng sự lưu thông tôm bố mẹ và tôm giống là nguyên nhân chủ yếu dẫn đến sự lan truyền của WSSV trên phạm vi không gian lớn (Chương 6).

Tóm lại, các vùng biến đổi gen khác nhau trong bộ gen WSSV được đánh giá là các yếu tố đánh dấu di truyền thích hợp cho nghiên cứu dịch tễ học phân tử ở các qui mô không gian và thời gian khác nhau. Sự kết hợp giữa số liệu phân tử và phân tích định lượng đơn giản đã đưa ra một mô hình lan truyền chi tiết của WSSV ở Việt Nam và các ý tưởng về lộ trình tiến hóa của sự tiến hóa kích thước bộ gen virut. Các kết quả thể hiện ý tưởng cho thiết kế nghiên cứu dịch tễ học phân tử cũng như các chiến lược ngăn ngừa WSSV và các yếu tố gây bệnh khác trên động vật thủy sản.

Acknowledgments

Professor Just Vlak, thanks for giving me the opportunity to work under your expert supervision. Although you are very busy with many students, you were always on time whenever I needed you. I can recognize the special way you treat us Asian students based on your deep understanding of our culture. You make us feel more comfortable and with less stress to work in a foreign place. It was a difficult challenge to work independently at the beginning, but you did not only help me to be more self-confident but you also learned me the way to supervise students more efficiently. I do not have enough words to say thanks for all your help and for what I have learned from you.

Dr. Mark Zwart, I am very lucky to have had you as my supervisor at the end of my PhD studies. With your help my molecular data became much more interesting and important when combined with mathematical and computational modeling. Thanks for your nice and stimulating words whenever I got stressed during writing the thesis. Thanks again for all your kind support.

I very much appreciated Prof. Rob Goldbach who gave me the opportunity to work at the Virology chair group. He supported our first paper and it is sad that he could not see the thesis as the end product because of his untimely death. He was very kind, helpful and friendly, and I was lucky to work in his laboratory.

Great thanks to professor Duong, former Head of BIRDI, who encouraged me to work on WSSV and who established links with Wageningen. Your wisdom and mentorship was very important for me. Dr. Rommert van den Bos who, through the MHO-7 project, gave me the opportunity to start the virus research with Wageningen University and get into this project. Thank you very much for your support. I also would like to thank the leadership of BIRDI to allow me to embark on this PhD project and for their continuous support during my PhD by accepting my frequent absence for the cause. I am very indebted to the shrimp group in BIRDI for helping in the sample collection.

The whispovirus group throughout three years, with Angela, Hendrik, Jeroen, and all the students. Thanks you all for kindly help during my stay in our group. It was an unforgettable time we worked together. Special words of thanks go to Hendrik for your help to get the first paper done, which led me to the PhD project and for your further support, even though you have left Virology for Nijmegen.

My friends and colleagues at Virology, Monique, Jan, Janneke, Thea, Els, Dick Lohuis, Hanke, Dick Peters and Wout, thank you all for hospitality. With your friendly help I felt less homesick and lonely in this foreign country, as I am very sensitive to a positive personal atmosphere. A special thanks to Thea and Janneke, with whom I could share all the feelings and personal problems and who gave me kindly help. I am very lucky to work with all of you in the same laboratory.

Christina, Esther, Afshin, Agah, Agata, Jeomdog, Liliana, Fang and Ke, I very much appreciated your friendly and nicely support. Thanks for your contribution to the the friendly atmosphere in the lab. It makes me stronger to overcome homesickness when working so far from home from time to time.

Jannie and Bert Wennekes, thank you very much for your friendship and hospitality. You both were so important for me during my stay in Wageningen. Your family helped me when feeling lonely in a nice but foreign country. You are my second family in Wageningen as you are always with me whenever I got a problem or needed joined memories. I enjoyed every time with both of you and these moments will stay in my memory forever.

RESCOPAR students, Gigi, Desrina, Bambang, Thu Ha, Nghia, Tuyen, Hoa, Phung Ha, thanks for the nice time in the group.

Mr. and Mrs. Minh, Mr. Thanh and Mrs. Thuy, Mr. Ke family, Huong family, thank you very much for your friendly support during my time in Wageningen. It was nice to meet and to become friends with all of you.

I would like to say thanks to the Vietnamese student community in Wageningen. Mr. Phong, Tin, Nhan, Hieu Trung, Lan Huong, Mai Huong, Cuc, Ai, Anh, Dung, Han, Thuy, Tram, Loan, I enjoyed the sweet time with you all in our activities to bring all Vietnamese students together. Every weekend in the open market and barbecue together next to the Bornsesteeg. These moments will be unforgettable. Special words of thanks go to Phung Ha and My Duyen, who have taken care of me, when I got sick in Wageningen, in the absence of my family.

Now that the thesis project is coming to an end, I would like to thank my dear family for their continuous support and their care for Trang when I was abroad. I am deeply grateful for their patience and confidence, for allowing my dream to come through. Without you I would not have made it so far. Special words of thanks go to my

youngest uncle, who always supported me during my study abroad. Finally, dear Trang, I have deprived you from a lot of quality time together in the past years. Thank you for your understanding and I promise you to make up for this in the many years to come. I am so glad that I don't have to miss you any longer for such long periods.

Bui Thi Minh Dieu
Wageningen, 1 June 2010

Curriculum vitae

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- MSc. Molecular Biology, Free University of Brussels (VUB), Belgium (2000-2002), Cum Laude
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- PhD Wageningen University (2004 – present), under supervision of Prof.dr. J.M. Vlak and co-supervision of Dr. M.P. Zwart, on a project entitled “Epidemiology and evolution of white spot syndrome virus”, initially sponsored by Wageningen University (PhD sandwich grant) and later by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

Work experience

- Research scientist at the Biotechnological Research and Development Institute (BIRDI), Can Tho University (January 1985 - present)

- Technical manager in the ‘Animal supplied mineral and vitamin premix factory’, Biotechnological Research and Development Institute, Can Tho University (1988-1996)
- Lecturer and Teacher practical courses in General Microbiology at Can Tho university rom 1988-1996 and 2003
- Lecturer “General Virology” in Vietnamese and English to Biotechnology undergraduate and graduate students in Can Tho University (2008-2010)
- Thesis supervision of undergraduate students (2007-2010)
- Manager of the BIRDI Molecular Biology laboratory (2008-present)
- Current position: Research Scientist at BIRDI responsible for molecular technology in food sciences and aquaculture

Publication Account

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PE&RC PhD Education Certificate

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

Review of Literature (5.6 ECTS)

- Molecular markers in epidemiology and evolution of white spot syndrome virus (WSSV) in shrimp (2005)

Laboratory Training and Working Visits (2.8 ECTS)

- Molecular methods to map the epidemiology of the white spot syndrome virus in Vietnamese shrimp (+ oral presentation); Michigan State University (2006)

Post-Graduate Courses (5.7 ECTS)

- Fish Immunology / Vaccination; WIAS/ Dr. ir. G.F. Wiegertjes (2005)
- Bioinformatics (BITS); Institute of technical Biochemistry University of Stuttgart, Germany (2006)
- Confocal/Electron Microscopy; WUR/Virology (2005)

Deficiency, Refresh, Brush-up Courses (1.4 ECTS)

- Bioinformatics; Institute of technical Biochemistry University of Stuttgart, Germany (2006)

Competence Strengthening / Skills Courses (3.6 ECTS)

- Project and time management; PE&RC (2007)
- Techniques for writing and presenting a scientific paper; PE&RC (2006)
- Media training for PhD students; PE&RC (2005)

Discussion Groups / Local Seminars and Other Scientific Meetings (9.6 ECTS)

- Genetic resource and diversity in production ecology (2006)
- Rescopar project meeting in sustainability of shrimp production (2007)
- Regular scientific meetings on diseases in aquaculture in Vietnam (2006, 2007, 2008, 2009)
- Scientific meetings in Vietnam with other PhD students of Rescopar (2007-2008)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.5 ECTS)

- PE&RC Weekend (2005)
- PE&RC Day meeting (2006)
- PE&RC Day symposium (2007)

International Symposia, Workshops and Conferences (7.6 ECTS)

- DAAVI Conference: oral presentation: “Molecular epidemiology and heterogeneity of White spot syndrome virus in shrimp in Vietnam” (2005)
- Rescopar conference: oral presentation: “Molecular epidemiology and heterogeneity of White spot syndrome virus in shrimp in Vietnam” (2006)
- DAAVII Conference: oral presentation: “Determining Genetic Factors Influencing WSSV Virulence” (2008)

The research described in this thesis was financially supported by NUFFIC (internship), Wageningen University (partial sandwich PhD fellowship) and by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO) (fellowhsip grant WB 83-207).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Printed by Wöhrmann Print Service, Zutphen, the Netherlands.