RNA INTERFERENCE MEDIATED INHIBITION OF TAURA SYNDROME VIRUS REPLICATION

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ABSTRACT

Pacific white shrimp (*Penaeus vannamei*) is an economically important farmed penaeid shrimp species in many countries, including Thailand. One of the most important diseases of the white shrimp is caused by Taura syndrome virus (TSV), which has recently affected shrimp cultivation throughout the world. At present, there is no known treatment or vaccine available for this viral disease.

TSV were isolated from three different cultivation areas of Thailand. Analysis of nucleotide and deduced amino acid sequences in open reading frame 1 (ORF1) of the TSV revealed that this region of the viral genome is highly conserved with overall identity. Subregions of the ORF1 (helicase, protease and polymerase) were chosen as targets for RNAi mediated viral suppression study. The feasibility of RNAi-based technique to prevent TSV infection was assessed in the pacific white shrimp. Shrimps were injected with TSV specific dsRNAs, followed by virus infection. Shrimp treated with specific dsRNA targeting the nonstructural gene of TSV (ORF1) showed a reduction in the viral level as compared with untreated shrimp. The dsRNA targeting helicase domain exhibited the strongest inhibitory effect while the domain targeting polymerase exhibited the least effect. Robalino *et al*. found that either virus sequence-specific or nonspecific dsRNA could afford antiviral immunity in shrimp. In agreement, these results suggested that virus specific dsRNAs could suppress TSV replication in white shrimp.

KEY WORDS: *Penaeus vannamei* / TAURA SYNDROME VIRUS (TSV) / RNA INTERFERENCE (RNAi) / DOUBLE-STRANDED RNA (dsRNA) / RT-PCR

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การยับยั้งการเพิ่มจำนวนของเชื้อไวรัสทอร่าด้วยกระบวนการยับยั้งการแสดงออกของยีน
(RNA INTERFERENCE MEDIATED INHIBITION OF TAURA SYNDROME VIRUS REPLICATION)

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บทคัดย่อ

กุ้งขาวแปซิฟิก (*Penaeus vannamei*) เป็นสายพันธุ์กุ้งทะเลที่มีความสำคัญทางเศรษฐกิจในหลายประเทศทั่วโลกรวมทั้งประเทศไทย โรคติดเชื้อที่สำคัญของกุ้งขาวแปซิฟิกคือโรคจากไวรัสทอร่า ซึ่งที่ผ่านมาได้ทำความเสียหายอย่างมากต่ออุตสาหกรรมการเลี้ยงกุ้งทั่วโลก ในปัจจุบันนี้ยังไม่พบวิธีการรักษาหรือวัคซีนสำหรับโรคติดเชื้อไวรัสทอร่าในกุ้งทะเล

ในการศึกษาครั้งนี้ได้แยกเชื้อไวรัสทอร่าจากตัวอย่างกุ้งจากแหล่งเพาะเลี้ยงกุ้งขาว 3 แห่งในประเทศไทย การวิเคราะห์ลำดับเบสและกรดอะมิโนในบริเวณที่มีการสร้างโปรตีน (open reading frame) ของยีนที่ 1 (ORF1) ของไวรัสทอร่าแสดงให้เห็นว่าในบริเวณนี้ของจีโนมไวรัสมีการอนุรักษ์สูง (conserved region) มาก ลำดับเบสใน ORF1 บางส่วนถูกเลือกเป็นบริเวณเป้าหมายสำหรับศึกษาการยับยั้งการเพิ่มจำนวนของเชื้อไวรัสโดยเทคนิคกระบวนการยับยั้งการแสดงออกของยีนที่เรียกว่า RNAi เพื่อศึกษาถึงความเป็นไปได้ในการประยุกต์ใช้กลไก RNAi นี้ป้องกันโรคติดเชื้อจากไวรัสทอร่าในกุ้งขาวแปซิฟิก การทดลองในกุ้งขาวแปซิฟิกโดยการฉีดอาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีนของไวรัสทอร่า (TSV-specific dsRNA) เข้าสู่ตัวกุ้งจากนั้นติดตามว่าสามารถยับยั้งการเพิ่มจำนวนของเชื้อไวรัสได้หรือไม่ ซึ่งพบว่ากุ้งที่ได้รับอาร์เอ็นเอสายคู่ที่มีลำดับจำเพาะกับยีนที่ไม่ใช่ยีนโปรตีนโครงสร้าง (nonstructural gene) ของไวรัสทอร่า คือยีน helicase protease และ polymerase ก่อนได้รับเชื้อไวรัส พบว่าระดับของเชื้อไวรัสทอร่าในกุ้งกลุ่มนี้ลดลงเมื่อเทียบกับกุ้งที่ไม่ได้รับอาร์เอ็นเอสายคู่ โดยอาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีน helicase ของไวรัสพบมีสามารถในการยับยั้งการเพิ่มจำนวนของเชื้อไวรัสมากที่สุด ขณะที่อาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีน polymerase นั้นแสดงความสามารถในการยับยั้งการเพิ่มจำนวนของเชื้อไวรัสได้น้อยที่สุด จากผลการทดลองแสดงให้เห็นว่า อาร์เอ็นเอสายคู่ที่มีลำดับเบสจำเพาะกับยีนของไวรัสทอร่าสามารถยับยั้งการเพิ่มจำนวนของเชื้อไวรัสได้ในกุ้งที่ติดเชื้อ ซึ่งสอดคล้องกับผลการทดลองของ Robalinoและคณะ ที่พบว่าอาร์เอ็นเอสายคู่ทั้งที่มีลำดับเบสจำเพาะและไม่จำเพาะนั้นแสดงความสามารถในการต้านทานต่อไวรัสในกุ้งขาวได้

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LIST OF ABBREVIATIONS

ATP = adenosine triphosphate
BIR = buculovirus inhibitor of apoptosis repeat
bp = base pair (s)
°C = degree Celsius
cDNA = complementary DNA
DEPC = Diethyl pyrocarbonate
DNA = deoxyribonucleic acid
dNTPs = dATP, dCTP, dGTP and dTTP
dsRNA = double-stranded ribonucleic acid
E. coli = *Escherichia coli*
EDTA = ethylenediamine tetreacetic acid
EtBr = ethidium bromide
et al. = and others
g = gravity or centrifugal force
GFP = green fluorescent protein
hr = hour (s)
IPTG = isopropyl-thio-β-D-galactopyranoside
kb = kilobase pair (s)
kDa = kilodalton (s)
lacZ = β-galactosidase gene
LB = Luria-Bertani medium
M = molar
mg = milligram
min = minute (s)
ml = milliliter
mM = milimolar
µg = microgram

LIST OF ABBREVIATIONS (Continued)

µl = microliter
µM = micromolar
ng = nanogram
nm = nanometer
nt = nucleotide (s)
OD = optical density
ORF = open reading frame
PBS = phosphate buffer saline
PCR = polymerase chain reaction
P. monodon = *Penaeus monodon*
ppt = parts per thousand
P. vannamei = *Penaeus vannamei*
RdRp = RNA dependent RNA polymerase
RNA = ribonucleic acid
RNase = ribonuclease
RT-PCR = reverse transcription- polymerase chain reaction
s = second (s)
SME = shrimp meat extract
T_m = melting temperature
TAE = Tris-acetate-EDTA
Tris-HCl = Tris-(hydroxymethyl)-aminoethane hydrochloric acid
TS = Taura syndrome
TSV = Taura syndrome virus
U = unit (s)
V = volt
v/v = volume / volume
WSSV = white spot syndrome virus
w/v = weight / volume
X-gal = 5-bromo-4-chloro-3-inolyl-β-D-galactopyranoside
YHV = yellow head virus

CHAPTER I

INTRODUCTION

Shrimp is an aquatic animal with high economic value of world aquaculture production. For many countries, shrimp cultivation has been seen as a cost-effective activity in the coastal area offering jobs and incomes for its populations. Shrimp cultivation has provided many countries with substantial sum of foreign currency earnings. In Thailand, a study of 1994 reported that farmer could increase his income by a factor of ten by switching from rice grower to shrimp farmer (1). Shrimp farming in Thailand has begun in the late 1970s, and its production increased sharply, with the growing demand of USA, Europe and Japan. Shrimp imports into Europe continue to grow and Spain is the main market, followed by France and UK. (2).

Although there are hundreds of marine shrimp species of the world, most are not suitable for human consumption. Shrimps caught by trawlers and farms belong to the Penaeidae family of decapod crustaceans and are referred to as penaeids. The family of penaeid shrimps consists of approximately 110 species, of which about dozen species are important for commercial culture. Of all these species, *Penaeus monodon*, *Penaeus vannamei* and *Penaeus chinensis* are the most widely cultured species (3). *P.vannamei* culture is discussed as the following.

1.1 Cultivation of *Penaeus vannamei*

P. vannamei (recently renamed *Litopenaeus vannamei*) is a scientific name of pacific white shrimp. It is one of the main commercial farmed marine shrimp species worldwide, especially in the America continent. Particularly, following the incidence of sustained outbreak of viral disease in black tiger shrimp, many countries in which shrimp farming in East and Southeast Asia shifted towards *P. vannamei*.

Early efforts on penaeid culture in the Americas concentrated on indigenous species such as *P. setiferus* and *P. stylirostris* in Panama; *P. aztecus* and *P.*

occidentalis in Honduras; *P. aztecus* and *P. duorarum* in southern USA and *P. schmitti* and *P. brasiliensis* in Brazil, whilst *P. vannamei* is native to the western Pacific coast of Latin America from Peru to Mexico. In early 1970s, *P. vannamei* was first introduced to the Pacific Islands, where French researchers developed techniques for intensive breeding and rearing of various penaeid shrimp species including *P. vannamei*. During the late 1970s and early 1980s, *P. vannamei* was introduced to the Hawaii and Eastern Atlantic coast of the Americas from South Carolina and Texas in the North to Central America and as far as Brazil in the South America (3). To date, *P. vannamei* has become the commercially important species of shrimp farmed in these countries over the past 20-25 years because the culture of *P. vannamei* gave a better production than other species.

P. vannamei has been introduced into Asian countries in the late 1970s starting in Philippines then China and Taiwan (3). In 1996, Taiwan began to import SPF broodstock of *P. vannamei* from Hawaii (4). The initial success in the culture of this non-native species had led to a huge demand for broodstock and to the introduction of wild broodstock of *P. vannamei* from many sources in Latin America later. Unfortunately, TSV, imported with wild broodstock from Latin America, caused dramatical mortality of juvenile shrimp in ponds in Taiwan in 1999 (5-6). Moreover, WSSV was also caused mortalities, and runt deformity syndrome (RDS) and slow growth due to IHHNV was common. The similar scenarios have occurred in China as in Taiwan. Despite disease problems, *P. vannamei* is the dominant cultured species in China, constituting approximately 70 percent of the country's total shrimp production. In addition, China now leads the world's production of farmed shrimp, surpassing Thailand, which held the top for over a decade. Subsequently, the introductions of *P. vannamei* have spread to the Philippines, Indonesia, Vietnam, Thailand, Malaysia and India.

1.1.1 Taxonomy and general biology

P. vannamei is a member of the kingdom *Animalia*, phylum *Arthropoda*, subphylum *Crustacea* class *Malacostraca*, subclass *Eumalacostraca* superorder *Eucarida*, order *Decapoda*, suborder *dendrobranchiata*, super family *Penaeiodae*, family *Penaeidae* and genus *Penaeus*. Place side by side, this shrimp appears grayishwhite in color. Its distribution ranges from the North of Peru to the North of Mexico. The species is now normally cultured around the world. *P. vannamei* is mostly found on mud bottoms, down to a depth of 75 meters. Females grow to a maximum weight of approximately 120 g whilst males are relatively small.

A main characteristic of the decapods is that they have an exoskeleton which is periodically shed (molting) to allow further growth. The shrimp body consists of the anterior head, middle thorax and posterior abdomen with six segments (Fig. 1). The head and entire thorax are covered dorsally and laterally by a fold of body wall called carapace. The head bears a pairs of eyestalk beside the base of the saw-like spine; the rostrum and several antennas. In all decapod crustaceans the first three anterior thoracic segments are fused with the head to form cephalothorax. The appendages of these segments are modified to function as mouthparts and are called maxillipeds. The remaining five segments of the thorax are not fused with the head. Their appendages are known by the general name of walking legs or pereopods. The abdomen has six segments. Its six segments are numbers from anterior to posterior. The last segment of the abdomen bears the uropods laterally and the unpaired median telson posteriorly, which allows the shrimp to swim/jump backwards. Under the abdomen, each segment except the telson, carries one pair of appendages; the pleopods or swimming legs. The different species can be recognized well by the number of teeth on the rostrum, by the presence and the shape of grooves on the thorax, or by the shape of the antenna. *P. vannamei* does not have grooves along the upper midline of the head and the upper midline of the lower region of the abdomen, but typically have longer antennae and a long rostrum. It has 8-9 teeth on the dorsal rostrum and 1-3 on the ventral side (8-9).

1.1.2 Life cycle of penaeid shrimp

Penaeid shrimp have much the same life cycle. Spawning usually occurs in the oceans from near the beaches to several miles offshore. Males and recently molted females express a courtship behavior by which they swim around and cling together, usually at night. During mating the male transfers the sperm package, called spermatophore, to the female. The spermatophore is glued to the underside the females. The female can carry the sperm with her until she spawns the eggs. After the

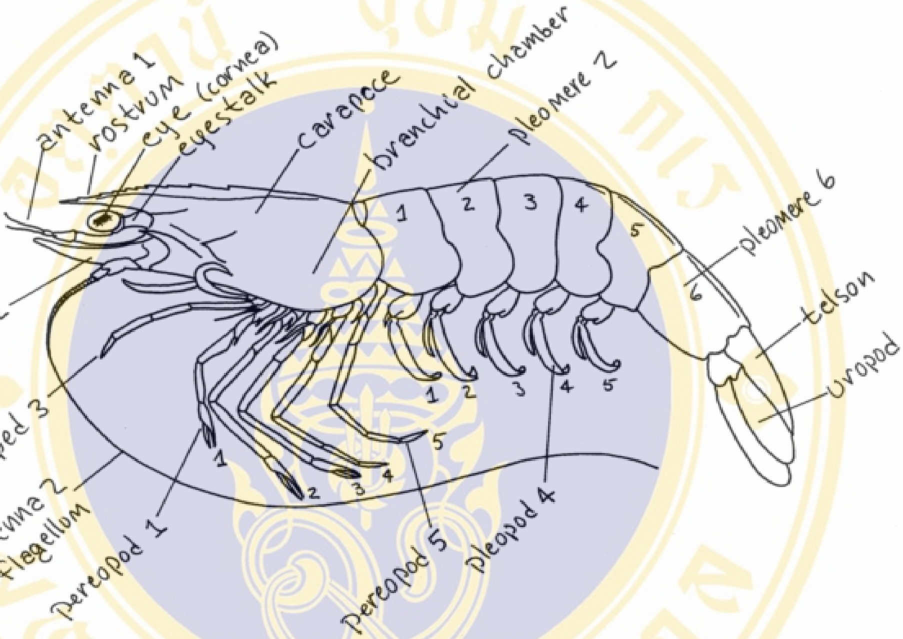


Figure 1: Schematic view of penaeid shrimp (8)

eggs are released from the body, they pass the sperms and are fertilized. Eggs, which are fertilized, are floated in the ocean currents and developed into nauplii. It does not look like the adult shrimps; they are almost round in shape with feather-like arms protruding in all directions. After the nauplii stages, there are protaozoea stages, mysis stages and then the postlarval stages (10). The postlarval stage looks like miniature versions of adult shrimp (Fig. 2). All those stages are planktonic and drift at sea, feeding on algae and small plankton. The postlarvae finally move into a coastal area. The juvenile grow up near the coast, in more inland estuaries. Here they are exposed to fluctuating salinities and temperatures. They feed on decaying plant material, plankton and small bottom organisms.

1.1.3 *Penaeus vannamei* farming in Thailand

Although black tiger shrimp or *P. monodon* is the dominant cultured species of shrimp in Thailand for decades, culturing of black tiger shrimp faces to many difficulties particularly the decline of its slow growth rate and survival primarily due to the stress of high IHHN viral loading in the broodstock passing to their offspring. *P. vannamei* was became the substituting species of shrimp farming in Thailand. First, it has been introduced in 1998 for experimental culture on a very small scale (3). Until 2001, Thailand began importing *P. vannamei* for commercial culture when shrimp farmers encountered slow-growth problems of *P. monodon*. Moreover, Thai government prohibits the cultivation of *P. monodon* in freshwater areas in 2000 (7). However, there are many reasons for the introduction of this shrimp species into areas where they are not originated. Reasons for the introduction of *P. vannamei* are that *P. vannamei* is easier to culture in a very high populated pond than *P. monodon* and they tolerate to a wide range of salinities or low temperature more than *P. monodon*. In addition, *P. vannamei* requires low protein feed than *P. monodon*, resulting in a reduction in production costs. For these reasons, many Thai farmers shifted to cultivation of *P. vannamei* for alternatives. As concerned about the possibility of the introduction of infected broodstock of *P. vannamei*, Thai government allowed official importations of certified SPF broodstock only to such qualified hatcheries with restriction.

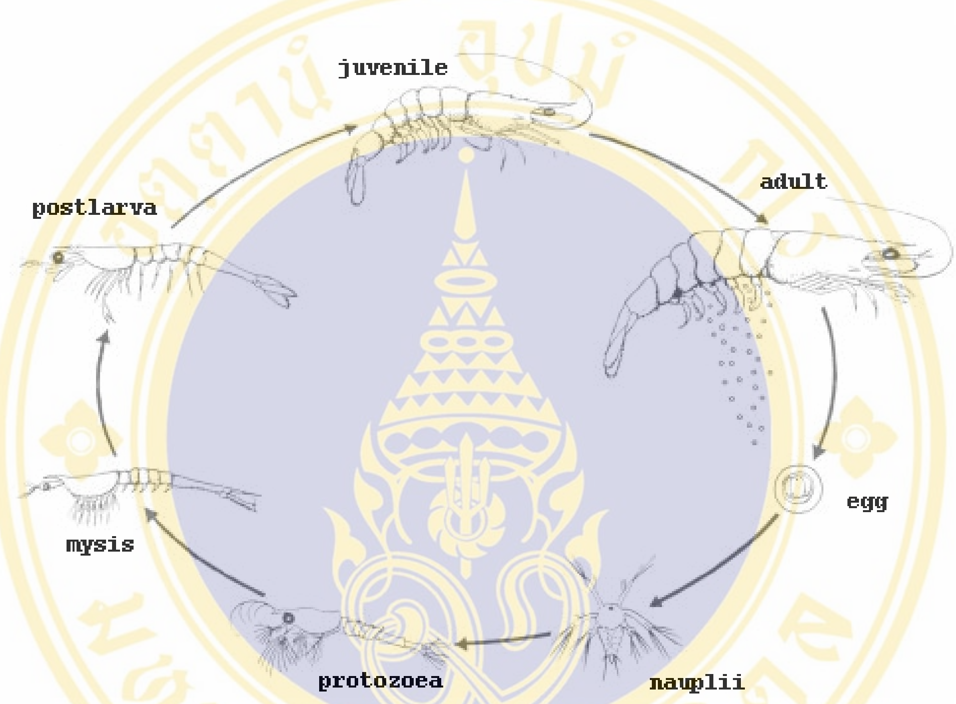


Figure 2: Life cycle of shrimp (11)

Despite the safeguards, the first sign of TSV occurred in the early 2003 in Thailand (7). This resulted in an official ban on the importation of *P. vannamei* and it remains to this date. But, due to the huge demand for postlarvae, large numbers of *P. vannamei* from Asian countries and from the Americas were illegally imported into Thailand. Although the official ban, Thailand was expected to increase amount of pacific white shrimp production (3).

1.2 Infectious diseases in *Penaeus vannamei*

Shrimp diseases have impacted shrimp farming industry negatively by affecting shrimp survival and growth, resulting in significant economic losses. These represent the major problem to the shrimp farming industry. Several disease outbreaks have caused problems in recent years. One of those was the marked decline in the shrimp production in many countries. There are a number of infectious agents that cause infections and mortalities to shrimps including, protozoa, fungi, bacteria and virus. Among a variety of infectious diseases that affect shrimps, diseases caused by viral infections have been suggested to play a very important influence on shrimp farming industry. More than 20 viruses were identified as having affected wild stocks and cultured shrimps, but a small number of viral diseases are considered to be transmissible and of socio-economic importance. These viral diseases are White spot syndrome, Infectious hypodermal and haematopoietic necrosis, Yellow head disease and Taura syndrome disease.

Likewise, different types of pathogens can cause infectious diseases of economic importance to cultured *P. vannamei*. This exotic shrimp species is known to be carriers of these following viral diseases:

White spot syndrome is a disease caused by white spot syndrome virus (WSSV) and is a highly infectious agent for *P. vannamei*. The virus was first reported in farmed *P. japonicus* from Japan (12). Then, it was discovered in cultured *P. monodon* in Taiwan (13). It spread throughout Asia and then to the Americas. It has a wide host range and is highly lethal, leading to mortality rates of 100% within a few days of infection. Symptoms include white spot on the carapace and reddish colouration of the hepatopancreas. Infected shrimp become lethargic before they die (13).

Infectious hypodermal and haematopoietic necrosis is a disease caused by infectious hypodermal and haematopoietic necrosis virus. IHHNV infection in *P. vannamei* can cause severe deformations called RDS, which typically results in cuticular deformities, slow growth and poor feed conversion (3). This small DNA virus has had significant negative impact for cultured farm of *P. vannamei* in the Americas. However, some strains of IHHNV have been found to be infectious for *P. vannamei* (14-15).

Besides virus, other pathogens are also important in *P. vannamei* farming industry, particularly various infectious bacteria. One of bacterial disease that affected *P. vannamei* is necrotising hepatopancreatitis (NHP). Gross signs detected include low feeding and growing, a soft shell and weak bodies (16).

Since Taura syndrome disease is known to be a highly virulent disease for *P. vannamei* causing mass mortality as much as 95%, it is described in detail.

1.2.1 Taura syndrome disease

Taura syndrome or TS was first recognized in penaied shrimp farms located near the mouth of the Taura River in the gulf of Guayaquil, Ecaudor in 1992. Initially, the cause of TS was uncertain. It thought to be involved in a fungicide used in banana farming, but TS appears to have a viral etiology due to a previously unknown virus now named Taura syndrome virus (TSV). Subsequent to the discovery of TSV, researchers at the University of Arizona have demonstrated TSV as the direct cause of TS (17). The disease is now well known for shrimp farmers throughout Ecuador as well as in The Americas and USA. TS is perhaps the biggest concern to Asian countries because the introduction of infected broodstock of *P. vannamei* resulted in mass mortality in cultured shrimps.

1.2.1.1 Classification and genome organization of TSV

Although TSV was tentatively classified in the family *Picornaviridae* based on its morphological characteristics, the study of the genome analysis by Mari J. *et al*. (2002) suggested that it should be considered as a member of the genus *Cricket paralysis-like viruses*. TSV is a non-enveloped icosahedral particle with diameter of 31-32 nm. Its buoyant density is 1.338 g/ml in CsCl. Its capsid comprised of three major (55, 40 and 24 kDa) and one minor (58 kDa) polypeptides.

The TSV genome consists of a linear, positive-sense single stranded RNA of approximately 10.2 kb in length (Fig. 3). It contains two large open reading frames (ORFs) that are separated by an intergenic region of 207 nucleotides. The ORFs are flanked by a 377 nucleotide 5’ untranslated region (UTR) and a 266 nucleotide 3’ UTR followed by a poly A tail (17-20). The predicted amino acid sequence of ORF1 revealed sequence motifs characteristic of a helicase, a protease and an RNA-dependent RNA polymerase. In addition, a short amino acid sequence located in the N-terminus of ORF1 presented a significant similarity with a baculovirus IAP repeat (BIR) domain. The anti-apoptotic activity of the IAP protein is found in animals and DNA virus. The function of this BIR-like sequence in a single stranded RNA virus is unknown. Amino acid sequences on the N-terminus of three TSV capsid proteins were mapped in the ORF2, which possesses a start codon upstream of the structural genes. The intergenic region shows nucleotide sequence similarity with those of the genus *CrPV-like viruses*, suggesting a mechanism of non-AUG-mediated translation initiation (20).

1.2.1.2 General pathology of Taura syndrome disease

TS is a virulent disease for *P. vannamei* with cumulative mortalities varies from 5 to more than 95%. The disease generally attacks juvenile *P. vannamei* (0.1-5.0 g) within two to four weeks after stocking in growout ponds. The causative virus (TSV) is principally targets the tissue just underneath the shell, the cuticle epidermis, in shrimps because it locates itself in the cytoplasm of infected epithelial cells of shrimp cuticle. TS could be divided into acute and chronic phases, which are easily distinguished. Shrimp afflicted with the acute phase of TS appear weak, have a soft shell, an empty digestive tract and may have diffuse expansion of the red chromatophores (pigment spot) in the appendages, especially in uropods, telson and pleopods. Shrimp with severe acute TS typically die during the molting process (21). Individuals that survive the acute phase of TS progress into the chronic phase of disease and show sign of recovery. Shrimp in the chronic phase of TS have scattered, pitted, melanized (black spot) lesions along their outer skin of shell (21). Once, the shell is shed during a subsequent molt cycle, gross lesion may be lacking, however, the shrimp are often asymptomatic carriers of TSV (22).

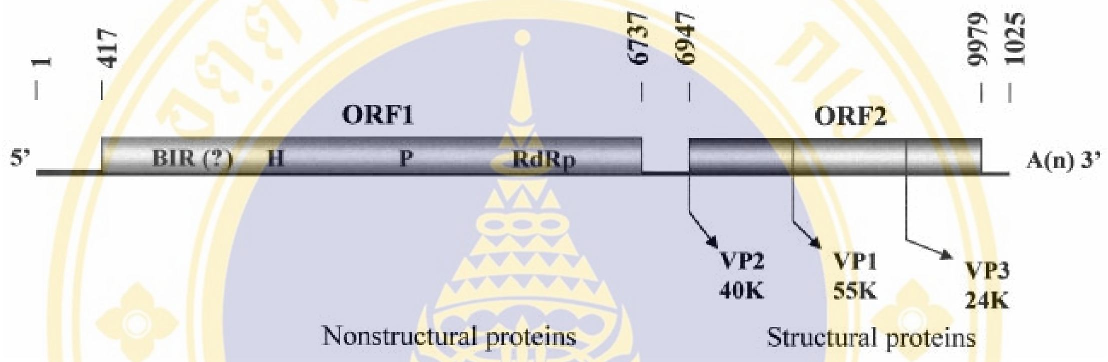


Figure 3: Schematic diagram of the genome of TSV (20)

The figure showed the gene organization of TSV RNA genome. Number indicates nucleotide positions. Open reading frames (ORFs) 1 and 2 are shown as open boxes and untranslated regions (UTRs) as a single line. The approximate positions of the BIR-like sequence (BIR), helicase (H), protease (P) and RNA-dependent RNA polymerase (RdRp) are indicated. Arrows represent the N-terminal of the capsid proteins.

1.2.1.3 Laboratory diagnosis of Taura syndrome disease

The current diagnostic and detection methods for TSV include histopathology, dot blot and in situ hybridization assay with TSV specific cDNA probes, RT-PCR, antibody-based methods and transmission electron microscopy (13, 23-26). Diagnostic histophatology for TS may be applied to diagnose the disease affected shrimps in the acute phase that display multifocal areas of the cuticular epithelium of the general body surface. The lesion is characterized by the presence of numerous, variably sized eosinophilic to basophilic cytoplasmic inclusion bodies (18, 21, 27). The electron microscopy method demonstrates the presence of spherical inclusions of aggregates of presumed TSV particles in the cytoplasm of affected cells.

1.2.2 Prevention and treatment

During evolution, there are two types of immune systems to protect multicellular organisms from foreign particles, namely innate immunity and adaptive immunity. Innate immune system is found in all multicellular organisms ranging from humans to plants. It is now suggested that invertebrates have relied on innate immune system to recognize and protect themselves from non-self particles, including invading pathogens (28-29). Some crustacean species contain soluble molecules that can bind to pathogen-associated molecules of bacterial/fungal cell walls leading to cell lysis. A few of these antibacterial molecules were active against some specific viruses (30). Furthermore, these antiviral molecules are non-specific viral inhibitors.

Traditional treatment of shrimp diseases bases on chemical and antibiotic products. Chemical treatment is toxic because of chemical waste is produced and allowed to collect in the environment for a long time. In addition, continuous use of antibiotics can induce resistance in virus and accumulation in shrimp or human consumers (31). Because of limitations, more researches must be conducted in order to develop new methods to control the disease, especially viral diseases since virus infections are common among invertebrates including shrimp. Currently, there are no known specific treatments or vaccines available for viral diseases, including TS. Many strategies have been used to try to control virus through preventive measures.

1.3 RNA interference

Posttranscriptional gene silencing (PTGS), virus induced gene silencing (VIGS), RNA interference (RNAi) and quelling are forms of RNA silencing that effect sequence-specific RNA degradation in the cytoplasm of eukaryotic cells. Pioneering observation on PTGS came as co-suppression of homologous transgenes in plants. Also, plants that carrying various sequences derived from RNA viruses could resist to both corresponding and homologous viruses: (VIGS). Related events were described as quelling in fungi and RNA interference in animals.

1.3.1 Mechanism of RNA interference (RNAi)

In recent years, a large number of research works have described the RNA silencing phenomenon in various organisms. Identifications of various components involved in silencing pathway by genetic mutant screens in plants and nematodes and uncovering the biochemistry of gene silencing led to a model to explain how RNAi works (32). As shown in figure 4, a simplified model for the RNAi pathway is based on two steps. The initiation step, the long dsRNAs enter a cellular where they are cleaved by the RNase III-like enzyme Dicer to generate discrete 21 to 25 nucleotide small RNA fragments containing 2 nucleotides 3' overhangs and 5' phosphate group. These small RNA fragments are called small interfering RNA (siRNAs). The cleavage is thought to be catalyzed by its tandem RNaseIII domain (33-37). Some Dicers contain an ATP-binding motif, suggesting that the production of siRNAs from dsRNA requires ATP (38-39). In the effector step, siRNAs are loaded into the multicomponent nuclease protein complex to form a RNA induced silencing complex (RISC) (37). The entire components of RISC have not yet been identified, but they might include an endonuclease, and exonuclease, a helicase and a homology searching activity. One of the protein components of this complex was identified as a member of the Argonaute gene family (40-41). Recently, two RNA binding proteins, the Vasa intronic gene and dFMR proteins, were identified as additional components of the RISC (42). During RISC assembly, this complex might undergo activation in the presence of ATP so that the antisense strand of the unwound siRNA becomes exposed. This antisense siRNAs in the activated RISC subsequently guide the RISC to complementary mRNA targets by Watson-Crick base pairing. This interaction induced an endonuclease specific

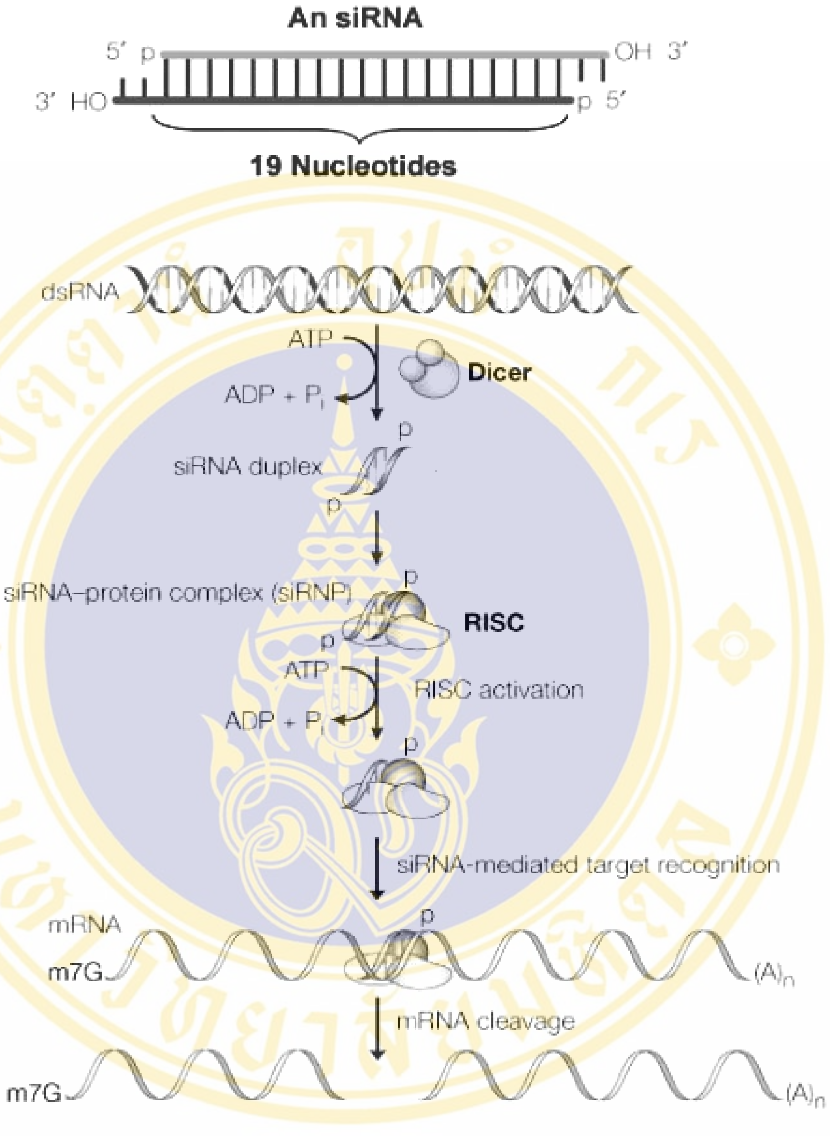


Figure 4: A model for RNAi mechanism (modified from 43)

First, the long dsRNAs get processed into 21-25 nucleotide (nt) small interfering RNA (siRNA) by RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into multicomponent nuclease protein complex known as RNA induced silencing complex (RISC), unwinding the siRNA duplex. The antisense siRNA subsequently guide the RISC to target complementary mRNA molecule, where RISC cleave and destroy the full-length cognate mRNA (effector step).

cleavage of the targeted mRNAs approximately in the middle of the duplex region (44). Degradation of the homologous mRNAs is achieved by the action of RNaseH activity in Argonaute (45). Finally, RISC is liberated from the cleaved mRNA and is recycled for the next rounds of catalysis. This property of RISC is responsible for the potent nature of the silencing effect. Furthermore in certain organisms those contain RNA-dependent RNA polymerase (RdRp), this enzyme plays an important role in amplification of siRNA molecule thus amplifying RNAi effect in the cells (46-47). In certain organisms such as mammals, the introduction of dsRNA of ≥ 30 nucleotides leads to the induction of the interferon response (48-49). This non-specific response to dsRNA results in global changes in cellular gene expression and apoptosis, masking the specific silencing effect of RNAi. However, the potent and specific gene silencing could be achieved by introduction of siRNAs, bypassing the step of generation siRNA (50). Since its discovery, RNAi phenomenon has been observed in various organisms including, protozoa, flies, nematodes, insects, parasites, mouse and humans suggesting that RNAi is evolutionarily conserved among eukaryotic organisms.

Presently, RNAi has been recognized as machinery involved in the control of developmental stages by posttranscriptional and transcriptional gene silencing mechanisms (51-53). RNAi also limits transposon mobilization, presumably by degrading transposon encoded mRNA and by ensuring and shaping chromosomal functions (54-55). Only a few years ago, RNAi was mostly considered to be a cellular defense mechanism against incoming foreign and parasitic genetic information and against aberrant endogenous mRNA. It is now generally accepted that RNAi silencing is a major antiviral mechanism in plants (56). Furthermore, RNAi also contributes to antiviral defense in invertebrates. In this respect, RNAi can be observed as an innate antiviral response.

1.3.2 Application of RNAi

Since the discovery of RNA interference, the field of RNAi has progressed at a striking rate in a period of seven years. It has been shown as a powerful tool to biologists. Genome sequencing projects generates an unknown amount of gene data. For researches attempt to get biological function of genes, they have applied RNAi technology to analyze the functions of a number of genes in wide range of organisms.

Because RNAi is easy to apply, whole genome screens by RNAi may become a useful method in the near future.

Given the sequence specific features of RNAi, it provides a new idea that this technique will play an important role in medical applications such as the use of RNAi-based therapeutic strategy to target virus and viral gene expression. To date, RNAi has been used effectively to inhibit the replication of several viruses in cultured cell lines, including human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), hepatitis C virus (HCV) and poliovirus (57-61). The inhibition of virus by RNAi required very high level of sequence homology since escape of resistant strain of poliovirus was appeared as a result of single mismatch between siRNA and targeted mRNA (58). Although single mismatches have been shown to render a siRNA ineffective, it may be tolerate by the use of multiple siRNAs targeting of multiple regions or regions that are very important for viral replication (60). Recently, the therapeutic potential of siRNA treatment has been demonstrated *in vivo* in mouse models infected with HCV (62).

Although much researches on RNAi remains to be intensively investigated, application of RNAi seems to play an important role in determining cellular gene function and shows a great deal of promise as a potential medical therapeutic agent.

CHAPTER II

OBJECTIVES

2.1 Rationale

Since the introduction of economically important exotic shrimp *P. vannamei* into Asia, the culture industry for this species has begun to increase within Asian countries including Thailand. Unfortunately, the importation of cheaper, non-disease free broodstock has resulted in the introduction of penaeid viral pathogens, particularly TSV, into Thailand. At present, there are no known specific treatments or vaccines for viral disease including Taura syndrome disease. Preventive measures have been used only to control the spread of viruses. The feasibility of using of RNAi technique to inhibit viral replication in primary lymphoid cell culture of *P. monodon* has been established in our group. The virus-specific dsRNA was capable of efficiently inhibiting viral replication in shrimp cells. In the same scenario, this study will evaluate the inhibiting effects of TSV-specific dsRNAs on viral replication in shrimp. Such this advance molecular technique may offer a promising method to develop for preventing or treating viral diseases in shrimp.

2.2 Objective

This thesis study aims to analyze nucleotide sequence in the nonstructural gene of 3 isolates of TSV from Thailand then evaluate the potency of using virus specific dsRNA to inhibit TSV replication in live *P. vannamei*.

CHAPTER III

MATERIALS

3.1 Source of TSV specimens

Pacific white shrimp, *Penaeus vannamei*, infected with TSV was collected from shrimp farms in Chanthaburi, Rayong and Samut Sakorn provinces, Thailand. Samples were stored at -80 °C until used.

3.2 Chemicals and enzymes

All chemicals used in this study were analytical grade purchased from various manufacturers such as Ambion, BDH Laboratory Supplies, Merck, Molecular Research Center. Enzymes were from Invitrogen, New England Biolabs, Promega, Sigma and United States Biological.

3.3 Bacterial strain

Escherichia coli strain DH5α: *supE*44, Δ*lacU*169 (ϕ80 *lacZ* ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1 obtained from Invitrogen was used as host for plasmid propagation.

3.4 Bacterial culture media

Luria-Bertani (LB) broth containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl was prepared as descried by Molecular Cloning: a laboratory manual (63). LB agar plate was LB broth containing 1.5% (w/v) agar.

3.5 Oligonucleotide primers

All primers used in this study listed in Table 2 were synthesized by PROLIGO Primers and Probes, Singapore.

Table 1. Description of the primers used in this study

Name	Sequence 5’to 3’	T_m (ºC)
F1	GAA CCC CTG TTG CCG ACC GAG C	65
R1	CTC GAC TAC CAT CAC CAC ATG TCA G	57
F2	CAC GAC ACG GTA GAT GCT AAT GTG C	60
R2	GGC ATC AAC AAC ATT CGT CCA TAC GCG	64
F3	CCA GAC TAT CAG GTG GAG TGG ACC G	62
R3	CGA TGC AGA TGG GTA ACC AAT ACA TGG	63
F1.2	CAT ATC ACA CTA TGG GAG TTT CAG G	54
R1.2	CAG TGA TTC CAA GAC ACA TGA CTG C	57
F2.2	CAA AAG CCA AAT CCT GAG CTA TTC G	59
R2.2	GTA ACA CCT TCC GAG CGA TTT CGG	62
F3.2	GAC TTT GTA GCC AAG TAC ATT GCT GC	57
R3.2	GAG GAA GGT AAC CTC ATT TAA GGA GC	56
Fhel	GGA GGC ACG CAA TTC AGT TAA GG	59
Rhel	GAT GAC CCT TAG ATC CAG CTA CG	54
Fpro	GTA GAG TGG ACC GAT TTG AGA ACT G	55
Rpro	GTC ACG CGA CCT GCA TGA GTT GTC G	66
Fpoly	GTT TCT TGG ACC ATG TGA TGA CG	56
Rpoly	CTC CAC ATG CAC ATA TCT TCA ATC G	57
TSV-stem-Hel-PstI	GAA TCT GCA GGG CAC GCA ATT CAG TTA AGG	68
TSV-stem-Hel-SpeI	ATA TAC TAG TGG ATT TAC GTT TGA GGT TGC	57
TSV-stem-Poly-PstI	AAT TCT GCA GGA CCA TGT GAT GAC GAA CAG	66
TSV-stem-Poly-SpeI	GGG TAC TAG TAT GCT GGT TGA ACC ATT CAC	61
Pv actinF	GAC TGC TAC GTC GGC GAC GAG G	63
Pv actinR	GCA CGG TGG TCA TCT CCT GCT CG	68
YHV-Hel-Sense-1	CAA GGA CCA CCT GGT ACC GGT AAG AC	62
YHV-Hel-Anti-1	GCG GAA ACG ACT GAC GGC TAC ATT CAC	66
RR1 sense	AAC TCG GTA CCC GGT CCA CCC TCG GAA CTT G	73
RR1 antisense	AAT CCA AGC TTT ATT TTC CTA TAC GTC TTC TCG G	64

3.6 Miscellaneous

TRI REAGENT®	Molecular Research Center
TRI REAGENT®-LS	Molecular Research Center
QIAquick Gel Extraction Kit	QIAGEN
QIAprep Spin Miniprep Kit	QIAGEN
QIAGEN Plasmid Maxi Kit	QIAGEN
RiboMAX™ Large Scale RNA Production Systems	Promega
Lambda DNA/*Hind* III markers	Promega
100 bp DNA Ladder	Invitrogen
1 Kb Plus DNA Ladder	Invitrogen

3.7 Plasmid vectors

pGEM®-Teasy vector (Promega, USA) is used for cloning of PCR products. The vector provides 3’ terminal thymidine at both ends which facilitate ligation of a PCR product with 5’ overhangs adenine. Additionally, pGEM®-Teasy vector contains T7 and SP6 RNA polymerase promoter flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase (Fig 5).

LITMUS™ 28i vector (NEB Inc., USA) is a small, high copy number plasmid vector designed for efficient transcription of double stranded RNA. It contains the origin of replication from pUC19, ampicillin selectable marker, M13 origin of replication and T7 RNA polymerase promoters flanking on both ends of the multiple cloning sites (Fig 6).

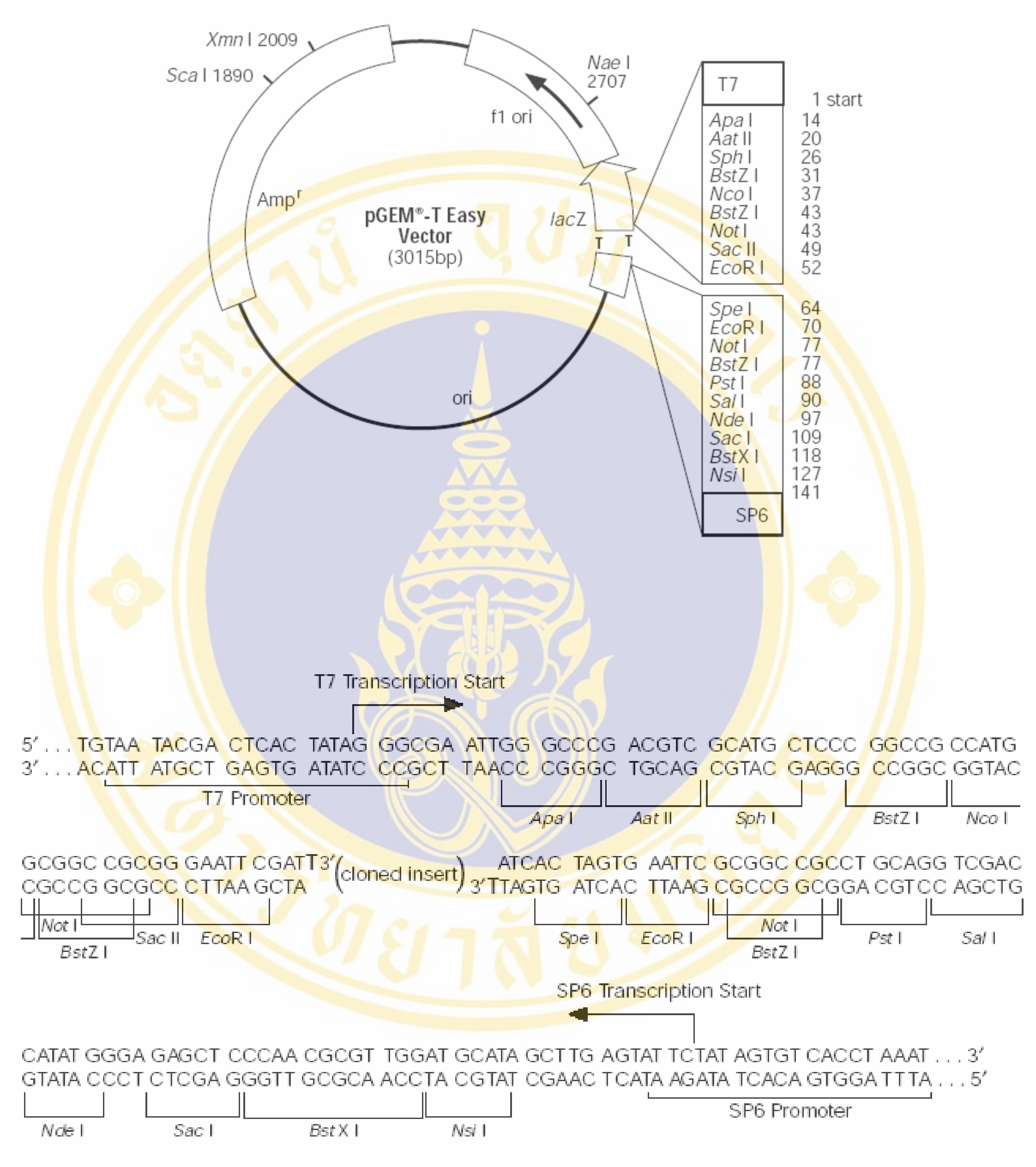


Figure 5. Physical map and multiple cloning sequence of the pGEM®-T Easy Vector

T7 and SP6 are T7 and SP6 RNA polymerase promoters, repectively, Amp^r is β-lactamase coding region and Ori is origin of replication. (Reproduced from the Promega Technical Manual).

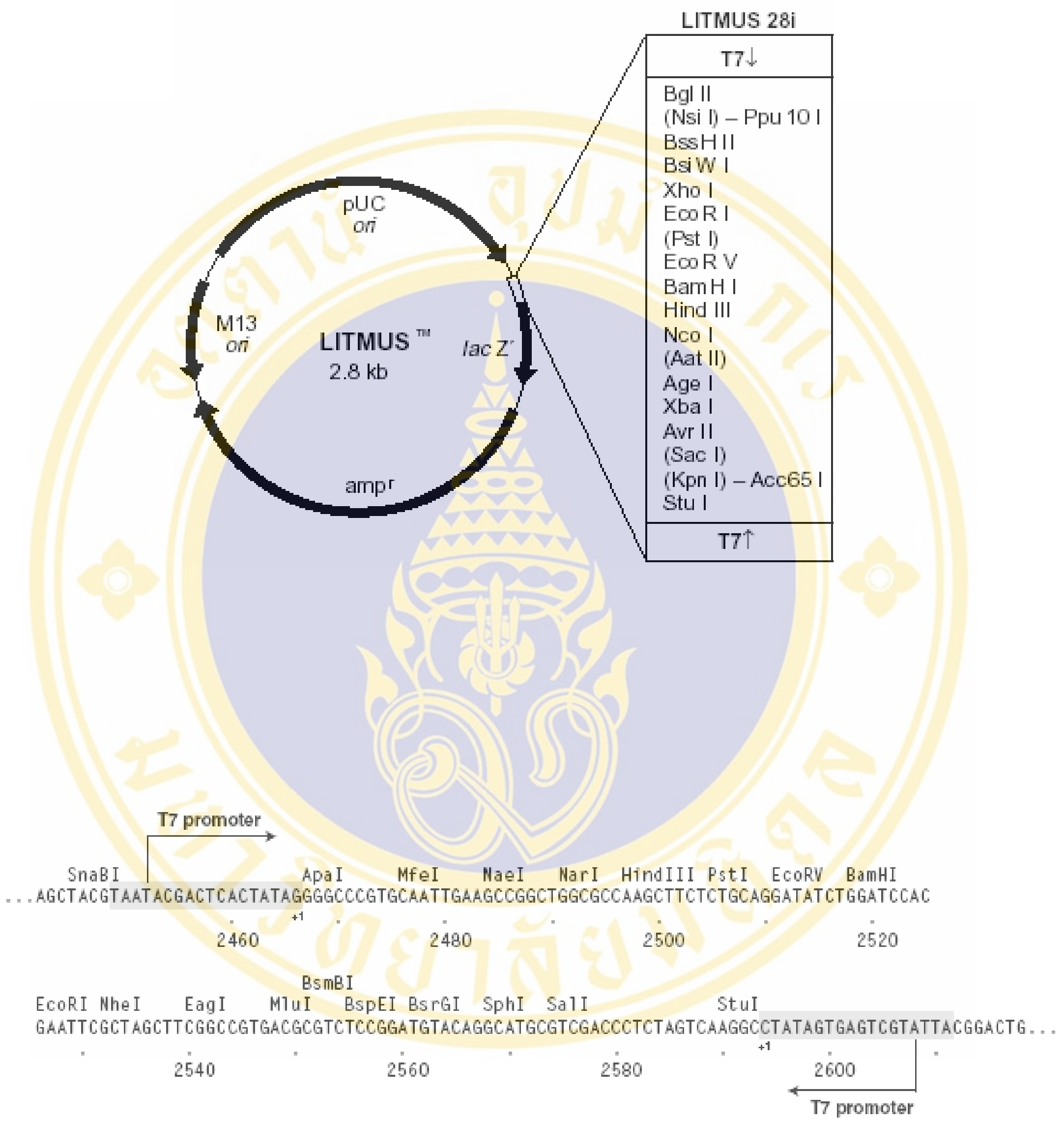


Figure 6. Physical map and multiple cloning sequence of the Litmus 28i Vector

T7 is T7 RNA polymerase promoter, Amp^r is an ampicillin selectable marker and pUC ori is origin of replication form pUC19 (Reproduced from the New England Biolabs catalog).

CHAPTER IV
METHODS

4.1 Screening for TSV, YHV and WSSV infection

4.1.1 Total RNA isolation

Total RNA was isolated from gill of TSV infected shrimps. The sample was ground in TN buffer (0.4 M NaCl, 0.01 M Tris-HCl pH 7.4) in liquid nitrogen as described by Bonami *et al*. (14). Tissue debris was eliminated by centrifugation at 5000 x g for 10 min at 4 ºC, 250 µl of supernatant was mixed in 750 µl of TRI REAGENT®-LS (Molecular Research Center, Inc., USA). The homogenate was then supplemented with 200 µl of chloroform then vigorously shaked and centrifuged at 12,000 x g for 15 min to separate RNA into the aqueous phase. Subsequently, RNA was precipitated with 500 µl of isopropanol and washed with 1 ml of ice-cold 75% ethanol. The RNA pellet was dissolved in 10 µl DEPC (Diethylpyrocarbonate) treated water and stored at -80 ºC.

4.1.2 Crude DNA preparation

50 µl of supernatant from gill sample in TN buffer was added with 1 µg Proteinase K and then incubated at 50 ºC for 2 hr.

4.1.3 Determination of concentration and purity of nucleic acid

The concentration and purity of isolated nucleic acid was determined at the absorbance of 260 and 280 nm using double beam spectrophotometer (U-2000, Hitachi, Ltd., Japan). The absorbance reading at 260 nm allows calculation of the concentration of nucleic acid. An OD of 1 corresponds to approximately 50 µl/ml for double-stranded DNA and 40 µl/ml for single stranded RNA. Therefore, DNA and RNA concentration was calculated by the following formula:

$$\text{Concentration of DNA } (\mu g/\mu l) = \frac{50 \times A_{260} \times \text{dilution factor}}{1000}$$

$$\text{Concentration of RNA } (\mu g/\mu l) = \frac{40 \times A_{260} \times \text{dilution factor}}{1000}$$

Additionally, Ratio of O.D. at 260 and 280 nm [A_{260}/A_{280}] provides a rough estimate of purity of the nucleic acid. Pure preparations of DNA and RNA have A_{260}/A_{280} values of 1.8 or higher.

4.1.4 Reverse transcription

1 µg of total RNA was added to 4 µl of DEPC treated water, 1 µl of 10 µM R1 reverse primer and 1 µl of 10 µM YHV-Hel-Sense-1 primer (Table 1). The mixture was heated at 80 ºC for 3 min then directly cooled down on ice. The mixture of 4.1 µl of DEPC treated water, 4 µl of ImProm-II™ 5X Reaction buffer (Promega, USA), 2.4 µl of 25 mM $MgCl_2$ (Promega, USA), 1 µl of 10 mM dNTPs mix (Promega, USA), 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor (Promega, USA) and 1 µl of ImProm-II™ Reverse Transcriptase (Promega, USA) was added. The reverse transcription (RT) reaction was carried out at 25 ºC for 5 min, followed by 42 ºC for 60 min. The reaction was terminated by incubation at 70 ºC for 15 min.

4.1.5 Polymerase chain reaction

Two microlitres of first stranded cDNAs from 4.1.4 were added to PCR reaction composing of 15.5 µl of DEPC treated water, 2.5 µl of 10X thermophilic polymerase reaction buffer (Promega, USA), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs mixture, 0.5 µl of 10 µM F2 primer, 0.5 µl of 10 µM R1 primer, 0.5 µl of 10 µM YHV-Hel-Sense-1 primer, 0.5 µl of 10 µM YHV-Hel-Anti-1 primer (Table 1), and 0.5 µl of *Taq* polymerase (Promega, USA) for TSV and YHV detection. In addition, 2 µl of crude DNA were added to PCR reaction composing of 16.5 µl of DEPC treated water, 2.5 µl of 10X thermophilic polymerase reaction buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs mix, 0.5 µl of 10 µM RR1 sense primer, 0.5 µl of 10 µM RR1 antisense primer (Table 1), and 0.5 µl of *Taq* polymerase (Promega, USA) for WSSV detection. Polymerase chain reaction (PCR) amplification was performed as follows: a first denaturation at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 15 s and extension at 72 ºC for 45 s, followed by final extension at 72 ºC for 7 min. Following RT-PCR, 10 µl of the amplicons were electrophoresed in 1.2% TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) agarose gel. The gel was stained with ethidium bromide and visualized by UV transilluminator.

4.2 Characterization of TSV ORF1 sequence

4.2.1 RT-PCR amplification of TSV ORF1 sequence as an overlapping fragments

Isolated RNA of TSV positive shrimp was used for the reverse transcription-polymerase chain reaction (RT-PCR) amplification and sequencing of the TSV ORF1 sequence. Overlapping cDNA fragments were transcribed separately by ImProm-II™ Reverse Transcriptase using TSV specific reverse primers (R1, R2 and R3; Table 1). RT conditions were described as above, with the exception of extension time of 80 min. After reverse transcription, 8 µl of each first stranded cDNA template was used for PCR amplification with TSV-specific primers (F1/R1, F2/R2 and F3/R3; Table 1) designed from TSV genomic sequence of Hawaii isolate. PCR amplifications were performed according to a bulletin supplied by the manufacturer of the $\text{Vent}_R^{®}$ DNA polymerase (NEB Inc., USA) of high fidelity. The PCR cycle was as follows: a first denaturation at 94 ºC for 5 min, followed by 40 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 15 s and extension at 72 ºC for 5 min, followed by final extension at 72 ºC for 7 min. Finally, 1 µl of *Taq* DNA polymerase was added to PCR reaction for the A-tailing and ligation reaction.

4.2.2 Purification of PCR product

PCR product was purified using QIAquick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's instruction. Initially, PCR products were electrophoresed in 0.8% agarose gel. PCR product with expected size was then excised from gel and transferred to 1.5 ml microcentrifuge tube. 3 volumes of buffer QG were then added to 1 volume of gel (0.1 g ~ 100 µl). The gel slice was incubated 50 ºC for 10 min and mixed by vortexing the tube every 2-3 min. After the gel was completely melted, 1 gel volume of isopropanol was added to the mixture and mixed. The mixture was transferred to QIAquick spin column and centrifuged at 17,900 x g for 1 min. The flow-through was discarded and 0.5 ml of buffer QG was added to column. The column was centrifuged at 17,900 x g for 1 min. Then 0.75 ml of buffer PE was added and centrifuged at 17,900 x g for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min at 17,900 x g. The column was placed into a new 1.5 ml centrifuge tube and 30 μl of sterile milli-Q water were added to elute DNA. The column was left to stand for 1 min before centrifugation at 19,400 x g for 1 min. The purified DNA was finally collected.

4.2.3 Ligation of DNA fragment into pGEM®T-easy vector

Purified PCR product was cloned into pGEM®T-easy vector (Promega, USA) at T cloning site using 3:1 molar ratio of the insert DNA to the vector as recommended by manufacturer's instruction. Ligation reaction was incubated overnight with T4 DNA ligase (Promega, USA) at 4 ºC in 1X Rapid Ligation Buffer (Promega, USA) in 10 μl of total volume.

4.2.4 Competent cell preparation by simple and efficient method (SEM)

Single colony of *E. coli* DH5α was inoculated into 250 ml SOB medium [1% 9 (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$] in 2 liters flask and grow at 18°C with vigorously shaking until the O.D. at 600 nm reached 0.6. The culture was chilled on ice for 10 minutes then transferred to 500 ml centrifuge tube and collected by centrifugation at 3,700 x g for 7 min at 4°C. The supernatant was discarded and cell pellet was washed with 80 ml ice-cold TB solution (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$ and 250 mM KCl). The cell was chilled on ice for 10 minutes then centrifugation at 3,700 x g for 7 min at 4°C and the cell pellet was resuspended in 20 ml ice-cold TB solution. The DMSO was slowly added to a 7% final concentration and cooled on ice for 10 minutes. Finally, 100 μl of competent cells were aliquot to 1.5 ml microtube then immediately frozen in liquid nitrogen. The competent cells were kept at -80°C (64).

4.2.5 Transformation of plasmid DNA by heat shock method

A hundred µl of *E. Coli* DH5α competent cells were thawed until completely dissolved. Two microlitres of ligation reaction were added and mixed gently before chill on ice for 30 min. The mixture was then heat shocked at 42 ºC for 90 s exactly in waterbath and immediately cooled down on ice at least 5 min. Then, 800 µl of LB broth were added into transformed cells and incubated at 37 ºC for 45 min by shaking. Finally, 70 µl of transformed cells were spreaded onto LB agar plate containing ampicillin and incubated at 37 ºC for 16-18 h.

In case of blue white colony selection, 20 µl of 20 mg/ml X-gal (5-bromo-4-chloro-3-inolyl-β-D-galactopyranoside) and 4µl of 200 mg/ml IPTG (isopropyl-thio-β-D-galactopyranoside) were spreaded onto LB agar plate containing ampicillin prior to spread transformed cells.

4.2.6 Rapid size screening for recombinant clones

A single colony of each candidate clone was picked and lysed in 25 µl of pre-warmed lysis buffer [100 mM NaOH, 60 mM KCl, 5 mM EDTA, 10% (w/v) D-sucrose, 0.25% (w/v) SDS and 0.05% (w/v) bromophenol blue] in microcentrifuge tube. The mixture was incubated at 37 ºC for 5 min and centrifuged at 19,400 x g for 5 min at room temperature. Then, 20 µl of supernatant were loaded for run gel electrophoresis. Candidate clones with larger plasmid size compared to empty plasmid were selected for further plasmid DNA purification.

4.2.7 Purification of plasmid DNA using QIAprep Spin Miniprep Kit

The plasmid DNA was purified by following manufacturer's handbook. The selected clone was inoculated in 3 ml LB broth containing ampicillin and grown overnight at 37 ºC for 16-18 h by shaking. The bacterial cultured cell was centrifuged at 19,400 x g for 1 min at room temperature and discarded supernatant. The cell pellet was resuspended in 250 µl of pre-chilled buffer P1 (50 mM Tris-HCl pH8.0, 10 mM EDTA and 100 µl/ml RNase A). Subsequently, the 250 µl of buffer P2 [200 mM NaOH and 1% (w/v) SDS] were added and inverted gently for 4-6 times. The 350 µl of buffer N3 were added and inverted gently after incubation for 5 min. The mixture was centrifuged at 19,400 x g for 10 min. The supernatant was transferred to the

QIAprep spin column and centrifuged at 19,400 x g for 1 min. The column was washed by adding 750 µl of buffer PB and centrifuged at 19,400 x g for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min at 17,900 x g. The QIAprep column was placed into a new 1.5 ml centrifuge tube and 50 µl of sterile milli-Q water were added to elute DNA. The column was left to stand for 1 min before centrifugation at 19,400 x g for 1 min. Finally, the plasmid DNA from flow through was collected for further analysis.

4.2.8 Restriction enzyme digestion of recombinant clones

The recombinant clone was digested with *Eco*RI restriction enzyme to determine insert DNA of expected size. The restriction enzyme reaction was set up in a volume of 20 µl. The reaction contained 1 µg of plasmid DNA, 1X restriction buffer H (Promega, USA), 2 µg acetylated BSA (Promega, USA), and 2 U of *Eco*RI restriction enzyme (Promega, USA). After incubation at 37 ºC for 3-4 h, the banding pattern was determined by gel electrophoresis.

4.2.9 DNA sequencing

Nucleotide sequencing of insert DNA fragment was performed at sequencing service (Macrogen Inc., Soeul, Korea) on 96-capillary 3730xl DNA analyzer (Applied Biosystem Inc., USA) using an ABI PRISM® BigDye™ Terminator V3.1 Cycle Sequencing Kit (Applied Biosystem Inc., USA). Sequencing was done on both ends of the entire fragment by using either universal primers (T7 and SP6 primer) or TSV specific primers (F1.2, R1.2, F2.2, R2.2, F3.2 and R3.2 primer; Table 1). The DNA sequence of each fragment was determined from two independent clones.

4.2.10 Computer analysis of sequence data

Sequences were assembled into a contiguous sequence and compared with each other as well as the reference sequence using AlignX program. Deduced amino acid sequences were also analyzed. The percentages of nucleotide sequences and amino acid sequences identities among different TSV isolates were determined using vector NTI software. The reference sequence used in this work is Taura syndrome virus (TSV) accession number NC003005.

4.3 dsRNA synthesis

4.3.1 dsRNA design

The dsRNAs were designed based on the TSV sequence of Thai isolates to target three regions of the helicase, protease and polymerase within the ORF1 of TSV.

4.3.2 Preparation of DNA templates for *in vitro* transcription

To generate recombinant plasmids for conventional dsRNA synthesis, three primer pairs (Fhel/Rhel, Fpro/Rpro, and Fpoly/Rpoly; Table 1) were used to PCR amplify DNA fragments using the Vent$_R$$^®$DNA polymerase. After amplification, DNA fragments were gel purified and cloned into pGEM$^®$T-easy vector. To facilitates *In vitro* transcription, DNA fragments from pGEM$^®$T-easy vector were cut with *Eco*RI restriction enzyme and subcloned into LITMUS 28i vector at the same site.

For stem loop dsRNAs, two primer pairs (TSV-stem-Hel-PstI/TSV-stem-Hel-SpeI and TSV-stem-Poly-PstI/TSV-stem-Poly-SpeI; Table 1) were synthesized to amplify additional DNA fragments of helicase and polymerase, repectively. The DNA fragments were then cloned into *Spe*I and *Sal*I restriction site of previous pGEM$^®$T-easy vector containing corresponding DNA fragment in antisense direction.

4.3.3 Purification of plasmid DNA using QIAGEN Plasmid Maxi Kit

Since high quantity and quality of DNA template are need for production of large amounts of RNA by *in vitro* transcription. The recombinant plasmid with designated insert was therefore prepared and purified by using QIAGEN Plasmid Maxi Kit (Promega, USA) according to the manufacturer's handbook. A single colony of bacterial cells harboring recombinant plasmid was inoculated a starter culture of 3 ml LB medium containing ampicillin and grown at 37 ºC for 8 h with vigorous shaking. The starter was then diluted into LB medium containing ampicillin at 1:500 dilution and grown at 37 ºC for 14-16 h with vigorous shaking. The bacterial cultured cells were transferred to a 250 ml centrifuge tube and harvested by centrifugation at 6000 x g for 15 min at 4 ºC. The cell pellet was resuspended in 10 ml of buffer P1 by vortexing. Then, 10 ml of buffer P2 were added and the mixture was mixed gently by inverting 4-6 times. After incubation at room temperature for 5 min, 10 ml of pre-chilled buffer P3 (3 M potassium acetate pH 5.5) were added and mixed immediately but gently by inverting 4-6 times followed by incubation on ice for 20 min. The mixture was centrifuged at 20,000 x g for 30 min at 4 ºC. The supernatant containing recombinant plasmid was transferred to a 30 ml centrifuge tube and centrifuged at 20,000 x g for 15 min at 4 ºC. The QIAGEN-tip 500 was equilibrated with 10 ml of buffer QBT [750 mM NaCl, 50 mM MOPS pH 7.0, 15% (V/V) isopropanol and 0.15% (V/V) Triton X] and allowed the column to empty by gravity flow before the supernatant was applied to the column. The column was then allowed to empty by gravity flow and washed twice with 30 ml of buffer QC [1 M NaCl, 50 mM MOPS pH 7.0 and 15% (V/V) isopropanol]. Subsequently, the plasmid DNA was eluted with 15 ml of buffer QF [1.25 M NaCl, 50 mM Tris-HCl pH 8.5 and 15% (V/V) isopropanol] into a new 30 ml centrifuge tube and precipitated with 10.5 ml of isopropanol. The mixture was mixed and centrifuged at 15,000 x g for 30 min at 4 ºC. The supernatant was discarded and the DNA pellet was washed with 5 ml of 70% ethanol by centrifugation at 15,000 x g for 10 min at 4 ºC. Finally, the DNA pellet was air dried for 10 min and dissolved in 300 μl of sterile milli-Q water.

4.3.4 Production of conventional dsRNA using *in vitro* transcription

The recombinant plasmids used as DNA templates were linearized with restriction enzymes to generate 5’-overhang (Table 2). Sense and antisense strands were transcribed *in vitro* by using RiboMAX™ Large Scale RNA Production Systems (Promega, USA) according to the protocol provided in Technical Bulletin No.166 with slight modifcations. The *in vitro* transcription reaction (20 μl) contained 1-2 μg of linear DNA template, 1X T7 transcription buffer (80 mM HEPES-KOH pH 7.5, 24 mM $MgCl_2$, 40 mM DTT and 2 mM spermidine) 25 mM rNTPs and 2 μl of T7 enzyme mix (Promega,USA) in a volume of 20 μl. The reaction was mixed gently and incubated at 37 ºC for 4 h. DNA templates were then removed by digestion with 1-2 U of DNase at 37 ºC for 15 min. The reaction was adjusted to 100 μl with DEPC treated water. The RNA was extracted with 1 volume of TE-saturated phenol:chloroform: isoamyl alcohol (25:24:1). The mixture was vortexed for 1 min and centrifuged at 13,000 x g for 15 min at 4 ºC. The aqueous phase containing RNA was transferred to a new 1.5 ml centrifuge tube and 1 volume of chloroform:isoamyl alcohol (24:1) was added. Subsequently, the mixture was vortexed for 1 min and centrifuged at 13,000 x g at 4 ºC for 15 min. The RNA was transferred to a new 1.5 ml centrifuge tube and 0.1 volume of 3M sodium acetate (pH 5.2) and 1 volume of isopropanol was added. The mixture was mixed and precipitated at -30 ºC for 2 h followed by centrifugation at 13,000 x g at 4 ºC for 20 min. The RNA pellet was washed with 1 ml of 70% ethanol by centrifugation at 7500 x g at 4 ºC for 5 min and air dried for 5 min. The RNA pellet was resuspended in DEPC treated water.

To generate conventional dsRNA, equal amounts of *in vitro* transcribed sense and antisense stranded RNAs (100 µg) were added to annealing buffer at 1X concentration (20 mM potassium acetate, 6 mM HEPES-KOH pH 7.4 and 0.4 mM magnesium acetate). The sample was heated at 80°C for 3 minutes then cooled down to room temperature and left for additional 1 h at room temperature to allow efficient annealing of the complementary strands. The RNA concentration was quantitated easily by ultraviolet light absorbance as described in method 4.1.3.

Table 2. Recombinant plasmids with their restriction enzyme(s) and dsRNAs size

Recombinant plasmids	Restriction enzyme(s)	dsRNA size (bp)
limus28i-Helicase	Sense: *Bam*H I/*Stu* I	700
	Antisense: *Sna*B I/*Xho* I	
limus28i-Protease	Sense: *Hind* III/*Stu* I	890
	Antisense: *Bgl* II/*Xho* I	
limus28i-Polymerase	Sense: *Bam*H I/*Stu* I	840
	Antisense: *Bgl* II/*Xho* I	
pBluescript II KS-senseGFP	*Sma* I	800
pBluescript II KS-antisenseGFP	*Sma* I	
pGEMTeasy-Helicase-stem	*Sal* I	400
pGEMTeasy-Polymerase-stem	*Sal* I	500

4.3.5 Production of stem loop dsRNA using *in vitro* transcription

The T7 transcription template was prepared by digesting with a restriction enzyme that linearized the DNA downstream from the insert (Table 2). *In vitro* transcription reaction was performed as above. The single stranded RNA that was produced in the 4 hours reaction annealed spontaneously to form dsRNA. Following transcription, The DNA template was removed as described above.

4.3.6 Characterization of dsRNA

To verify the formation of dsRNAs, both conventional dsRNAs and stem loop dsRNAs were digested with RNase A and III ribonuclease enzymes and compared to undigested dsRNAs on agarose gel electrophoresis. For RNase A digestion reaction, 2 µg of dsRNA were incubated with 0.01 µg of RNase A in 1X RNase A buffer (300 mM sodium acetate, 10 mM Tris, 5 mM EDTA) in a total volume of 10 µl. For RNase III digestion reaction, 2 µg of dsRNA were incubated with 0.5 U of RNase III in a final 1X RNase III buffer (10 mM Tris, 0.1 mM $CaCl_2$ and 2.5 mM $MgCl_2$) concentration. The digestion reactions were incubated at 37 ºC for 5 min and immediately loaded onto gel.

4.4 Preparation of TSV

To prepare viral stock used in the study, pacific white shrimp, *Penaeus vannamei,* infected with TSV was minced then grinded with mortar and pestle in 5 ml of TN buffer. The mixture prepared from the infected tissue was divided into several aliquots of equal volume. All aliqouts were centrifuged at 5000 x g for 10 min at 4 ºC and the supernatant containing virus from each aliquot was then carefully recovered so as not to disturb the pellet to a new tube. The supernatant was kept at -80 ºC as a viral stock.

4.5 Infectivity of TSV

4.5.1 Preparation of pacific white shrimp

Pacific white shrimps were maintained in a 80 liters tank containing 40 liters artificial seawater with 10 ppt salinity at room temperature. Each tank was fitted with individual vinyl tubing, airstone and covered with tank cover. Shrimps were fed once a day with commercial diet pellet in the evening.

4.5.2 *In vivo* infection of TSV

A preliminary experiment was conducted to evaluate whether Taura syndrome virus prepared from TSV infected shrimp is infective. If it is infective, we would like to know whether a nonnative host, *P. monodon*, was susceptible to TSV infection. Therefore, two species of shrimps: *P. vanamei*, a highly susceptible target host, and *P. monodon* were inoculated with a viral stock at a 1:50 dilution by intramuscular injection. Control groups of both shrimp species were injected with PBS. After 5 days postinfection, haemolymph samples were drawn and shrimps were sacrified to be analyzed by RT-PCR.

In the subsequent infectivity experiment, a total of 15 shrimps were divided into three groups. The TSV stock was prepared as a 1:10, 1:50 and 1:100 dilutions in PBS to inject intramuscularly into abdominal muscle of the three groups. Deaths were observed 2 times a day from Day 0 to Day 4, the termination. During the experiment, the affected shrimps were collected and frozen at -80 ºC for further RNA isolation.

4.5.3 Preparation of primary cell culture from lymphoid organ of *Penaeus monodon*

The primary cell culture from lymphoid (Oka) organ of *P. monodon* was prepared as described by Kasornchandra *et al.* and Assavalapsakul *et al.* (65-66). Briefly, shrimps were killed and lymphoid organs were excised and placed into washing solution [2X Leibovitz's L-15 medium, 1% D-glucose, 0.5% NaCl, 200 IU/ml penicillin and 200 µg/ml streptomycin]. Lymphoid organs were washed for ten times with the washing solution and minced into small pieces. The ground tissue were transferred to a 50 ml centrifuged tube containing approximately 15 ml of working medium [2X Leibovitz's L-15 medium, 1% D-glucose, 0.5% NaCl, 200 IU/ml penicillin and 200 µg/ml streptomycin supplement with 15% (v/v) fetal bovine serum, 15% (v/v) shrimp meat extract and 5% (v/v) lactabumin] and left at room temperature for 5-10 min. The upper cell suspension was transferred to a new 50 ml centrifuged tube and seeded into 24-well tissue culture plate for monolayer culture and incubated at 26-28 ºC for 3 days. Cultured cells were observed daily with an inverted microscope (Nikon) for the propagation of cells.

4.5.4 *In vitro* infection of TSV and sample preparation

Primary lymphoid cells were inoculated with 1:1 filtered TSV in LHM [10.1 g Minimum Essential Medium with L-glutamine, 3 mM $Na_2HPO_4{\cdot}7H_2O$, 28 mM $MgSO_4{\cdot}7H2O$, 15 mM $CaCl_2{\cdot}2H_2O$, 10 mM KCl, 500 mM NaCl, 5 mM $MgCl_2{\cdot}6H_2O$, 36 mM $NaHCO_3$ and 3 mM D-glucose] on Day 3 after plating. Cells were incubated at 28 ºC for 14 h and washed 2 times with washing solution after inoculation. Cells were periodically observed under a microscope for any sign of cytopathic effect as well as 50 µl of culture medium were collected at 0, 24, 48, 72 and 96 h. Cells were lysed with 250 µl of TRI REAGENT®-LS for RNA extraction at the same time points.

4.5.5 RT-PCR for TSV RNA

Total RNA was extracted from culture medium and cell lysates by TRI REAGENT®-LS according to protocol supplied by manufacturer. cDNA was transcribed from 1µg of total RNA or 2 µl of culture medium using ImProm-II™ Reverse Transcriptase. The BIR region of TSV was amplified by PCR using F2 and R1 primers as described above. The products were then resolved by gel electrophoresis.

4.6 RNA interference assay

4.6.1 Preparation of experimental shrimp

Shrimps were kept in tanks (5-7 shrimps/tank) containing artificial seawater with 10 ppt salinity at room temperature and fed with commercial feed.

4.6.2 Experimental design

There were eight experimental groups including negative and positive control group. Four of eight groups were administered with 25 or 35 µg in 100 µl of each conventional dsRNA of helicase, protease, polymerase or green fluorescent protein through haemolymph. Two of eight groups were administered with 25 or 35 µg in 100 µl of each stem dsRNA of helicase and polymerase. Negative and positive control group were administered with PBS. Twenty-four hours after administeration, shrimps were infected by intramuscular injection at 1:50 dilution except negative control group. Injection volumes were 100 µl. Finally, haemolymph was drawn from individual shrimp and shrimps were sacrified at 2 days post infection for RT-PCR analysis.

4.6.3 RNA isolation and RT-PCR analysis

Total RNA were extracted from haemolymph and gill samples by TRI REAGENT®-LS and TRI REAGENT, respectively according to protocol supplied by manufacturer. Total RNA was kept at -80 ºC until used. Reverse transcription was performed with 1 µg of total RNA, 1 µl of 10 µM R1 primer and 1 µl of 10 µM Pv actinR primer (Table 1) in a total volume of 7 µl, at 80 ºC for 3 min. The mixture was then cooled down on ice at least 5 min and a reaction mixture composed of 4.4 µl of DEPC treated water, 4 µl of ImProm-II™ 5X Reaction buffer, 2.4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs mix, 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor and 1 µl of ImProm-II™ Reverse Transcriptase was added. The reverse transcription (RT) reaction was carried out at 25 ºC for 5 min, 42 ºC for 80 min followed by 70 ºC for 15 min. After the RT reaction, the cDNA of TSV within BIR region and β-actin were then amplified in separable reaction. PCR reaction composed of 16.5 µl of DEPC treated water, 2.5 µl of 10X thermophilic polymerase reaction buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs mix, 0.5 µl of *Taq* polymerase, 0.5 µl of 10 µM either F2 primer or Pv actinF primer and 0.5 µl of 10 µM either R1 primer or Pv actinR primer (Table 1). PCR conditions were performed as follows: TSV BIR region, 94 ºC for 5 min for first denaturation, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 15 s and extension at 72 ºC for 45 s, followed by final extension at 72 ºC for 7 min; β-actin, 94 ºC for 5 min for first denaturation, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 57 ºC for 15 s and extension at 72 ºC for 45 s, followed by final extension at 72 ºC for 7 min. Amplified products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. The size of amplified products was 214 bp for TSV BIR region and 542 bp for β-actin mRNA.

CHAPTER V
RESULTS

RNA interference is a phenomenon in which the introduction of double stranded RNA into cells induces degradation of homologous mRNA in a sequence specific manner. Due to its potency and specificity in inhibiting the target RNA, this method has been employed to inhibit replication of pathogenic viruses in various organisms, including shrimp (62, 67-68). To investigate whether specific double-stranded RNA could mediate inhibition of TSV replication, dsRNAs corresponding to the helicase, protease and polymerase genes were designed and tested. Since the specificity of dsRNA sequence plays an important role in silencing targeted mRNA, therefore, the first part of this work aims to determine and analyze the nucleotide sequence of TSV in the ORF1 covering helicase, protease and polymerase genes. Then, the conserved cDNA region of each gene was amplified and constructed as recombinant plasmids for dsRNAs synthesis. The recombinant plasmid was used as a DNA template to generate dsRNA. Finally, the dsRNAs were tested for their ability to inhibit replication of the virus in white shrimps.

5.1 Isolation of TSV Thai isolate from infected shrimps

To identify whether shrimps specimen collected from several region of Thailand were infected with any viruses, either reverse transcription-polymerase chain reaction (RT-PCR) or polymerase chain reaction (PCR) were employed (21, 69-70). In this study, the presence of Taura syndrome virus (TSV), Yellow head virus (YHV) and White spot syndrome virus (WSSV) were monitored. Therefore, an assay using a single tube, RT-PCR and PCR were performed to detect TSV/YHV and WSSV, respectively. Two sets of primers, F2/R1 and YHV-Hel-Sense-1/YHV-Hel-Anti-1, were mixed at a ratio of 1:1 to amplify a specific fragment of the TSV or YHV in the RT-PCR reaction whereas the primer set, RR1 sense/RR1 antisense, was used for the amplification of the specific fragment of WSSV in the PCR reaction. The designed primer pairs are listed in Table 3 and displayed the product sizes of each virus. The expected size of RT-PCR/PCR products are 214 bp, 850 bp and 338 bp (as shown in positive control) indicating the presence of the TSV, YHV and WSSV. In this study, shrimps suspected of TSV infection were collected from different areas as follows: Samut Sakorn, Chanthaburi and Rayong provinces. Although these shrimp showed no pathological signs of TSV infection, the presence of intense specific 214 bp product after the RT-PCR amplification in these shrimps indicated that they were infected with TSV. In contrast, there were no specific amplified PCR products of 850 bp of YHV or 338 bp of WSSV detected in these shrimp samples (Fig. 7). These results suggested that all these shrimp samples were infected with TSV but not with YHV or WSSV.

Table 3. Primers and their expected size of PCR product for each primer pair when used to amplify an appropriate viral template

Virus	Primers	Product size (bp)
TSV	F2: (5’-CAC GAC ACG GTA GAT GCT AAT GTG C-3’) R1: (5’- CTC GAC TAC CAT CAC CAC ATG TCA G-3’)	214
YHV	YHV-Hel-Sense-1: (5’-CAA GGA CCA CCT GGT ACC GGT AAG AC-3’) YHV-Hel-Anti-1: (5’-GCG GAA ACG ACT GAC GGC TAC ATT CAC-3’)	850
WSSV	RR1 sense: (5’-AAC TCG GTA CCC GGT CCA CCT CGG AAC TTG-3’) RR1 antisense: (5’-AAT CCA ACT TTA TTT TCC TAT ACG TCT TCT CGG-3’)	338

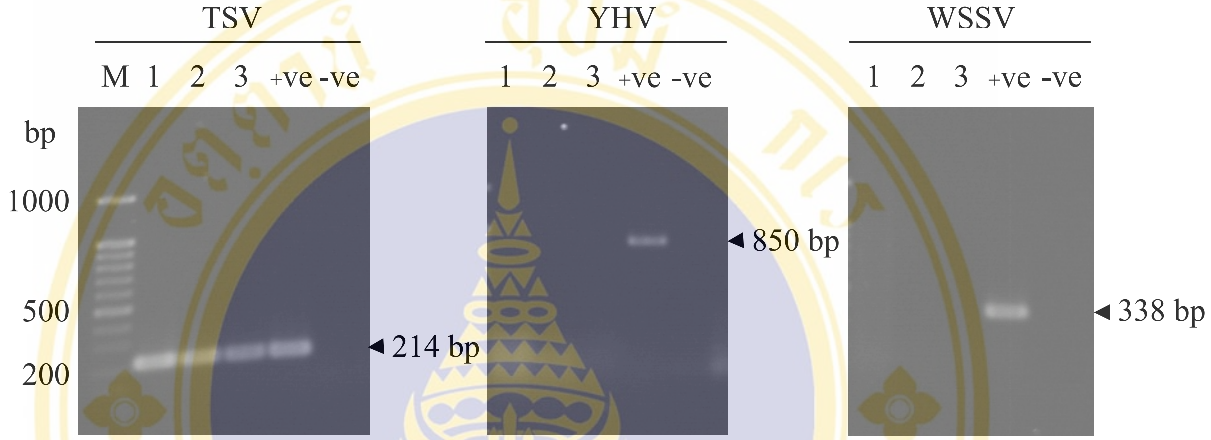


Figure 7: Detection of multiple viruses in cultured shrimps

RT-PCR and PCR analysis were performed by using virus-specific primer pairs and shrimp samples collected from Chanthaburi, Rayong and Samut Sakorn provinces for the presence of TSV and YHV genomic RNA and WSSV genomic DNA. The amplified product was separated on agarose gel and visualized by staining with ethidium bromide. The 214, 850 and 338 bp band indicate virus specific amplification products of TSV, YHV and WSSV, respectively.

Lane M: 100 bp DNA ladder
Lane 1: Shrimp sample collected from Samut Sakorn
Lane 2: Shrimp sample collected from Chanthaburi
Lane 3: Shrimp sample collected from Rayong
Lane +ve: Positive control
Lane -ve: Negative control

5.2 Determination and analysis of nucleotide sequence in Open Reading Frame 1 gene from TSV Thai isolates

To determine nucleotide sequence and its variations in ORF1 of TSV from naturally infected shrimps of different areas of Thailand, 6 kb cDNA covering this region was PCR amplified into overlapping fragments, cloned and subjected to sequence. The RT-PCR amplification of viral RNA used TSV specific primers as described in Table 2, a total of three overlapping fragments representing most of the ORF1 of TSV have been amplified (Fig. 8 and 9). These RT-PCR amplified fragments were cloned into pGEM®T-easy vector and sequenced from both directions. The sequences were shown to be the majority of sequence of the individual virus isolates. The sequences from those three overlapping fragments were assembled into a contiguous sequence. In this study, we determined the nucleotide sequence of TSV ORF1 gene of the three different isolates originated from Samut Sakorn, Chanthaburi and Rayong provinces. Thereafter, we compare the nucleotide and amino acid sequence within the Thai isolates, and between the Thai isolates (3 isolates) and the reference strain of Hawaii isolate within the TSV ORF1 gene.

5.2.1 Determination of nucleotide sequence in the Open Reading Frame 1 (ORF1) of TSV genome

The nucleotide sequences of the Thai isolates were aligned with each other as well as with the reference sequence of Hawaii isolate (Fig. 10). Comparison between the nucleotide sequences of the ORF1 gene of Thai isolates and those of Hawaii isolate revealed 104 nucleotide substitutions, of which changed nucleotides were conserved among Thai isolates, and the substitutions seem to be randomly distributed throughout the gene. As summarized in table 4, the nucleotide identities between ORF1 gene of Thai isolates and Hawaii isolate ranged from 96.4 to 97.0% whereas the identities among Thai isolates were higher and ranged from 97.8 to 99.2 %. Of three Thai isolates, the Chanthaburi isolate showed the lowest identity of 96.4% and the Samut Sakorn isolate showed the highest identity of 97.0%, when compared to the reference Hawaii isolate, suggesting that the Samut Sakorn isolate was more closely related to the Hawaii isolate than to either the Chanthaburi isolate or the Rayong isolate.

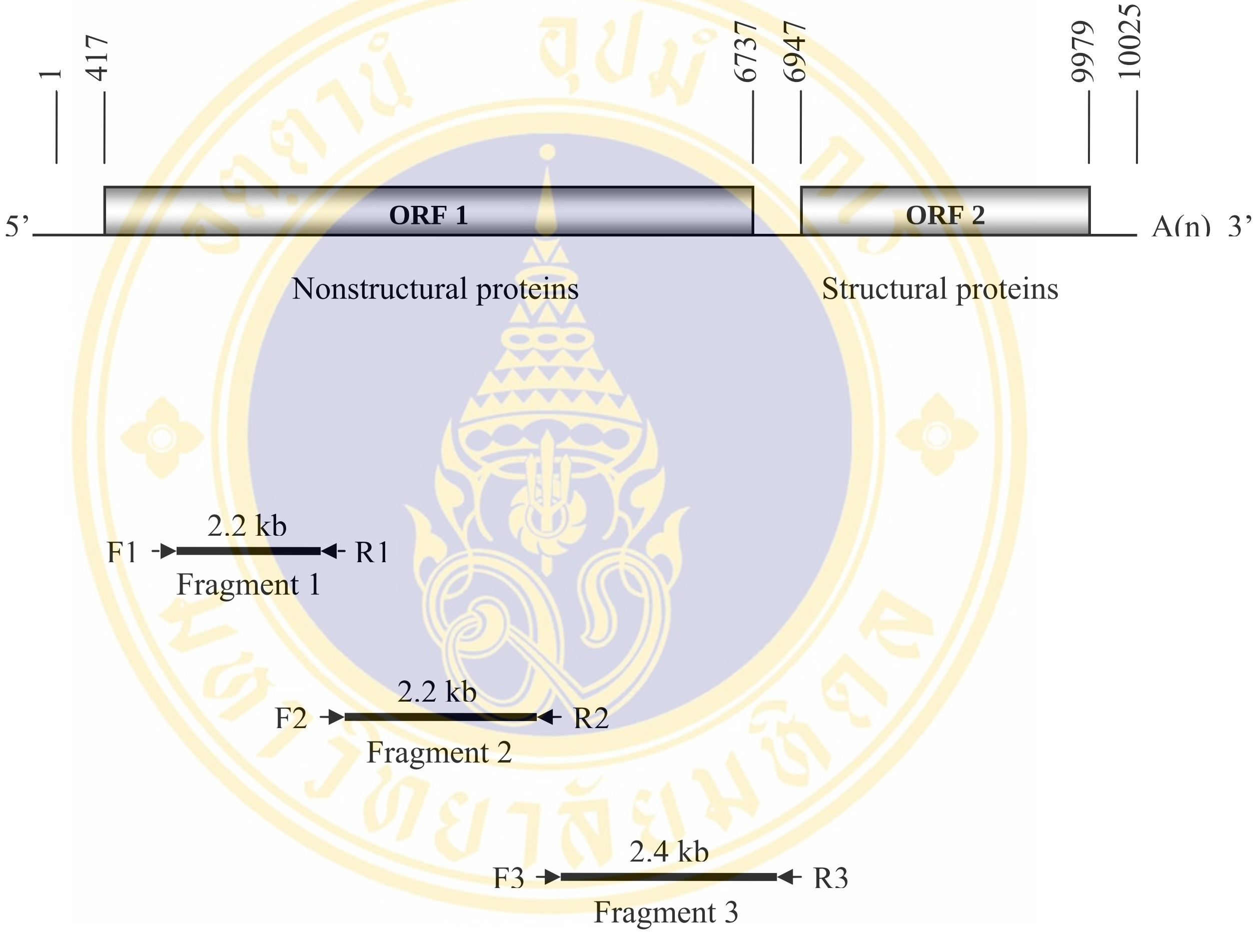


Figure 8: A schematic representation of Taura syndrome virus genomic RNA along with the location of primers used in the RT-PCR

The three pairs of primer were used to amplify the overlapping fragments of the TSV ORF1 gene by reverse transcriptase-polymerase chain reaction (RT-PCR).

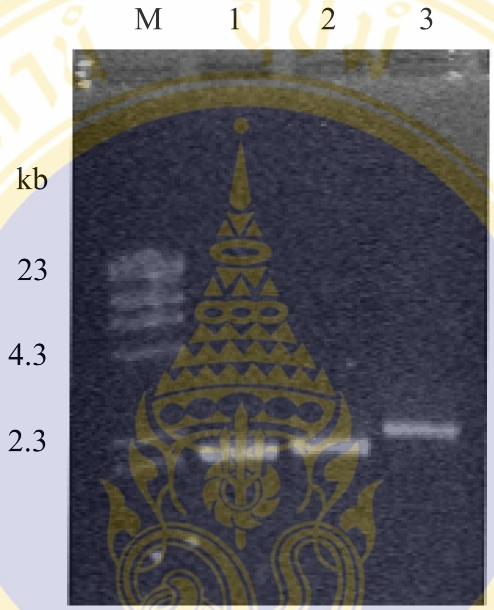


Figure 9: RT-PCR amplification of fragments representing the TSV ORF1 gene of Samut Sakorn sample

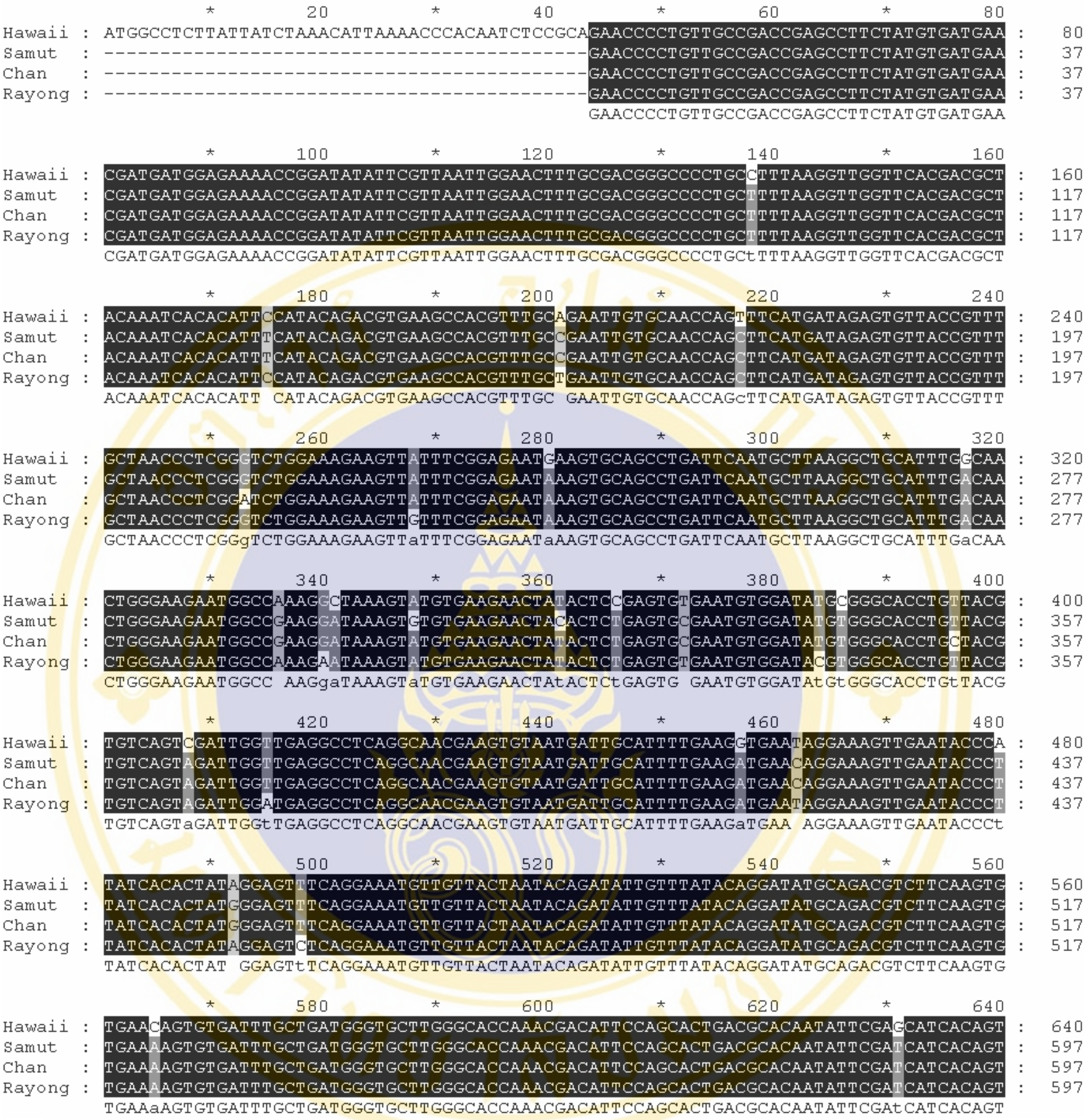
The ORF1 gene of TSV was amplified as overlapping fragments with TSV-specific primer pairs.

Lane M: λ *Hin*d III marker

Lane 1: The 2.2 kb amplification products of fragment 1

Lane 2: The 2.2 kb amplification products of fragment 2

Lane 3: The 2.4 kb amplification products of fragment 3



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                  *        20         *        40         *        60         *        80
Hawaii : ATGGCCTCTTATTATCTAAACATTAAAACCCACAATCTCCGCAGAACCCCTGTTGCCGACCGAGCCTTCTATGTGATGAA :   80
Samut  : -------------------------------------------GAACCCCTGTTGCCGACCGAGCCTTCTATGTGATGAA :   37
Chan   : -------------------------------------------GAACCCCTGTTGCCGACCGAGCCTTCTATGTGATGAA :   37
Rayong : -------------------------------------------GAACCCCTGTTGCCGACCGAGCCTTCTATGTGATGAA :   37
                                                    GAACCCCTGTTGCCGACCGAGCCTTCTATGTGATGAA

                  *       100         *       120         *       140         *       160
Hawaii : CGATGATGGAGAAAACCGGATATATTCGTTAATTGGAACTTTGCGACGGGCCCCTGCCTTTAAGGTTGGTTCACGACGCT :  160
Samut  : CGATGATGGAGAAAACCGGATATATTCGTTAATTGGAACTTTGCGACGGGCCCCTGCTTTTAAGGTTGGTTCACGACGCT :  117
Chan   : CGATGATGGAGAAAACCGGATATATTCGTTAATTGGAACTTTGCGACGGGCCCCTGCTTTTAAGGTTGGTTCACGACGCT :  117
Rayong : CGATGATGGAGAAAACCGGATATATTCGTTAATTGGAACTTTGCGACGGGCCCCTGCTTTTAAGGTTGGTTCACGACGCT :  117
         CGATGATGGAGAAAACCGGATATATTCGTTAATTGGAACTTTGCGACGGGCCCCTGCtTTTAAGGTTGGTTCACGACGCT

                  *       180         *       200         *       220         *       240
Hawaii : ACAAATCACACATTCCATACAGACGTGAAGCCACGTTTGCAGAATTGTGCAACCAGTTTCATGATAGAGTGTTACCGTTT :  240
Samut  : ACAAATCACACATTTCATACAGACGTGAAGCCACGTTTGCCGAATTGTGCAACCAGCTTCATGATAGAGTGTTACCGTTT :  197
Chan   : ACAAATCACACATTTCATACAGACGTGAAGCCACGTTTGCCGAATTGTGCAACCAGCTTCATGATAGAGTGTTACCGTTT :  197
Rayong : ACAAATCACACATTCCATACAGACGTGAAGCCACGTTTGCTGAATTGTGCAACCAGCTTCATGATAGAGTGTTACCGTTT :  197
         ACAAATCACACATT CATACAGACGTGAAGCCACGTTTGC GAATTGTGCAACCAGcTTCATGATAGAGTGTTACCGTTT

                  *       260         *       280         *       300         *       320
Hawaii : GCTAACCCTCGGGTCTGGAAAGAAGTTATTTCGGAGAATGAAGTGCAGCCTGATTCAATGCTTAAGGCTGCATTTGGCAA :  320
Samut  : GCTAACCCTCGGGTCTGGAAAGAAGTTATTTCGGAGAATAAAGTGCAGCCTGATTCAATGCTTAAGGCTGCATTTGACAA :  277
Chan   : GCTAACCCTCGGATCTGGAAAGAAGTTATTTCGGAGAATAAAGTGCAGCCTGATTCAATGCTTAAGGCTGCATTTGACAA :  277
Rayong : GCTAACCCTCGGGTCTGGAAAGAAGTTGTTTCGGAGAATAAAGTGCAGCCTGATTCAATGCTTAAGGCTGCATTTGACAA :  277
         GCTAACCCTCGGgTCTGGAAAGAAGTTaTTTCGGAGAATaAAGTGCAGCCTGATTCAATGCTTAAGGCTGCATTTGaCAA

                  *       340         *       360         *       380         *       400
Hawaii : CTGGGAAGAATGGCCAAAGGCTAAAGTATGTGAAGAACTATACTCCGAGTGTGAATGTGGATATGCGGGCACCTGTTACG :  400
Samut  : CTGGGAAGAATGGCCGAAGGATAAAGTGTGTGAAGAACTACACTCTGAGTGCGAATGTGGATATGTGGGCACCTGTTACG :  357
Chan   : CTGGGAAGAATGGCCGAAGGATAAAGTATGTGAAGAACTATACTCTGAGTGCGAATGTGGATATGTGGGCACCTGCTACG :  357
Rayong : CTGGGAAGAATGGCCAAAGAATAAAGTATGTGAAGAACTATACTCTGAGTGTGAATGTGGATACGTGGGCACCTGTTACG :  357
         CTGGGAAGAATGGCC AAGgaTAAAGTaTGTGAAGAACTAtACTCtGAGTG GAATGTGGATAtGtGGGCACCTGtTACG

                  *       420         *       440         *       460         *       480
Hawaii : TGTCAGTCGATTGGTTGAGGCCTCAGGCAACGAAGTGTAATGATTGCATTTTGAAGGTGAATAGGAAAGTTGAATACCCA :  480
Samut  : TGTCAGTAGATTGGTTGAGGCCTCAGGCAACGAAGTGTAATGATTGCATTTTGAAGATGAACAGGAAAGTTGAATACCCT :  437
Chan   : TGTCAGTAGATTGGTTGAGGCCTCAGGCAACGAAGTGTAATGATTGCATTTTGAAGATGAACAGGAAAGTTGAATACCCT :  437
Rayong : TGTCAGTAGATTGGATGAGGCCTCAGGCAACGAAGTGTAATGATTGCATTTTGAAGATGAATAGGAAAGTTGAATACCCT :  437
         TGTCAGTaGATTGGtTGAGGCCTCAGGCAACGAAGTGTAATGATTGCATTTTGAAGaTGAA AGGAAAGTTGAATACCCt

                  *       500         *       520         *       540         *       560
Hawaii : TATCACACTATAGGAGTTTCAGGAAATGTTGTTACTAATACAGATATTGTTTATACAGGATATGCAGACGTCTTCAAGTG :  560
Samut  : TATCACACTATGGGAGTTTCAGGAAATGTTGTTACTAATACAGATATTGTTTATACAGGATATGCAGACGTCTTCAAGTG :  517
Chan   : TATCACACTATGGGAGTTTCAGGAAATGTTGTTACTAATACAGATATTGTTTATACAGGATATGCAGACGTCTTCAAGTG :  517
Rayong : TATCACACTATAGGAGTCTCAGGAAATGTTGTTACTAATACAGATATTGTTTATACAGGATATGCAGACGTCTTCAAGTG :  517
         TATCACACTAT GGAGTtTCAGGAAATGTTGTTACTAATACAGATATTGTTTATACAGGATATGCAGACGTCTTCAAGTG

                  *       580         *       600         *       620         *       640
Hawaii : TGAACAGTGTGATTTGCTGATGGGTGCTTGGGCACCAAACGACATTCCAGCACTGACGCACAATATTCGAGCATCACAGT :  640
Samut  : TGAAAAGTGTGATTTGCTGATGGGTGCTTGGGCACCAAACGACATTCCAGCACTGACGCACAATATTCGATCATCACAGT :  597
Chan   : TGAAAAGTGTGATTTGCTGATGGGTGCTTGGGCACCAAACGACATTCCAGCACTGACGCACAATATTCGATCATCACAGT :  597
Rayong : TGAAAAGTGTGATTTGCTGATGGGTGCTTGGGCACCAAACGACATTCCAGCACTGACGCACAATATTCGATCATCACAGT :  597
         TGAAaAGTGTGATTTGCTGATGGGTGCTTGGGCACCAAACGACATTCCAGCACTGACGCACAATATTCGAtCATCACAGT
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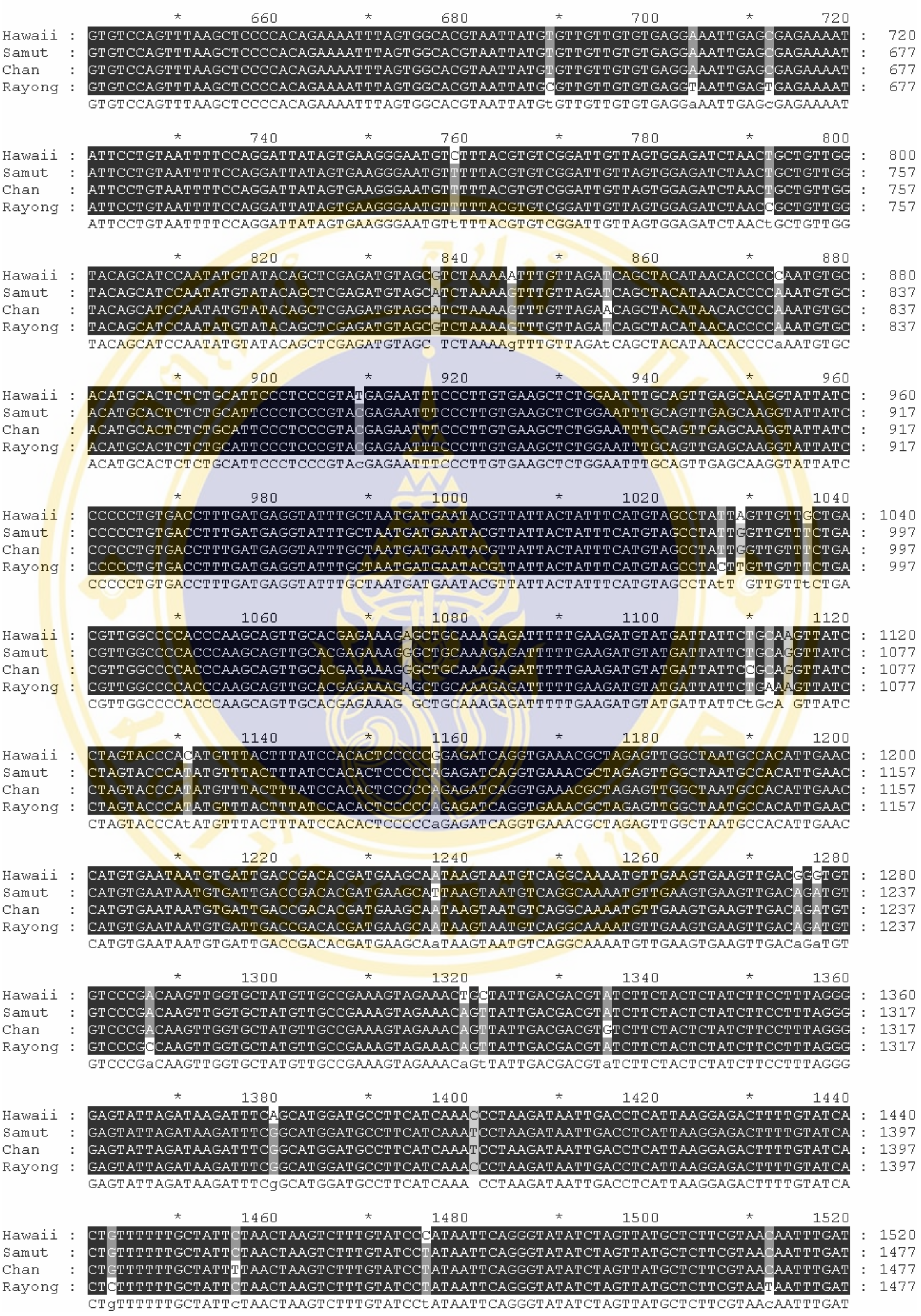
Figure 10: Multiple sequences alignment of Thai TSV isolates

The nucleotide sequences of the ORF 1 gene of TSV were compared between three Thai isolates and the Hawaii isolate. The first 43 nucleotides at the 5'end were not determined. The Hawaii sequence is shown in the top line. The followings are sequences from Samut Sakorn (Samut), Chanthaburi (Chan) and Rayong (Rayong) isolates. Black shadings represent matching nucleotides. Gray shadings represent matching nucleotides between two or three sequences. Numbers on the right are position of nucleotide relative to the start codon of ORF1.



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                  *       660         *       680         *       700         *       720
Hawaii : GTGTCCAGTTTAAGCTCCCCACAGAAAATTTAGTGGCACGTAATTATGTGTTGTTGTGTGAGGAAATTGAGCGAGAAAAT :  720
Samut  : GTGTCCAGTTTAAGCTCCCCACAGAAAATTTAGTGGCACGTAATTATGTGTTGTTGTGTGAGGAAATTGAGCGAGAAAAT :  677
Chan   : GTGTCCAGTTTAAGCTCCCCACAGAAAATTTAGTGGCACGTAATTATGTGTTGTTGTGTGAGGAAATTGAGCGAGAAAAT :  677
Rayong : GTGTCCAGTTTAAGCTCCCCACAGAAAATTTAGTGGCACGTAATTATGCGTTGTTGTGTGAGGTAATTGAGTGAGAAAAT :  677
         GTGTCCAGTTTAAGCTCCCCACAGAAAATTTAGTGGCACGTAATTATGtGTTGTTGTGTGAGGaAATTGAGcGAGAAAAT

                  *       740         *       760         *       780         *       800
Hawaii : ATTCCTGTAATTTTCCAGGATTATAGTGAAGGGAATGTCTTTACGTGTCGGATTGTTAGTGGAGATCTAACTGCTGTTGG :  800
Samut  : ATTCCTGTAATTTTCCAGGATTATAGTGAAGGGAATGTTTTTACGTGTCGGATTGTTAGTGGAGATCTAACTGCTGTTGG :  757
Chan   : ATTCCTGTAATTTTCCAGGATTATAGTGAAGGGAATGTTTTTACGTGTCGGATTGTTAGTGGAGATCTAACTGCTGTTGG :  757
Rayong : ATTCCTGTAATTTTCCAGGATTATAGTGAAGGGAATGTTTTTACGTGTCGGATTGTTAGTGGAGATCTAACCGCTGTTGG :  757
         ATTCCTGTAATTTTCCAGGATTATAGTGAAGGGAATGTtTTTACGTGTCGGATTGTTAGTGGAGATCTAACtGCTGTTGG

                  *       820         *       840         *       860         *       880
Hawaii : TACAGCATCCAATATGTATACAGCTCGAGATGTAGCGTCTAAAAATTTGTTAGATCAGCTACATAACACCCCCAATGTGC :  880
Samut  : TACAGCATCCAATATGTATACAGCTCGAGATGTAGCATCTAAAAGTTTGTTAGATCAGCTACATAACACCCCAAATGTGC :  837
Chan   : TACAGCATCCAATATGTATACAGCTCGAGATGTAGCATCTAAAAGTTTGTTAGAACAGCTACATAACACCCCAAATGTGC :  837
Rayong : TACAGCATCCAATATGTATACAGCTCGAGATGTAGCGTCTAAAAGTTTGTTAGATCAGCTACATAACACCCCAAATGTGC :  837
         TACAGCATCCAATATGTATACAGCTCGAGATGTAGC TCTAAAAgTTTGTTAGAtCAGCTACATAACACCCCaAATGTGC

                  *       900         *       920         *       940         *       960
Hawaii : ACATGCACTCTCTGCATTCCCTCCCGTATGAGAATTTCCCTTGTGAAGCTCTGGAATTTGCAGTTGAGCAAGGTATTATC :  960
Samut  : ACATGCACTCTCTGCATTCCCTCCCGTACGAGAATTTCCCTTGTGAAGCTCTGGAATTTGCAGTTGAGCAAGGTATTATC :  917
Chan   : ACATGCACTCTCTGCATTCCCTCCCGTACGAGAATTTCCCTTGTGAAGCTCTGGAATTTGCAGTTGAGCAAGGTATTATC :  917
Rayong : ACATGCACTCTCTGCATTCCCTCCCGTACGAGAATTTCCCTTGTGAAGCTCTGGAATTTGCAGTTGAGCAAGGTATTATC :  917
         ACATGCACTCTCTGCATTCCCTCCCGTAcGAGAATTTCCCTTGTGAAGCTCTGGAATTTGCAGTTGAGCAAGGTATTATC

                  *       980         *      1000         *      1020         *      1040
Hawaii : CCCCCTGTGACCTTTGATGAGGTATTTGCTAATGATGAATACGTTATTACTATTTCATGTAGCCTATTAGTTGTTGCTGA : 1040
Samut  : CCCCCTGTGACCTTTGATGAGGTATTTGCTAATGATGAATACGTTATTACTATTTCATGTAGCCTATTGGTTGTTTCTGA :  997
Chan   : CCCCCTGTGACCTTTGATGAGGTATTTGCTAATGATGAATACGTTATTACTATTTCATGTAGCCTATTGGTTGTTTCTGA :  997
Rayong : CCCCCTGTGACCTTTGATGAGGTATTTGCTAATGATGAATACGTTATTACTATTTCATGTAGCCTACTTGTTGTTTCTGA :  997
         CCCCCTGTGACCTTTGATGAGGTATTTGCTAATGATGAATACGTTATTACTATTTCATGTAGCCTAtT GTTGTTtCTGA

                  *      1060         *      1080         *      1100         *      1120
Hawaii : CGTTGGCCCCACCCAAGCAGTTGCACGAGAAAGAGCTGCAAAGAGATTTTTGAAGATGTATGATTATTCTGCAAGTTATC : 1120
Samut  : CGTTGGCCCCACCCAAGCAGTTGCACGAGAAAGGGCTGCAAAGAGATTTTTGAAGATGTATGATTATTCTGCAGGTTATC : 1077
Chan   : CGTTGGCCCCACCCAAGCAGTTGCACGAGAAAGGGCTGCAAAGAGATTTTTGAAGATGTATGATTATTCCGCAGGTTATC : 1077
Rayong : CGTTGGCCCCACCCAAGCAGTTGCACGAGAAAGAGCTGCAAAGAGATTTTTGAAGATGTATGATTATTCTGAAAGTTATC : 1077
         CGTTGGCCCCACCCAAGCAGTTGCACGAGAAAG GCTGCAAAGAGATTTTTGAAGATGTATGATTATTCtGcA GTTATC

                  *      1140         *      1160         *      1180         *      1200
Hawaii : CTAGTACCCACATGTTTACTTTATCCACACTCCCCGGAGATCAGGTGAAACGCTAGAGTTGGCTAATGCCACATTGAAC : 1200
Samut  : CTAGTACCCATATGTTTACTTTATCCACACTCCCCAGAGATCAGGTGAAACGCTAGAGTTGGCTAATGCCACATTGAAC : 1157
Chan   : CTAGTACCCATATGTTTACTTTATCCACACTCCCCAGAGATCAGGTGAAACGCTAGAGTTGGCTAATGCCACATTGAAC : 1157
Rayong : CTAGTACCCATATGTTTACTTTATCCACACTCCCCAGAGATCAGGTGAAACGCTAGAGTTGGCTAATGCCACATTGAAC : 1157
         CTAGTACCCAtATGTTTACTTTATCCACACTCCCCaGAGATCAGGTGAAACGCTAGAGTTGGCTAATGCCACATTGAAC

                  *      1220         *      1240         *      1260         *      1280
Hawaii : CATGTGAATAATGTGATTGACCGACACGATGAAGCAATAAGTAATGTCAGGCAAAATGTTGAAGTGAAGTTGACGGGTGT : 1280
Samut  : CATGTGAATAATGTGATTGACCGACACGATGAAGCATTAAGTAATGTCAGGCAAAATGTTGAAGTGAAGTTGACAGATGT : 1237
Chan   : CATGTGAATAATGTGATTGACCGACACGATGAAGCAATAAGTAATGTCAGGCAAAATGTTGAAGTGAAGTTGACAGATGT : 1237
Rayong : CATGTGAATAATGTGATTGACCGACACGATGAAGCAATAAGTAATGTCAGGCAAAATGTTGAAGTGAAGTTGACAGATGT : 1237
         CATGTGAATAATGTGATTGACCGACACGATGAAGCAaTAAGTAATGTCAGGCAAAATGTTGAAGTGAAGTTGACaGaTGT

                  *      1300         *      1320         *      1340         *      1360
Hawaii : GTCCCGACAAGTTGGTGCTATGTTGCCGAAAGTAGAAACTGCTATTGACGACGTATCTTCTACTCTATCTTCCTTTAGGG : 1360
Samut  : GTCCCGACAAGTTGGTGCTATGTTGCCGAAAGTAGAAACAGTTATTGACGACGTATCTTCTACTCTATCTTCCTTTAGGG : 1317
Chan   : GTCCCGACAAGTTGGTGCTATGTTGCCGAAAGTAGAAACAGTTATTGACGACGTGTCTTCTACTCTATCTTCCTTTAGGG : 1317
Rayong : GTCCCGCCAAGTTGGTGCTATGTTGCCGAAAGTAGAAACAGTTATTGACGACGTATCTTCTACTCTATCTTCCTTTAGGG : 1317
         GTCCCGaCAAGTTGGTGCTATGTTGCCGAAAGTAGAAACaGtTATTGACGACGTaTCTTCTACTCTATCTTCCTTTAGGG

                  *      1380         *      1400         *      1420         *      1440
Hawaii : GAGTATTAGATAAGATTTCAGCATGGATGCCTTCATCAAACCCTAAGATAATTGACCTCATTAAGGAGACTTTTGTATCA : 1440
Samut  : GAGTATTAGATAAGATTTCGGCATGGATGCCTTCATCAAATCCTAAGATAATTGACCTCATTAAGGAGACTTTTGTATCA : 1397
Chan   : GAGTATTAGATAAGATTTCGGCATGGATGCCTTCATCAAATCCTAAGATAATTGACCTCATTAAGGAGACTTTTGTATCA : 1397
Rayong : GAGTATTAGATAAGATTTCGGCATGGATGCCTTCATCAAACCCTAAGATAATTGACCTCATTAAGGAGACTTTTGTATCA : 1397
         GAGTATTAGATAAGATTTCgGCATGGATGCCTTCATCAAA CCTAAGATAATTGACCTCATTAAGGAGACTTTTGTATCA

                  *      1460         *      1480         *      1500         *      1520
Hawaii : CTGTTTTTTGCTATTCTAACTAAGTCTTTGTATCCCATAATTCAGGGTATATCTAGTTATGCTCTTCGTAACAATTTGAT : 1520
Samut  : CTGTTTTTTGCTATTCTAACTAAGTCTTTGTATCCTATAATTCAGGGTATATCTAGTTATGCTCTTCGTAACAATTTGAT : 1477
Chan   : CTGTTTTTTGCTATTTTAACTAAGTCTTTGTATCCTATAATTCAGGGTATATCTAGTTATGCTCTTCGTAACAATTTGAT : 1477
Rayong : CTCTTTTTTGCTATTCTAACTAAGTCTTTGTATCCTATAATTCAGGGTATATCTAGTTATGCTCTTCGTAATAATTTGAT : 1477
         CTgTTTTTTGCTATTcTAACTAAGTCTTTGTATCCtATAATTCAGGGTATATCTAGTTATGCTCTTCGTAAcAATTTGAT
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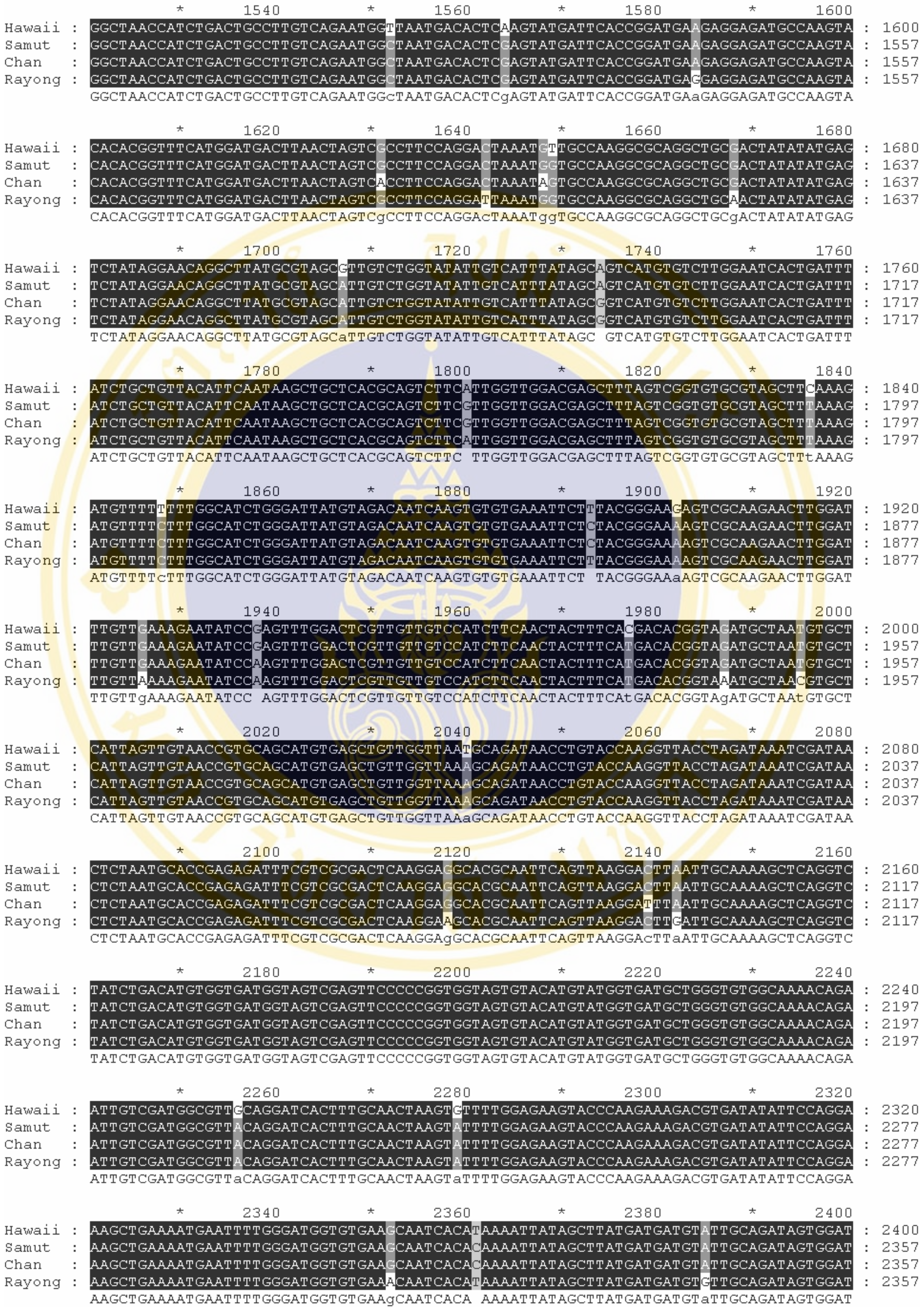
Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)



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                  *      1540         *      1560         *      1580         *      1600
Hawaii : GGCTAACCATCTGACTGCCTTGTCAGAATGGTTAATGACACTCAAGTATGATTCACCGGATGAAGAGGAGATGCCAAGTA : 1600
Samut  : GGCTAACCATCTGACTGCCTTGTCAGAATGGCTAATGACACTCGAGTATGATTCACCGGATGAAGAGGAGATGCCAAGTA : 1557
Chan   : GGCTAACCATCTGACTGCCTTGTCAGAATGGCTAATGACACTCGAGTATGATTCACCGGATGAAGAGGAGATGCCAAGTA : 1557
Rayong : GGCTAACCATCTGACTGCCTTGTCAGAATGGCTAATGACACTCGAGTATGATTCACCGGATGAGGAGGAGATGCCAAGTA : 1557
         GGCTAACCATCTGACTGCCTTGTCAGAATGGcTAATGACACTCgAGTATGATTCACCGGATGAaGAGGAGATGCCAAGTA

                  *      1620         *      1640         *      1660         *      1680
Hawaii : CACACGGTTTCATGGATGACTTAACTAGTCGCCTTCCAGGACTAAATGTTGCCAAGGCGCAGGCTGCGACTATATATGAG : 1680
Samut  : CACACGGTTTCATGGATGACTTAACTAGTCGCCTTCCAGGACTAAATGGTGCCAAGGCGCAGGCTGCGACTATATATGAG : 1637
Chan   : CACACGGTTTCATGGATGACTTAACTAGTCACCTTCCAGGACTAAATAGTGCCAAGGCGCAGGCTGCGACTATATATGAG : 1637
Rayong : CACACGGTTTCATGGATGACTTAACTAGTCGCCTTCCAGGATTAAATGGTGCCAAGGCGCAGGCTGCAACTATATATGAG : 1637
         CACACGGTTTCATGGATGACTTAACTAGTCgCCTTCCAGGAcTAAATggTGCCAAGGCGCAGGCTGCgACTATATATGAG

                  *      1700         *      1720         *      1740         *      1760
Hawaii : TCTATAGGAACAGGCTTATGCGTAGCGTTGTCTGGTATATTGTCATTTATAGCAGTCATGTGTCTTGGAATCACTGATTT : 1760
Samut  : TCTATAGGAACAGGCTTATGCGTAGCATTGTCTGGTATATTGTCATTTATAGCAGTCATGTGTCTTGGAATCACTGATTT : 1717
Chan   : TCTATAGGAACAGGCTTATGCGTAGCATTGTCTGGTATATTGTCATTTATAGCGGTCATGTGTCTTGGAATCACTGATTT : 1717
Rayong : TCTATAGGAACAGGCTTATGCGTAGCATTGTCTGGTATATTGTCATTTATAGCGGTCATGTGTCTTGGAATCACTGATTT : 1717
         TCTATAGGAACAGGCTTATGCGTAGCaTTGTCTGGTATATTGTCATTTATAGC GTCATGTGTCTTGGAATCACTGATTT

                  *      1780         *      1800         *      1820         *      1840
Hawaii : ATCTGCTGTTACATTCAATAAGCTGCTCACGCAGTCTTCATTGGTTGGACGAGCTTTAGTCGGTGTGCGTAGCTTCAAAG : 1840
Samut  : ATCTGCTGTTACATTCAATAAGCTGCTCACGCAGTCTTCGTTGGTTGGACGAGCTTTAGTCGGTGTGCGTAGCTTTAAAG : 1797
Chan   : ATCTGCTGTTACATTCAATAAGCTGCTCACGCAGTCTTCGTTGGTTGGACGAGCTTTAGTCGGTGTGCGTAGCTTTAAAG : 1797
Rayong : ATCTGCTGTTACATTCAATAAGCTGCTCACGCAGTCTTCATTGGTTGGACGAGCTTTAGTCGGTGTGCGTAGCTTTAAAG : 1797
         ATCTGCTGTTACATTCAATAAGCTGCTCACGCAGTCTTC TTGGTTGGACGAGCTTTAGTCGGTGTGCGTAGCTTtAAAG

                  *      1860         *      1880         *      1900         *      1920
Hawaii : ATGTTTTTTTTGGCATCTGGGATTATGTAGACAATCAAGTGTGTGAAATTCTTTACGGGAAGAGTCGCAAGAACTTGGAT : 1920
Samut  : ATGTTTTCTTTGGCATCTGGGATTATGTAGACAATCAAGTGTGTGAAATTCTCTACGGGAAAAGTCGCAAGAACTTGGAT : 1877
Chan   : ATGTTTTCTTTGGCATCTGGGATTATGTAGACAATCAAGTGTGTGAAATTCTCTACGGGAAAAGTCGCAAGAACTTGGAT : 1877
Rayong : ATGTTTTCTTTGGCATCTGGGATTATGTAGACAATCAAGTGTGTGAAATTCTTTACGGGAAAAGTCGCAAGAACTTGGAT : 1877
         ATGTTTTcTTTGGCATCTGGGATTATGTAGACAATCAAGTGTGTGAAATTCT TACGGGAAaAGTCGCAAGAACTTGGAT

                  *      1940         *      1960         *      1980         *      2000
Hawaii : TTGTTGAAAGAATATCCGAGTTTGGACTCGTTGTTGTCCATCTTCAACTACTTTCACGACACGGTAGATGCTAATGTGCT : 2000
Samut  : TTGTTGAAAGAATATCCGAGTTTGGACTCGTTGTTGTCCATCTTCAACTACTTTCATGACACGGTAGATGCTAATGTGCT : 1957
Chan   : TTGTTGAAAGAATATCCAAGTTTGGACTCGTTGTTGTCCATCTTCAACTACTTTCATGACACGGTAGATGCTAATGTGCT : 1957
Rayong : TTGTTAAAAGAATATCCAAGTTTGGACTCGTTGTTGTCCATCTTCAACTACTTTCATGACACGGTAAATGCTAACGTGCT : 1957
         TTGTTgAAAGAATATCC AGTTTGGACTCGTTGTTGTCCATCTTCAACTACTTTCAtGACACGGTAgATGCTAAtGTGCT

                  *      2020         *      2040         *      2060         *      2080
Hawaii : CATTAGTTGTAACCGTGCAGCATGTGAGCTGTTGGTTAATGCAGATAACCTGTACCAAGGTTACCTAGATAAATCGATAA : 2080
Samut  : CATTAGTTGTAACCGTGCAGCATGTGAGCTGTTGGTTAAAGCAGATAACCTGTACCAAGGTTACCTAGATAAATCGATAA : 2037
Chan   : CATTAGTTGTAACCGTGCAGCATGTGAGCTGTTGGTTAAAGCAGATAACCTGTACCAAGGTTACCTAGATAAATCGATAA : 2037
Rayong : CATTAGTTGTAACCGTGCAGCATGTGAGCTGTTGGTTAAAGCAGATAACCTGTACCAAGGTTACCTAGATAAATCGATAA : 2037
         CATTAGTTGTAACCGTGCAGCATGTGAGCTGTTGGTTAAaGCAGATAACCTGTACCAAGGTTACCTAGATAAATCGATAA

                  *      2100         *      2120         *      2140         *      2160
Hawaii : CTCTAATGCACCGAGAGATTTCGTCGCGACTCAAGGAGGCACGCAATTCAGTTAAGGACTTAATTGCAAAAGCTCAGGTC : 2160
Samut  : CTCTAATGCACCGAGAGATTTCGTCGCGACTCAAGGAGGCACGCAATTCAGTTAAGGACTTAATTGCAAAAGCTCAGGTC : 2117
Chan   : CTCTAATGCACCGAGAGATTTCGTCGCGACTCAAGGAGGCACGCAATTCAGTTAAGGATTTAATTGCAAAAGCTCAGGTC : 2117
Rayong : CTCTAATGCACCGAGAGATTTCGTCGCGACTCAAGGAAGCACGCAATTCAGTTAAGGACTTGATTGCAAAAGCTCAGGTC : 2117
         CTCTAATGCACCGAGAGATTTCGTCGCGACTCAAGGAgGCACGCAATTCAGTTAAGGAcTTaATTGCAAAAGCTCAGGTC

                  *      2180         *      2200         *      2220         *      2240
Hawaii : TATCTGACATGTGGTGATGGTAGTCGAGTTCCCCCGGTGGTAGTGTACATGTATGGTGATGCTGGGTGTGGCAAAACAGA : 2240
Samut  : TATCTGACATGTGGTGATGGTAGTCGAGTTCCCCCGGTGGTAGTGTACATGTATGGTGATGCTGGGTGTGGCAAAACAGA : 2197
Chan   : TATCTGACATGTGGTGATGGTAGTCGAGTTCCCCCGGTGGTAGTGTACATGTATGGTGATGCTGGGTGTGGCAAAACAGA : 2197
Rayong : TATCTGACATGTGGTGATGGTAGTCGAGTTCCCCCGGTGGTAGTGTACATGTATGGTGATGCTGGGTGTGGCAAAACAGA : 2197
         TATCTGACATGTGGTGATGGTAGTCGAGTTCCCCCGGTGGTAGTGTACATGTATGGTGATGCTGGGTGTGGCAAAACAGA

                  *      2260         *      2280         *      2300         *      2320
Hawaii : ATTGTCGATGGCGTTGCAGGATCACTTTGCAACTAAGTGTTTTGGAGAAGTACCCAAGAAAGACGTGATATATTCCAGGA : 2320
Samut  : ATTGTCGATGGCGTTACAGGATCACTTTGCAACTAAGTATTTTGGAGAAGTACCCAAGAAAGACGTGATATATTCCAGGA : 2277
Chan   : ATTGTCGATGGCGTTACAGGATCACTTTGCAACTAAGTATTTTGGAGAAGTACCCAAGAAAGACGTGATATATTCCAGGA : 2277
Rayong : ATTGTCGATGGCGTTACAGGATCACTTTGCAACTAAGTATTTTGGAGAAGTACCCAAGAAAGACGTGATATATTCCAGGA : 2277
         ATTGTCGATGGCGTTaCAGGATCACTTTGCAACTAAGTaTTTTGGAGAAGTACCCAAGAAAGACGTGATATATTCCAGGA

                  *      2340         *      2360         *      2380         *      2400
Hawaii : AAGCTGAAAATGAATTTTGGGATGGTGTGAAGCAATCACATAAAATTATAGCTTATGATGATGTATTGCAGATAGTGGAT : 2400
Samut  : AAGCTGAAAATGAATTTTGGGATGGTGTGAAGCAATCACACAAAATTATAGCTTATGATGATGTATTGCAGATAGTGGAT : 2357
Chan   : AAGCTGAAAATGAATTTTGGGATGGTGTGAAGCAATCACACAAAATTATAGCTTATGATGATGTATTGCAGATAGTGGAT : 2357
Rayong : AAGCTGAAAATGAATTTTGGGATGGTGTGAAACAATCACATAAAATTATAGCTTATGATGATGTGTTGCAGATAGTGGAT : 2357
         AAGCTGAAAATGAATTTTGGGATGGTGTGAAgCAATCACA AAAATTATAGCTTATGATGATGTaTTGCAGATAGTGGAT
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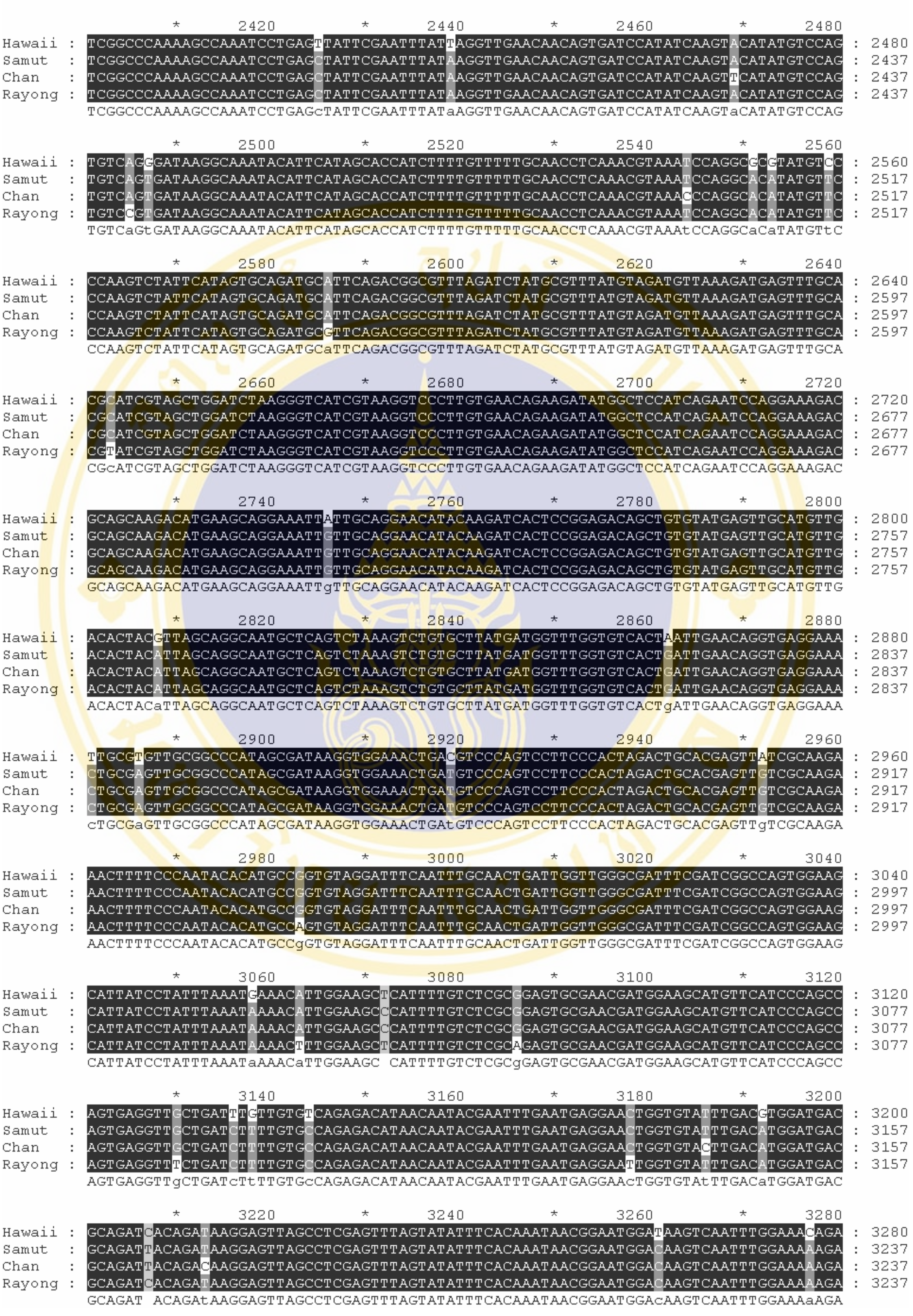
Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)



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                  *      2420         *      2440         *      2460         *      2480
Hawaii : TCGGCCCAAAAGCCAAATCCTGAGTTATTCGAATTTATTAGGTTGAACAACAGTGATCCATATCAAGTACATATGTCCAG : 2480
Samut  : TCGGCCCAAAAGCCAAATCCTGAGCTATTCGAATTTATAAGGTTGAACAACAGTGATCCATATCAAGTACATATGTCCAG : 2437
Chan   : TCGGCCCAAAAGCCAAATCCTGAGCTATTCGAATTTATAAGGTTGAACAACAGTGATCCATATCAAGTTCATATGTCCAG : 2437
Rayong : TCGGCCCAAAAGCCAAATCCTGAGCTATTCGAATTTATAAGGTTGAACAACAGTGATCCATATCAAGTACATATGTCCAG : 2437
         TCGGCCCAAAAGCCAAATCCTGAGcTATTCGAATTTATaAGGTTGAACAACAGTGATCCATATCAAGTaCATATGTCCAG

                  *      2500         *      2520         *      2540         *      2560
Hawaii : TGTCAGGGATAAGGCAAATACATTCATAGCACCATCTTTTGTTTTTGCAACCTCAAACGTAAATCCAGGCGCGTATGTCC : 2560
Samut  : TGTCAGTGATAAGGCAAATACATTCATAGCACCATCTTTTGTTTTTGCAACCTCAAACGTAAATCCAGGCACATATGTTC : 2517
Chan   : TGTCAGTGATAAGGCAAATACATTCATAGCACCATCTTTTGTTTTTGCAACCTCAAACGTAAACCCAGGCACATATGTTC : 2517
Rayong : TGTCCGTGATAAGGCAAATACATTCATAGCACCATCTTTTGTTTTTGCAACCTCAAACGTAAATCCAGGCACATATGTTC : 2517
         TGTCaGtGATAAGGCAAATACATTCATAGCACCATCTTTTGTTTTTGCAACCTCAAACGTAAAtCCAGGCaCaTATGTtC

                  *      2580         *      2600         *      2620         *      2640
Hawaii : CCAAGTCTATTCATAGTGCAGATGCATTCAGACGGCGTTTAGATCTATGCGTTTATGTAGATGTTAAAGATGAGTTTGCA : 2640
Samut  : CCAAGTCTATTCATAGTGCAGATGCATTCAGACGGCGTTTAGATCTATGCGTTTATGTAGATGTTAAAGATGAGTTTGCA : 2597
Chan   : CCAAGTCTATTCATAGTGCAGATGCATTCAGACGGCGTTTAGATCTATGCGTTTATGTAGATGTTAAAGATGAGTTTGCA : 2597
Rayong : CCAAGTCTATTCATAGTGCAGATGCGTTCAGACGGCGTTTAGATCTATGCGTTTATGTAGATGTTAAAGATGAGTTTGCA : 2597
         CCAAGTCTATTCATAGTGCAGATGCaTTCAGACGGCGTTTAGATCTATGCGTTTATGTAGATGTTAAAGATGAGTTTGCA

                  *      2660         *      2680         *      2700         *      2720
Hawaii : CGCATCGTAGCTGGATCTAAGGGTCATCGTAAGGTCCCTTGTGAACAGAAGATATGGCTCCATCAGAATCCAGGAAAGAC : 2720
Samut  : CGCATCGTAGCTGGATCTAAGGGTCATCGTAAGGTCCCTTGTGAACAGAAGATATGGCTCCATCAGAATCCAGGAAAGAC : 2677
Chan   : CGCATCGTAGCTGGATCTAAGGGTCATCGTAAGGTCCCTTGTGAACAGAAGATATGGCTCCATCAGAATCCAGGAAAGAC : 2677
Rayong : CGTATCGTAGCTGGATCTAAGGGTCATCGTAAGGTCCCTTGTGAACAGAAGATATGGCTCCATCAGAATCCAGGAAAGAC : 2677
         CGcATCGTAGCTGGATCTAAGGGTCATCGTAAGGTCCCTTGTGAACAGAAGATATGGCTCCATCAGAATCCAGGAAAGAC

                  *      2740         *      2760         *      2780         *      2800
Hawaii : GCAGCAAGACATGAAGCAGGAAATTATTGCAGGAACATACAAGATCACTCCGGAGACAGCTGTGTATGAGTTGCATGTTG : 2800
Samut  : GCAGCAAGACATGAAGCAGGAAATTGTTGCAGGAACATACAAGATCACTCCGGAGACAGCTGTGTATGAGTTGCATGTTG : 2757
Chan   : GCAGCAAGACATGAAGCAGGAAATTGTTGCAGGAACATACAAGATCACTCCGGAGACAGCTGTGTATGAGTTGCATGTTG : 2757
Rayong : GCAGCAAGACATGAAGCAGGAAATTGTTGCAGGAACATACAAGATCACTCCGGAGACAGCTGTGTATGAGTTGCATGTTG : 2757
         GCAGCAAGACATGAAGCAGGAAATTgTTGCAGGAACATACAAGATCACTCCGGAGACAGCTGTGTATGAGTTGCATGTTG

                  *      2820         *      2840         *      2860         *      2880
Hawaii : ACACTACGTTAGCAGGCAATGCTCAGTCTAAAGTCTGTGCTTATGATGGTTTGGTGTCACTAATTGAACAGGTGAGGAAA : 2880
Samut  : ACACTACATTAGCAGGCAATGCTCAGTCTAAAGTCTGTGCTTATGATGGTTTGGTGTCACTGATTGAACAGGTGAGGAAA : 2837
Chan   : ACACTACATTAGCAGGCAATGCTCAGTCTAAAGTCTGTGCTTATGATGGTTTGGTGTCACTGATTGAACAGGTGAGGAAA : 2837
Rayong : ACACTACATTAGCAGGCAATGCTCAGTCTAAAGTCTGTGCTTATGATGGTTTGGTGTCACTGATTGAACAGGTGAGGAAA : 2837
         ACACTACaTTAGCAGGCAATGCTCAGTCTAAAGTCTGTGCTTATGATGGTTTGGTGTCACTgATTGAACAGGTGAGGAAA

                  *      2900         *      2920         *      2940         *      2960
Hawaii : TTGCGTGTTGCGGCCCATAGCGATAAGGTGGAAACTGACGTCCCAGTCCTTCCCACTAGACTGCACGAGTTATCGCAAGA : 2960
Samut  : CTGCGAGTTGCGGCCCATAGCGATAAGGTGGAAACTGATGTCCCAGTCCTTCCCACTAGACTGCACGAGTTGTCGCAAGA : 2917
Chan   : CTGCGAGTTGCGGCCCATAGCGATAAGGTGGAAACTGATGTCCCAGTCCTTCCCACTAGACTGCACGAGTTGTCGCAAGA : 2917
Rayong : CTGCGAGTTGCGGCCCATAGCGATAAGGTGGAAACTGATGTCCCAGTCCTTCCCACTAGACTGCACGAGTTGTCGCAAGA : 2917
         cTGCGaGTTGCGGCCCATAGCGATAAGGTGGAAACTGAtGTCCCAGTCCTTCCCACTAGACTGCACGAGTTgTCGCAAGA

                  *      2980         *      3000         *      3020         *      3040
Hawaii : AACTTTTCCCAATACACATGCCGGTGTAGGATTTCAATTTGCAACTGATTGGTTGGGCGATTTCGATCGGCCAGTGGAAG : 3040
Samut  : AACTTTTCCCAATACACATGCCGGTGTAGGATTTCAATTTGCAACTGATTGGTTGGGCGATTTCGATCGGCCAGTGGAAG : 2997
Chan   : AACTTTTCCCAATACACATGCCGGTGTAGGATTTCAATTTGCAACTGATTGGTTGGGCGATTTCGATCGGCCAGTGGAAG : 2997
Rayong : AACTTTTCCCAATACACATGCCAGTGTAGGATTTCAATTTGCAACTGATTGGTTGGGCGATTTCGATCGGCCAGTGGAAG : 2997
         AACTTTTCCCAATACACATGCCgGTGTAGGATTTCAATTTGCAACTGATTGGTTGGGCGATTTCGATCGGCCAGTGGAAG

                  *      3060         *      3080         *      3100         *      3120
Hawaii : CATTATCCTATTTAAATGAAACATTGGAAGCTCATTTTGTCTCGCGGAGTGCGAACGATGGAAGCATGTTCATCCCAGCC : 3120
Samut  : CATTATCCTATTTAAATAAAACATTGGAAGCCCATTTTGTCTCGCGGAGTGCGAACGATGGAAGCATGTTCATCCCAGCC : 3077
Chan   : CATTATCCTATTTAAATAAAACATTGGAAGCCCATTTTGTCTCGCGGAGTGCGAACGATGGAAGCATGTTCATCCCAGCC : 3077
Rayong : CATTATCCTATTTAAATAAAACTTTGGAAGCTCATTTTGTCTCGCAGAGTGCGAACGATGGAAGCATGTTCATCCCAGCC : 3077
         CATTATCCTATTTAAATaAAACaTTGGAAGC CATTTTGTCTCGCgGAGTGCGAACGATGGAAGCATGTTCATCCCAGCC

                  *      3140         *      3160         *      3180         *      3200
Hawaii : AGTGAGGTTGCTGATTTGTTGTGTCAGAGACATAACAATACGAATTTGAATGAGGAACTGGTGTATTTGACGTGGATGAC : 3200
Samut  : AGTGAGGTTGCTGATCTTTTGTGCCAGAGACATAACAATACGAATTTGAATGAGGAACTGGTGTATTTGACATGGATGAC : 3157
Chan   : AGTGAGGTTGCTGATCTTTTGTGCCAGAGACATAACAATACGAATTTGAATGAGGAACTGGTGTACTTGACATGGATGAC : 3157
Rayong : AGTGAGGTTTCTGATCTTTTGTGCCAGAGACATAACAATACGAATTTGAATGAGGAATTGGTGTATTTGACATGGATGAC : 3157
         AGTGAGGTTgCTGATcTtTTGTGcCAGAGACATAACAATACGAATTTGAATGAGGAAcTGGTGTAtTTGACaTGGATGAC

                  *      3220         *      3240         *      3260         *      3280
Hawaii : GCAGATCACAGATAAGGAGTTAGCCTCGAGTTTAGTATATTTCACAAATAACGGAATGGATAAGTCAATTTGGAAACAGA : 3280
Samut  : GCAGATTACAGATAAGGAGTTAGCCTCGAGTTTAGTATATTTCACAAATAACGGAATGGACAAGTCAATTTGGAAAAAGA : 3237
Chan   : GCAGATTACAGACAAGGAGTTAGCCTCGAGTTTAGTATATTTCACAAATAACGGAATGGACAAGTCAATTTGGAAAAAGA : 3237
Rayong : GCAGATCACAGATAAGGAGTTAGCCTCGAGTTTAGTATATTTCACAAATAACGGAATGGACAAGTCAATTTGGAAAAAGA : 3237
         GCAGAT ACAGAtAAGGAGTTAGCCTCGAGTTTAGTATATTTCACAAATAACGGAATGGAcAAGTCAATTTGGAAAaAGA
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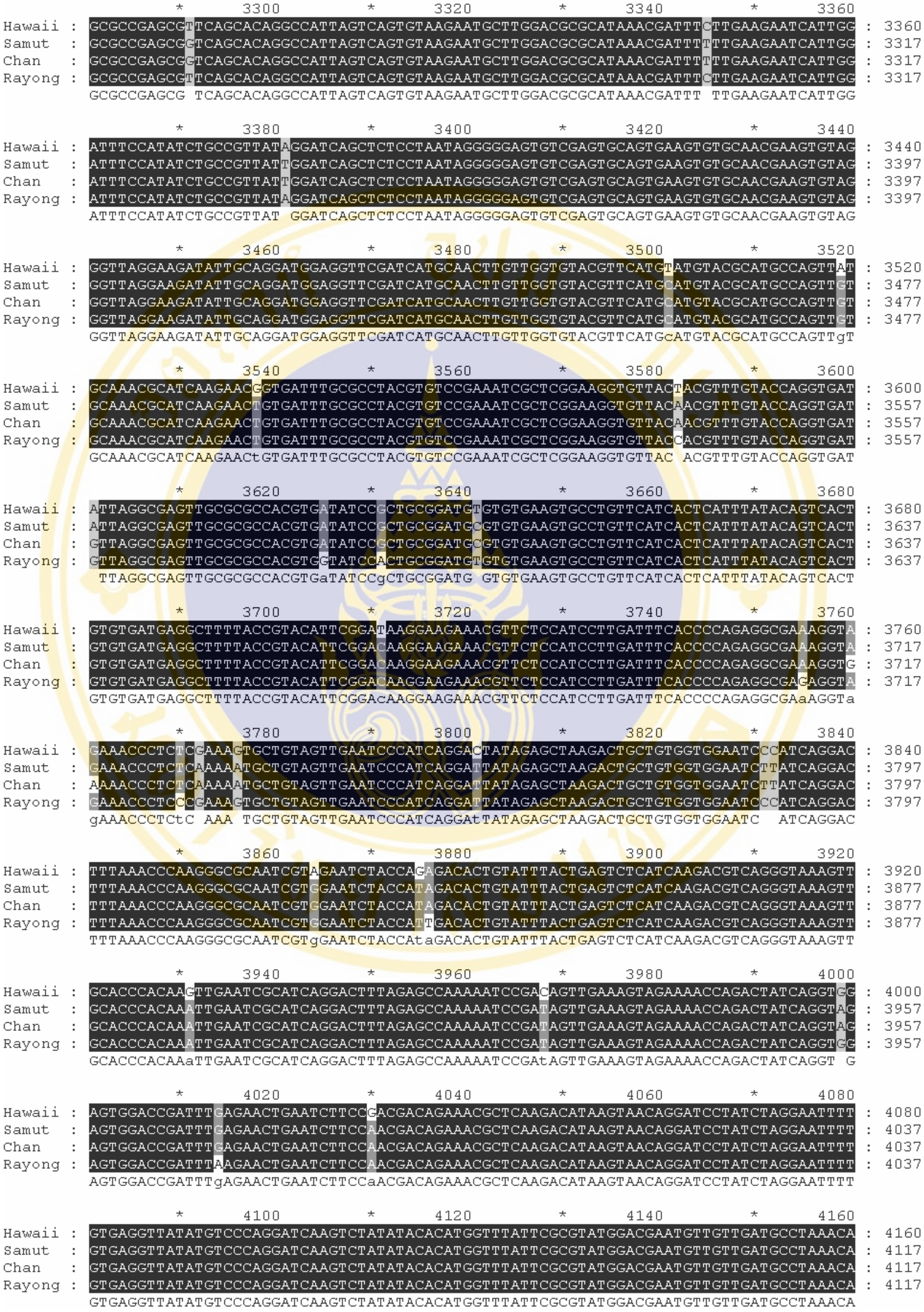
Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)



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                  *      3300         *      3320         *      3340         *      3360
Hawaii : GCGCCGAGCGTTCAGCACAGGCCATTAGTCAGTGTAAGAATGCTTGGACGCGCATAAACGATTTCTTGAAGAATCATTGG : 3360
Samut  : GCGCCGAGCGGTCAGCACAGGCCATTAGTCAGTGTAAGAATGCTTGGACGCGCATAAACGATTTTTTGAAGAATCATTGG : 3317
Chan   : GCGCCGAGCGGTCAGCACAGGCCATTAGTCAGTGTAAGAATGCTTGGACGCGCATAAACGATTTTTTGAAGAATCATTGG : 3317
Rayong : GCGCCGAGCGTTCAGCACAGGCCATTAGTCAGTGTAAGAATGCTTGGACGCGCATAAACGATTTCTTGAAGAATCATTGG : 3317
         GCGCCGAGCG TCAGCACAGGCCATTAGTCAGTGTAAGAATGCTTGGACGCGCATAAACGATTT TTGAAGAATCATTGG

                  *      3380         *      3400         *      3420         *      3440
Hawaii : ATTTCCATATCTGCCGTTATAGGATCAGCTCTCCTAATAGGGGGAGTGTCGAGTGCAGTGAAGTGTGCAACGAAGTGTAG : 3440
Samut  : ATTTCCATATCTGCCGTTATTGGATCAGCTCTCCTAATAGGGGGAGTGTCGAGTGCAGTGAAGTGTGCAACGAAGTGTAG : 3397
Chan   : ATTTCCATATCTGCCGTTATTGGATCAGCTCTCCTAATAGGGGGAGTGTCGAGTGCAGTGAAGTGTGCAACGAAGTGTAG : 3397
Rayong : ATTTCCATATCTGCCGTTATAGGATCAGCTCTCCTAATAGGGGGAGTGTCGAGTGCAGTGAAGTGTGCAACGAAGTGTAG : 3397
         ATTTCCATATCTGCCGTTAT GGATCAGCTCTCCTAATAGGGGGAGTGTCGAGTGCAGTGAAGTGTGCAACGAAGTGTAG

                  *      3460         *      3480         *      3500         *      3520
Hawaii : GGTTAGGAAGATATTGCAGGATGGAGGTTCGATCATGCAACTTGTTGGTGTACGTTCATGTATGTACGCATGCCAGTTAT : 3520
Samut  : GGTTAGGAAGATATTGCAGGATGGAGGTTCGATCATGCAACTTGTTGGTGTACGTTCATGCATGTACGCATGCCAGTTGT : 3477
Chan   : GGTTAGGAAGATATTGCAGGATGGAGGTTCGATCATGCAACTTGTTGGTGTACGTTCATGCATGTACGCATGCCAGTTGT : 3477
Rayong : GGTTAGGAAGATATTGCAGGATGGAGGTTCGATCATGCAACTTGTTGGTGTACGTTCATGCATGTACGCATGCCAGTTGT : 3477
         GGTTAGGAAGATATTGCAGGATGGAGGTTCGATCATGCAACTTGTTGGTGTACGTTCATGcATGTACGCATGCCAGTTgT

                  *      3540         *      3560         *      3580         *      3600
Hawaii : GCAAACGCATCAAGAACGGTGATTTGCGCCTACGTGTCCGAAATCGCTCGGAAGGTGTTACTACGTTTGTACCAGGTGAT : 3600
Samut  : GCAAACGCATCAAGAACTGTGATTTGCGCCTACGTGTCCGAAATCGCTCGGAAGGTGTTACAACGTTTGTACCAGGTGAT : 3557
Chan   : GCAAACGCATCAAGAACTGTGATTTGCGCCTACGTGTCCGAAATCGCTCGGAAGGTGTTACAACGTTTGTACCAGGTGAT : 3557
Rayong : GCAAACGCATCAAGAACTGTGATTTGCGCCTACGTGTCCGAAATCGCTCGGAAGGTGTTACCACGTTTGTACCAGGTGAT : 3557
         GCAAACGCATCAAGAACtGTGATTTGCGCCTACGTGTCCGAAATCGCTCGGAAGGTGTTAC ACGTTTGTACCAGGTGAT

                  *      3620         *      3640         *      3660         *      3680
Hawaii : ATTAGGCGAGTTGCGCGCCACGTGATATCCGCTGCGGATGTGTGTGAAGTGCCTGTTCATCACTCATTTATACAGTCACT : 3680
Samut  : ATTAGGCGAGTTGCGCGCCACGTGATATCCGCTGCGGATGCGTGTGAAGTGCCTGTTCATCACTCATTTATACAGTCACT : 3637
Chan   : GTTAGGCGAGTTGCGCGCCACGTGATATCCGCTGCGGATGCGTGTGAAGTGCCTGTTCATCACTCATTTATACAGTCACT : 3637
Rayong : GTTAGGCGAGTTGCGCGCCACGTGGTATCCACTGCGGATGTGTGTGAAGTGCCTGTTCATCACTCATTTATACAGTCACT : 3637
          TTAGGCGAGTTGCGCGCCACGTGaTATCCgCTGCGGATG GTGTGAAGTGCCTGTTCATCACTCATTTATACAGTCACT

                  *      3700         *      3720         *      3740         *      3760
Hawaii : GTGTGATGAGGCTTTTACCGTACATTCGGATAAGGAAGAAACGTTCTCCATCCTTGATTTCACCCCAGAGGCGAAAGGTA : 3760
Samut  : GTGTGATGAGGCTTTTACCGTACATTCGGACAAGGAAGAAACGTTCTCCATCCTTGATTTCACCCCAGAGGCGAAAGGTA : 3717
Chan   : GTGTGATGAGGCTTTTACCGTACATTCGGACAAGGAAGAAACGTTCTCCATCCTTGATTTCACCCCAGAGGCGAAAGGTG : 3717
Rayong : GTGTGATGAGGCTTTTACCGTACATTCGGACAAGGAAGAAACGTTCTCCATCCTTGATTTCACCCCAGAGGCGAGAGGTA : 3717
         GTGTGATGAGGCTTTTACCGTACATTCGGAcAAGGAAGAAACGTTCTCCATCCTTGATTTCACCCCAGAGGCGAaAGGTa

                  *      3780         *      3800         *      3820         *      3840
Hawaii : GAAACCCTCTCGAAAGTGCTGTAGTTGAATCCCATCAGGACTATAGAGCTAAGACTGCTGTGGTGGAATCCCATCAGGAC : 3840
Samut  : GAAACCCTCTCAAAAATGCTGTAGTTGAATCCCATCAGGATTATAGAGCTAAGACTGCTGTGGTGGAATCTTATCAGGAC : 3797
Chan   : AAAACCCTCTCAAAAATGCTGTAGTTGAATCCCATCAGGATTATAGAGCTAAGACTGCTGTGGTGGAATCTTATCAGGAC : 3797
Rayong : GAAACCCTCCCGAAAGTGCTGTAGTTGAATCCCATCAGGATTATAGAGCTAAGACTGCTGTGGTGGAATCCCATCAGGAC : 3797
         gAAACCCTCtC AAA TGCTGTAGTTGAATCCCATCAGGAtTATAGAGCTAAGACTGCTGTGGTGGAATC  ATCAGGAC

                  *      3860         *      3880         *      3900         *      3920
Hawaii : TTTAAACCCAAGGGCGCAATCGTAGAATCTACCAGAGACACTGTATTTACTGAGTCTCATCAAGACGTCAGGGTAAAGTT : 3920
Samut  : TTTAAACCCAAGGGCGCAATCGTGGAATCTACCATAGACACTGTATTTACTGAGTCTCATCAAGACGTCAGGGTAAAGTT : 3877
Chan   : TTTAAACCCAAGGGCGCAATCGTGGAATCTACCATAGACACTGTATTTACTGAGTCTCATCAAGACGTCAGGGTAAAGTT : 3877
Rayong : TTTAAACCCAAGGGCGCAATCGTGGAATCTACCATTGACACTGTATTTACTGAGTCTCATCAAGACGTCAGGGTAAAGTT : 3877
         TTTAAACCCAAGGGCGCAATCGTgGAATCTACCAtaGACACTGTATTTACTGAGTCTCATCAAGACGTCAGGGTAAAGTT

                  *      3940         *      3960         *      3980         *      4000
Hawaii : GCACCCACAAGTTGAATCGCATCAGGACTTTAGAGCCAAAAATCCGACAGTTGAAAGTAGAAAACCAGACTATCAGGTGG : 4000
Samut  : GCACCCACAAATTGAATCGCATCAGGACTTTAGAGCCAAAAATCCGATAGTTGAAAGTAGAAAACCAGACTATCAGGTAG : 3957
Chan   : GCACCCACAAATTGAATCGCATCAGGACTTTAGAGCCAAAAATCCGATAGTTGAAAGTAGAAAACCAGACTATCAGGTAG : 3957
Rayong : GCACCCACAAATTGAATCGCATCAGGACTTTAGAGCCAAAAATCCGATAGTTGAAAGTAGAAAACCAGACTATCAGGTGG : 3957
         GCACCCACAAaTTGAATCGCATCAGGACTTTAGAGCCAAAAATCCGAtAGTTGAAAGTAGAAAACCAGACTATCAGGT G

                  *      4020         *      4040         *      4060         *      4080
Hawaii : AGTGGACCGATTTGAGAACTGAATCTTCCGACGACAGAAACGCTCAAGACATAAGTAACAGGATCCTATCTAGGAATTTT : 4080
Samut  : AGTGGACCGATTTGAGAACTGAATCTTCCAACGACAGAAACGCTCAAGACATAAGTAACAGGATCCTATCTAGGAATTTT : 4037
Chan   : AGTGGACCGATTTGAGAACTGAATCTTCCAACGACAGAAACGCTCAAGACATAAGTAACAGGATCCTATCTAGGAATTTT : 4037
Rayong : AGTGGACCGATTTAAGAACTGAATCTTCCAACGACAGAAACGCTCAAGACATAAGTAACAGGATCCTATCTAGGAATTTT : 4037
         AGTGGACCGATTTgAGAACTGAATCTTCCaACGACAGAAACGCTCAAGACATAAGTAACAGGATCCTATCTAGGAATTTT

                  *      4100         *      4120         *      4140         *      4160
Hawaii : GTGAGGTTATATGTCCCAGGATCAAGTCTATATACACATGGTTTATTCGCGTATGGACGAATGTTGTTGATGCCTAAACA : 4160
Samut  : GTGAGGTTATATGTCCCAGGATCAAGTCTATATACACATGGTTTATTCGCGTATGGACGAATGTTGTTGATGCCTAAACA : 4117
Chan   : GTGAGGTTATATGTCCCAGGATCAAGTCTATATACACATGGTTTATTCGCGTATGGACGAATGTTGTTGATGCCTAAACA : 4117
Rayong : GTGAGGTTATATGTCCCAGGATCAAGTCTATATACACATGGTTTATTCGCGTATGGACGAATGTTGTTGATGCCTAAACA : 4117
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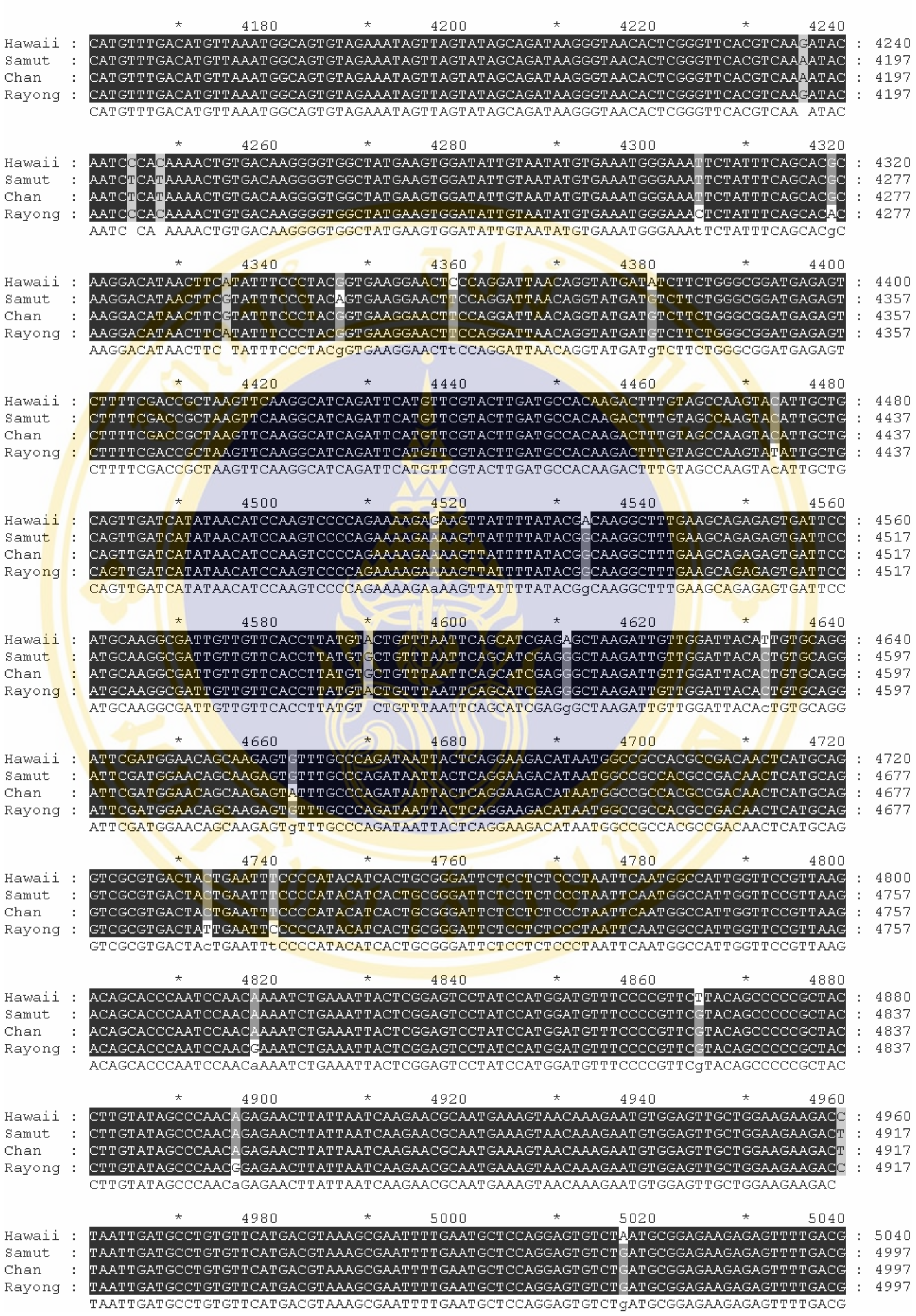
Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)



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                  *      4180         *      4200         *      4220         *      4240
Hawaii : CATGTTTGACATGTTAAATGGCAGTGTAGAAATAGTTAGTATAGCAGATAAGGGTAACACTCGGGTTCACGTCAAGATAC : 4240
Samut  : CATGTTTGACATGTTAAATGGCAGTGTAGAAATAGTTAGTATAGCAGATAAGGGTAACACTCGGGTTCACGTCAAAATAC : 4197
Chan   : CATGTTTGACATGTTAAATGGCAGTGTAGAAATAGTTAGTATAGCAGATAAGGGTAACACTCGGGTTCACGTCAAAATAC : 4197
Rayong : CATGTTTGACATGTTAAATGGCAGTGTAGAAATAGTTAGTATAGCAGATAAGGGTAACACTCGGGTTCACGTCAAGATAC : 4197
         CATGTTTGACATGTTAAATGGCAGTGTAGAAATAGTTAGTATAGCAGATAAGGGTAACACTCGGGTTCACGTCAA ATAC

                  *      4260         *      4280         *      4300         *      4320
Hawaii : AATCCCACAAAACTGTGACAAGGGGTGGCTATGAAGTGGATATTGTAATATGTGAAATGGGAAATTCTATTTCAGCACGC : 4320
Samut  : AATCTCATAAAACTGTGACAAGGGGTGGCTATGAAGTGGATATTGTAATATGTGAAATGGGAAATTCTATTTCAGCACGC : 4277
Chan   : AATCTCATAAAACTGTGACAAGGGGTGGCTATGAAGTGGATATTGTAATATGTGAAATGGGAAATTCTATTTCAGCACGC : 4277
Rayong : AATCCCACAAAACTGTGACAAGGGGTGGCTATGAAGTGGATATTGTAATATGTGAAATGGGAAACTCTATTTCAGCACAC : 4277
         AATC CA AAAACTGTGACAAGGGGTGGCTATGAAGTGGATATTGTAATATGTGAAATGGGAAAtTCTATTTCAGCACgC

                  *      4340         *      4360         *      4380         *      4400
Hawaii : AAGGACATAACTTCATATTTCCCTACGGTGAAGGAACTCCCAGGATTAACAGGTATGATATCTTCTGGGCGGATGAGAGT : 4400
Samut  : AAGGACATAACTTCGTATTTCCCTACAGTGAAGGAACTTCCAGGATTAACAGGTATGATGTCTTCTGGGCGGATGAGAGT : 4357
Chan   : AAGGACATAACTTCGTATTTCCCTACGGTGAAGGAACTTCCAGGATTAACAGGTATGATGTCTTCTGGGCGGATGAGAGT : 4357
Rayong : AAGGACATAACTTCATATTTCCCTACGGTGAAGGAACTTCCAGGATTAACAGGTATGATGTCTTCTGGGCGGATGAGAGT : 4357
         AAGGACATAACTTC TATTTCCCTACgGTGAAGGAACTtCCAGGATTAACAGGTATGATgTCTTCTGGGCGGATGAGAGT

                  *      4420         *      4440         *      4460         *      4480
Hawaii : CTTTTCGACCGCTAAGTTCAAGGCATCAGATTCATGTTCGTACTTGATGCCACAAGACTTTGTAGCCAAGTACATTGCTG : 4480
Samut  : CTTTTCGACCGCTAAGTTCAAGGCATCAGATTCATGTTCGTACTTGATGCCACAAGACTTTGTAGCCAAGTACATTGCTG : 4437
Chan   : CTTTTCGACCGCTAAGTTCAAGGCATCAGATTCATGTTCGTACTTGATGCCACAAGACTTTGTAGCCAAGTACATTGCTG : 4437
Rayong : CTTTTCGACCGCTAAGTTCAAGGCATCAGATTCATGTTCGTACTTGATGCCACAAGACTTTGTAGCCAAGTATATTGCTG : 4437
         CTTTTCGACCGCTAAGTTCAAGGCATCAGATTCATGTTCGTACTTGATGCCACAAGACTTTGTAGCCAAGTAcATTGCTG

                  *      4500         *      4520         *      4540         *      4560
Hawaii : CAGTTGATCATATAACATCCAAGTCCCCAGAAAAGAGAAGTTATTTTATACGACAAGGCTTTGAAGCAGAGAGTGATTCC : 4560
Samut  : CAGTTGATCATATAACATCCAAGTCCCCAGAAAAGAAAAGTTATTTTATACGGCAAGGCTTTGAAGCAGAGAGTGATTCC : 4517
Chan   : CAGTTGATCATATAACATCCAAGTCCCCAGAAAAGAAAAGTTATTTTATACGGCAAGGCTTTGAAGCAGAGAGTGATTCC : 4517
Rayong : CAGTTGATCATATAACATCCAAGTCCCCAGAAAAGAAAAGTTATTTTATACGGCAAGGCTTTGAAGCAGAGAGTGATTCC : 4517
         CAGTTGATCATATAACATCCAAGTCCCCAGAAAAGAaAAGTTATTTTATACGgCAAGGCTTTGAAGCAGAGAGTGATTCC

                  *      4580         *      4600         *      4620         *      4640
Hawaii : ATGCAAGGCGATTGTTGTTCACCTTATGTACTGTTTAATTCAGCATCGAGAGCTAAGATTGTTGGATTACATTGTGCAGG : 4640
Samut  : ATGCAAGGCGATTGTTGTTCACCTTATGTGCTGTTTAATTCAGCATCGAGGGCTAAGATTGTTGGATTACACTGTGCAGG : 4597
Chan   : ATGCAAGGCGATTGTTGTTCACCTTATGTGCTGTTTAATTCAGCATCGAGGGCTAAGATTGTTGGATTACACTGTGCAGG : 4597
Rayong : ATGCAAGGCGATTGTTGTTCACCTTATGTACTGTTTAATTCAGCATCGAGGGCTAAGATTGTTGGATTACACTGTGCAGG : 4597
         ATGCAAGGCGATTGTTGTTCACCTTATGT CTGTTTAATTCAGCATCGAGgGCTAAGATTGTTGGATTACAcTGTGCAGG

                  *      4660         *      4680         *      4700         *      4720
Hawaii : ATTCGATGGAACAGCAAGAGTGTTTGCCCAGATAATTACTCAGGAAGACATAATGGCCGCCACGCCGACAACTCATGCAG : 4720
Samut  : ATTCGATGGAACAGCAAGAGTGTTTGCCCAGATAATTACTCAGGAAGACATAATGGCCGCCACGCCGACAACTCATGCAG : 4677
Chan   : ATTCGATGGAACAGCAAGAGTATTTGCCCAGATAATTACTCAGGAAGACATAATGGCCGCCACGCCGACAACTCATGCAG : 4677
Rayong : ATTCGATGGAACAGCAAGAGTGTTTGCCCAGATAATTACTCAGGAAGACATAATGGCCGCCACGCCGACAACTCATGCAG : 4677
         ATTCGATGGAACAGCAAGAGTgTTTGCCCAGATAATTACTCAGGAAGACATAATGGCCGCCACGCCGACAACTCATGCAG

                  *      4740         *      4760         *      4780         *      4800
Hawaii : GTCGCGTGACTACTGAATTTCCCCATACATCACTGCGGGATTCTCCTCTCCCTAATTCAATGGCCATTGGTTCCGTTAAG : 4800
Samut  : GTCGCGTGACTACTGAATTTCCCCATACATCACTGCGGGATTCTCCTCTCCCTAATTCAATGGCCATTGGTTCCGTTAAG : 4757
Chan   : GTCGCGTGACTACTGAATTTCCCCATACATCACTGCGGGATTCTCCTCTCCCTAATTCAATGGCCATTGGTTCCGTTAAG : 4757
Rayong : GTCGCGTGACTATTGAATTCCCCCATACATCACTGCGGGATTCTCCTCTCCCTAATTCAATGGCCATTGGTTCCGTTAAG : 4757
         GTCGCGTGACTAcTGAATTtCCCCATACATCACTGCGGGATTCTCCTCTCCCTAATTCAATGGCCATTGGTTCCGTTAAG

                  *      4820         *      4840         *      4860         *      4880
Hawaii : ACAGCACCCAATCCAACAAAATCTGAAATTACTCGGAGTCCTATCCATGGATGTTTCCCCGTTCTTACAGCCCCCGCTAC : 4880
Samut  : ACAGCACCCAATCCAACAAAATCTGAAATTACTCGGAGTCCTATCCATGGATGTTTCCCCGTTCGTACAGCCCCCGCTAC : 4837
Chan   : ACAGCACCCAATCCAACAAAATCTGAAATTACTCGGAGTCCTATCCATGGATGTTTCCCCGTTCGTACAGCCCCCGCTAC : 4837
Rayong : ACAGCACCCAATCCAACGAAATCTGAAATTACTCGGAGTCCTATCCATGGATGTTTCCCCGTTCGTACAGCCCCCGCTAC : 4837
         ACAGCACCCAATCCAACaAAATCTGAAATTACTCGGAGTCCTATCCATGGATGTTTCCCCGTTCgTACAGCCCCCGCTAC

                  *      4900         *      4920         *      4940         *      4960
Hawaii : CTTGTATAGCCCAACAGAGAACTTATTAATCAAGAACGCAATGAAAGTAACAAAGAATGTGGAGTTGCTGGAAGAAGACC : 4960
Samut  : CTTGTATAGCCCAACAGAGAACTTATTAATCAAGAACGCAATGAAAGTAACAAAGAATGTGGAGTTGCTGGAAGAAGACT : 4917
Chan   : CTTGTATAGCCCAACAGAGAACTTATTAATCAAGAACGCAATGAAAGTAACAAAGAATGTGGAGTTGCTGGAAGAAGACT : 4917
Rayong : CTTGTATAGCCCAACGGAGAACTTATTAATCAAGAACGCAATGAAAGTAACAAAGAATGTGGAGTTGCTGGAAGAAGACC : 4917
         CTTGTATAGCCCAACaGAGAACTTATTAATCAAGAACGCAATGAAAGTAACAAAGAATGTGGAGTTGCTGGAAGAAGAC

                  *      4980         *      5000         *      5020         *      5040
Hawaii : TAATTGATGCCTGTGTTCATGACGTAAAGCGAATTTTGAATGCTCCAGGAGTGTCTAATGCGGAGAAGAGAGTTTTGACG : 5040
Samut  : TAATTGATGCCTGTGTTCATGACGTAAAGCGAATTTTGAATGCTCCAGGAGTGTCTGATGCGGAGAAGAGAGTTTTGACG : 4997
Chan   : TAATTGATGCCTGTGTTCATGACGTAAAGCGAATTTTGAATGCTCCAGGAGTGTCTGATGCGGAGAAGAGAGTTTTGACG : 4997
Rayong : TAATTGATGCCTGTGTTCATGACGTAAAGCGAATTTTGAATGCTCCAGGAGTGTCTGATGCGGAGAAGAGAGTTTTGACG : 4997
         TAATTGATGCCTGTGTTCATGACGTAAAGCGAATTTTGAATGCTCCAGGAGTGTCTgATGCGGAGAAGAGAGTTTTGACG
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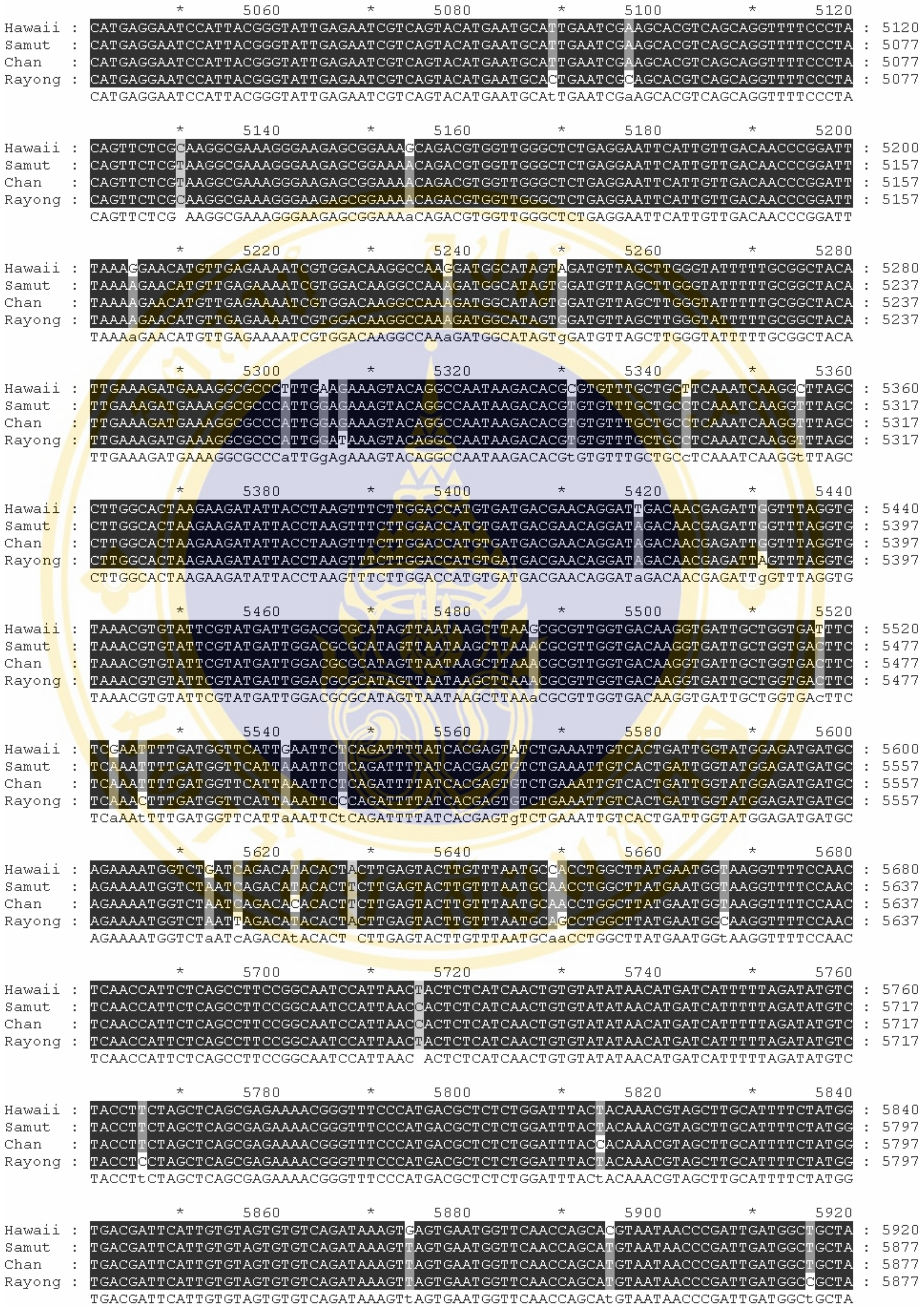
Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)



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                  *      5060          *      5080          *      5100          *      5120
Hawaii : CATGAGGAATCCATTACGGGTATTGAGAATCGTCAGTACATGAATGCATTGAATCGAAGCACGTCAGCAGGTTTTCCCTA : 5120
Samut  : CATGAGGAATCCATTACGGGTATTGAGAATCGTCAGTACATGAATGCATTGAATCGAAGCACGTCAGCAGGTTTTCCCTA : 5077
Chan   : CATGAGGAATCCATTACGGGTATTGAGAATCGTCAGTACATGAATGCATTGAATCGAAGCACGTCAGCAGGTTTTCCCTA : 5077
Rayong : CATGAGGAATCCATTACGGGTATTGAGAATCGTCAGTACATGAATGCACTGAATCGCAGCACGTCAGCAGGTTTTCCCTA : 5077
         CATGAGGAATCCATTACGGGTATTGAGAATCGTCAGTACATGAATGCAtTGAATCGaAGCACGTCAGCAGGTTTTCCCTA

                  *      5140          *      5160          *      5180          *      5200
Hawaii : CAGTTCTCGCAAGGCGAAAGGGAAGAGCGGAAAGCAGACGTGGTTGGGCTCTGAGGAATTCATTGTTGACAACCCGGATT : 5200
Samut  : CAGTTCTCGTAAGGCGAAAGGGAAGAGCGGAAAACAGACGTGGTTGGGCTCTGAGGAATTCATTGTTGACAACCCGGATT : 5157
Chan   : CAGTTCTCGTAAGGCGAAAGGGAAGAGCGGAAAACAGACGTGGTTGGGCTCTGAGGAATTCATTGTTGACAACCCGGATT : 5157
Rayong : CAGTTCTCGCAAGGCGAAAGGGAAGAGCGGAAAACAGACGTGGTTGGGCTCTGAGGAATTCATTGTTGACAACCCGGATT : 5157
         CAGTTCTCG AAGGCGAAAGGGAAGAGCGGAAAaCAGACGTGGTTGGGCTCTGAGGAATTCATTGTTGACAACCCGGATT

                  *      5220          *      5240          *      5260          *      5280
Hawaii : TAAAGGAACATGTTGAGAAAATCGTGGACAAGGCCAAGGATGGCATAGTAGATGTTAGCTTGGGTATTTTTGCGGCTACA : 5280
Samut  : TAAAAGAACATGTTGAGAAAATCGTGGACAAGGCCAAAGATGGCATAGTGGATGTTAGCTTGGGTATTTTTGCGGCTACA : 5237
Chan   : TAAAAGAACATGTTGAGAAAATCGTGGACAAGGCCAAAGATGGCATAGTGGATGTTAGCTTGGGTATTTTTGCGGCTACA : 5237
Rayong : TAAAAGAACATGTTGAGAAAATCGTGGACAAGGCCAAAGATGGCATAGTGGATGTTAGCTTGGGTATTTTTGCGGCTACA : 5237
         TAAAaGAACATGTTGAGAAAATCGTGGACAAGGCCAAaGATGGCATAGTgGATGTTAGCTTGGGTATTTTTGCGGCTACA

                  *      5300          *      5320          *      5340          *      5360
Hawaii : TTGAAAGATGAAAGGCGCCCTTTGAAGAAAGTACAGGCCAATAAGACACGCGTGTTTGCTGCTTCAAATCAAGGCTTAGC : 5360
Samut  : TTGAAAGATGAAAGGCGCCCATTGGAGAAAGTACAGGCCAATAAGACACGTGTGTTTGCTGCCTCAAATCAAGGTTTAGC : 5317
Chan   : TTGAAAGATGAAAGGCGCCCATTGGAGAAAGTACAGGCCAATAAGACACGTGTGTTTGCTGCCTCAAATCAAGGTTTAGC : 5317
Rayong : TTGAAAGATGAAAGGCGCCCATTGGATAAAGTACAGGCCAATAAGACACGTGTGTTTGCTGCCTCAAATCAAGGTTTAGC : 5317
         TTGAAAGATGAAAGGCGCCCaTTGgAgAAAGTACAGGCCAATAAGACACGtGTGTTTGCTGCcTCAAATCAAGGtTTAGC

                  *      5380          *      5400          *      5420          *      5440
Hawaii : CTTGGCACTAAGAAGATATTACCTAAGTTTCTTGGACCATGTGATGACGAACAGGATTGACAACGAGATTGGTTTAGGTG : 5440
Samut  : CTTGGCACTAAGAAGATATTACCTAAGTTTCTTGGACCATGTGATGACGAACAGGATAGACAACGAGATTGGTTTAGGTG : 5397
Chan   : CTTGGCACTAAGAAGATATTACCTAAGTTTCTTGGACCATGTGATGACGAACAGGATAGACAACGAGATTGGTTTAGGTG : 5397
Rayong : CTTGGCACTAAGAAGATATTACCTAAGTTTCTTGGACCATGTGATGACGAACAGGATAGACAACGAGATTAGTTTAGGTG : 5397
         CTTGGCACTAAGAAGATATTACCTAAGTTTCTTGGACCATGTGATGACGAACAGGATaGACAACGAGATTgGTTTAGGTG

                  *      5460          *      5480          *      5500          *      5520
Hawaii : TAAACGTGTATTCGTATGATTGGACGCGCATAGTTAATAAGCTTAAGCGCGTTGGTGACAAGGTGATTGCTGGTGATTTC : 5520
Samut  : TAAACGTGTATTCGTATGATTGGACGCGCATAGTTAATAAGCTTAAACGCGTTGGTGACAAGGTGATTGCTGGTGACTTC : 5477
Chan   : TAAACGTGTATTCGTATGATTGGACGCGCATAGTTAATAAGCTTAAACGCGTTGGTGACAAGGTGATTGCTGGTGACTTC : 5477
Rayong : TAAACGTGTATTCGTATGATTGGACGCGCATAGTTAATAAGCTTAAACGCGTTGGTGACAAGGTGATTGCTGGTGACTTC : 5477
         TAAACGTGTATTCGTATGATTGGACGCGCATAGTTAATAAGCTTAAaCGCGTTGGTGACAAGGTGATTGCTGGTGAcTTC

                  *      5540          *      5560          *      5580          *      5600
Hawaii : TCGAATTTTGATGGTTCATTGAATTCTCAGATTTTATCACGAGTATCTGAAATTGTCACTGATTGGTATGGAGATGATGC : 5600
Samut  : TCAAATTTTGATGGTTCATTAAATTCTCAGATTTTATCACGAGTGTCTGAAATTGTCACTGATTGGTATGGAGATGATGC : 5557
Chan   : TCAAATTTTGATGGTTCATTAAATTCTCAGATTTTATCACGAGTGTCTGAAATTGTCACTGATTGGTATGGAGATGATGC : 5557
Rayong : TCAAACTTTGATGGTTCATTAAATTCCCAGATTTTATCACGAGTGTCTGAAATTGTCACTGATTGGTATGGAGATGATGC : 5557
         TCaAAtTTTGATGGTTCATTaAATTCtCAGATTTTATCACGAGTgTCTGAAATTGTCACTGATTGGTATGGAGATGATGC

                  *      5620          *      5640          *      5660          *      5680
Hawaii : AGAAAATGGTCTGATCAGACATACACTACTTGAGTACTTGTTTAATGCCACCTGGCTTATGAATGGTAAGGTTTTCCAAC : 5680
Samut  : AGAAAATGGTCTAATCAGACATACACTTCTTGAGTACTTGTTTAATGCAACCTGGCTTATGAATGGTAAGGTTTTCCAAC : 5637
Chan   : AGAAAATGGTCTAATCAGACACACACTTCTTGAGTACTTGTTTAATGCAACCTGGCTTATGAATGGTAAGGTTTTCCAAC : 5637
Rayong : AGAAAATGGTCTAATTAGACATACACTACTTGAGTACTTGTTTAATGCAGCCTGGCTTATGAATGGCAAGGTTTTCCAAC : 5637
         AGAAAATGGTCTaATcAGACAtACACT CTTGAGTACTTGTTTAATGCaaCCTGGCTTATGAATGGtAAGGTTTTCCAAC

                  *      5700          *      5720          *      5740          *      5760
Hawaii : TCAACCATTCTCAGCCTTCCGGCAATCCATTAACTACTCTCATCAACTGTGTATATAACATGATCATTTTTAGATATGTC : 5760
Samut  : TCAACCATTCTCAGCCTTCCGGCAATCCATTAACCACTCTCATCAACTGTGTATATAACATGATCATTTTTAGATATGTC : 5717
Chan   : TCAACCATTCTCAGCCTTCCGGCAATCCATTAACCACTCTCATCAACTGTGTATATAACATGATCATTTTTAGATATGTC : 5717
Rayong : TCAACCATTCTCAGCCTTCCGGCAATCCATTAACTACTCTCATCAACTGTGTATATAACATGATCATTTTTAGATATGTC : 5717
         TCAACCATTCTCAGCCTTCCGGCAATCCATTAAC ACTCTCATCAACTGTGTATATAACATGATCATTTTTAGATATGTC

                  *      5780          *      5800          *      5820          *      5840
Hawaii : TACCTTCTAGCTCAGCGAGAAAACGGGTTTCCCATGACGCTCTCTGGATTTACTACAAACGTAGCTTGCATTTTCTATGG : 5840
Samut  : TACCTTCTAGCTCAGCGAGAAAACGGGTTTCCCATGACGCTCTCTGGATTTACTACAAACGTAGCTTGCATTTTCTATGG : 5797
Chan   : TACCTTCTAGCTCAGCGAGAAAACGGGTTTCCCATGACGCTCTCTGGATTTACCACAAACGTAGCTTGCATTTTCTATGG : 5797
Rayong : TACCTCCTAGCTCAGCGAGAAAACGGGTTTCCCATGACGCTCTCTGGATTTACTACAAACGTAGCTTGCATTTTCTATGG : 5797
         TACCTtCTAGCTCAGCGAGAAAACGGGTTTCCCATGACGCTCTCTGGATTTACtACAAACGTAGCTTGCATTTTCTATGG

                  *      5860          *      5880          *      5900          *      5920
Hawaii : TGACGATTCATTGTGTAGTGTGTCAGATAAAGTGAGTGAATGGTTCAACCAGCACGTAATAACCCGATTGATGGCTGCTA : 5920
Samut  : TGACGATTCATTGTGTAGTGTGTCAGATAAAGTTAGTGAATGGTTCAACCAGCATGTAATAACCCGATTGATGGCTGCTA : 5877
Chan   : TGACGATTCATTGTGTAGTGTGTCAGATAAAGTTAGTGAATGGTTCAACCAGCATGTAATAACCCGATTGATGGCTGCTA : 5877
Rayong : TGACGATTCATTGTGTAGTGTGTCAGATAAAGTTAGTGAATGGTTCAACCAGCATGTAATAACCCGATTGATGGCCGCTA : 5877
         TGACGATTCATTGTGTAGTGTGTCAGATAAAGTtAGTGAATGGTTCAACCAGCAtGTAATAACCCGATTGATGGCtGCTA
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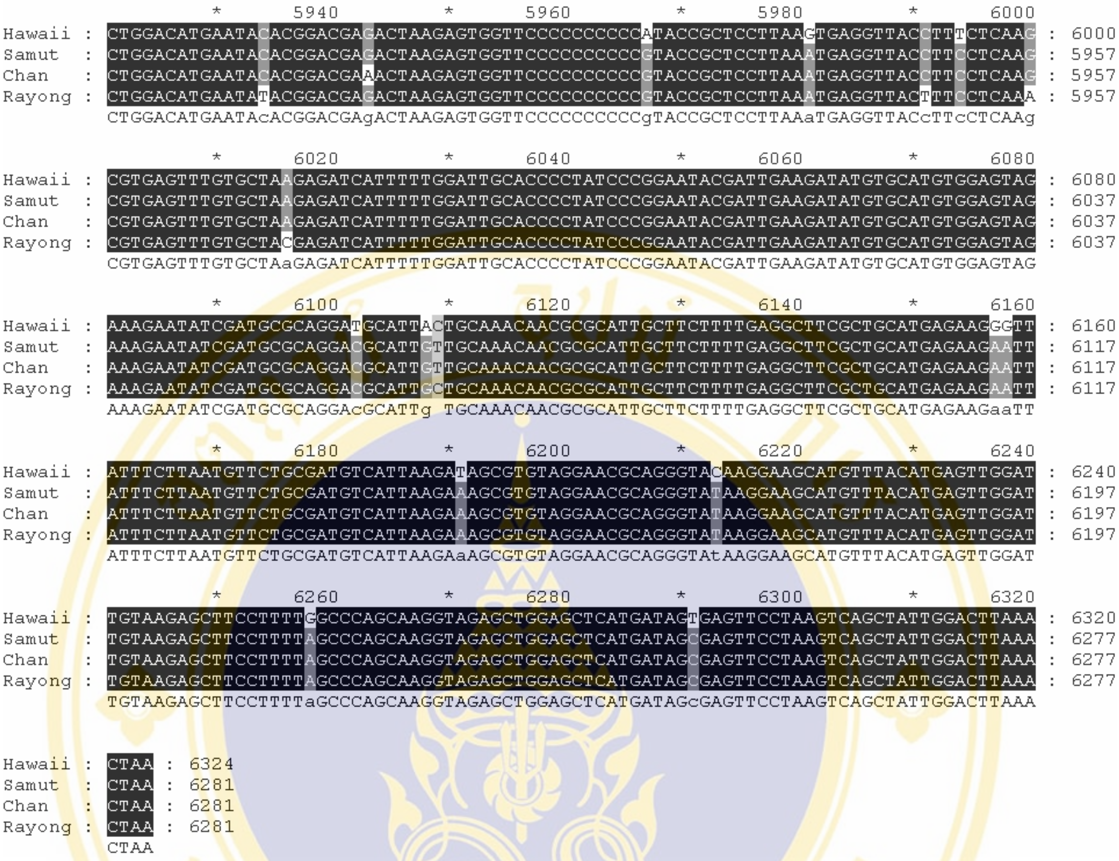
Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)



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                      *        5940           *        5960           *        5980           *        6000
Hawaii : CTGGACATGAATACACGGACGAGACTAAGAGTGGTTCCCCCCCCCCATACCGCTCCTTAAGTGAGGTTACCTTTCTCAAG : 6000
Samut  : CTGGACATGAATACACGGACGAGACTAAGAGTGGTTCCCCCCCCCCGTACCGCTCCTTAAATGAGGTTACCTTCCTCAAG : 5957
Chan   : CTGGACATGAATACACGGACGAAACTAAGAGTGGTTCCCCCCCCCCGTACCGCTCCTTAAATGAGGTTACCTTCCTCAAG : 5957
Rayong : CTGGACATGAATATACGGACGAGACTAAGAGTGGTTCCCCCCCCCCGTACCGCTCCTTAAATGAGGTTACTTTCCTCAAA : 5957
         CTGGACATGAATAcACGGACGAgACTAAGAGTGGTTCCCCCCCCCCgTACCGCTCCTTAAaTGAGGTTACcTTcCTCAAg

                      *        6020           *        6040           *        6060           *        6080
Hawaii : CGTGAGTTTGTGCTAAGAGATCATTTTTGGATTGCACCCCTATCCCGGAATACGATTGAAGATATGTGCATGTGGAGTAG : 6080
Samut  : CGTGAGTTTGTGCTAAGAGATCATTTTTGGATTGCACCCCTATCCCGGAATACGATTGAAGATATGTGCATGTGGAGTAG : 6037
Chan   : CGTGAGTTTGTGCTAAGAGATCATTTTTGGATTGCACCCCTATCCCGGAATACGATTGAAGATATGTGCATGTGGAGTAG : 6037
Rayong : CGTGAGTTTGTGCTACGAGATCATTTTTGGATTGCACCCCTATCCCGGAATACGATTGAAGATATGTGCATGTGGAGTAG : 6037
         CGTGAGTTTGTGCTAaGAGATCATTTTTGGATTGCACCCCTATCCCGGAATACGATTGAAGATATGTGCATGTGGAGTAG

                      *        6100           *        6120           *        6140           *        6160
Hawaii : AAAGAATATCGATGCGCAGGATGCATTACTGCAAACAACGCGCATTGCTTCTTTTGAGGCTTCGCTGCATGAGAAGGGTT : 6160
Samut  : AAAGAATATCGATGCGCAGGACGCATTGTTGCAAACAACGCGCATTGCTTCTTTTGAGGCTTCGCTGCATGAGAAGAATT : 6117
Chan   : AAAGAATATCGATGCGCAGGACGCATTGTTGCAAACAACGCGCATTGCTTCTTTTGAGGCTTCGCTGCATGAGAAGAATT : 6117
Rayong : AAAGAATATCGATGCGCAGGACGCATTGCTGCAAACAACGCGCATTGCTTCTTTTGAGGCTTCGCTGCATGAGAAGAATT : 6117
         AAAGAATATCGATGCGCAGGAcGCATTg TGCAAACAACGCGCATTGCTTCTTTTGAGGCTTCGCTGCATGAGAAGaaTT

                      *        6180           *        6200           *        6220           *        6240
Hawaii : ATTTCTTAATGTTCTGCGATGTCATTAAGATAGCGTGTAGGAACGCAGGGTACAAGGAAGCATGTTTACATGAGTTGGAT : 6240
Samut  : ATTTCTTAATGTTCTGCGATGTCATTAAGAAAGCGTGTAGGAACGCAGGGTATAAGGAAGCATGTTTACATGAGTTGGAT : 6197
Chan   : ATTTCTTAATGTTCTGCGATGTCATTAAGAAAGCGTGTAGGAACGCAGGGTATAAGGAAGCATGTTTACATGAGTTGGAT : 6197
Rayong : ATTTCTTAATGTTCTGCGATGTCATTAAGAAAGCGTGTAGGAACGCAGGGTATAAGGAAGCATGTTTACATGAGTTGGAT : 6197
         ATTTCTTAATGTTCTGCGATGTCATTAAGAaAGCGTGTAGGAACGCAGGGTAtAAGGAAGCATGTTTACATGAGTTGGAT

                      *        6260           *        6280           *        6300           *        6320
Hawaii : TGTAAGAGCTTCCTTTTGGCCCAGCAAGGTAGAGCTGGAGCTCATGATAGTGAGTTCCTAAGTCAGCTATTGGACTTAAA : 6320
Samut  : TGTAAGAGCTTCCTTTTAGCCCAGCAAGGTAGAGCTGGAGCTCATGATAGCGAGTTCCTAAGTCAGCTATTGGACTTAAA : 6277
Chan   : TGTAAGAGCTTCCTTTTAGCCCAGCAAGGTAGAGCTGGAGCTCATGATAGCGAGTTCCTAAGTCAGCTATTGGACTTAAA : 6277
Rayong : TGTAAGAGCTTCCTTTTAGCCCAGCAAGGTAGAGCTGGAGCTCATGATAGCGAGTTCCTAAGTCAGCTATTGGACTTAAA : 6277
         TGTAAGAGCTTCCTTTTaGCCCAGCAAGGTAGAGCTGGAGCTCATGATAGcGAGTTCCTAAGTCAGCTATTGGACTTAAA


Hawaii : CTAA : 6324
Samut  : CTAA : 6281
Chan   : CTAA : 6281
Rayong : CTAA : 6281
         CTAA
```

Figure 10: Multiple sequences alignment of Thai TSV isolates (Continued)

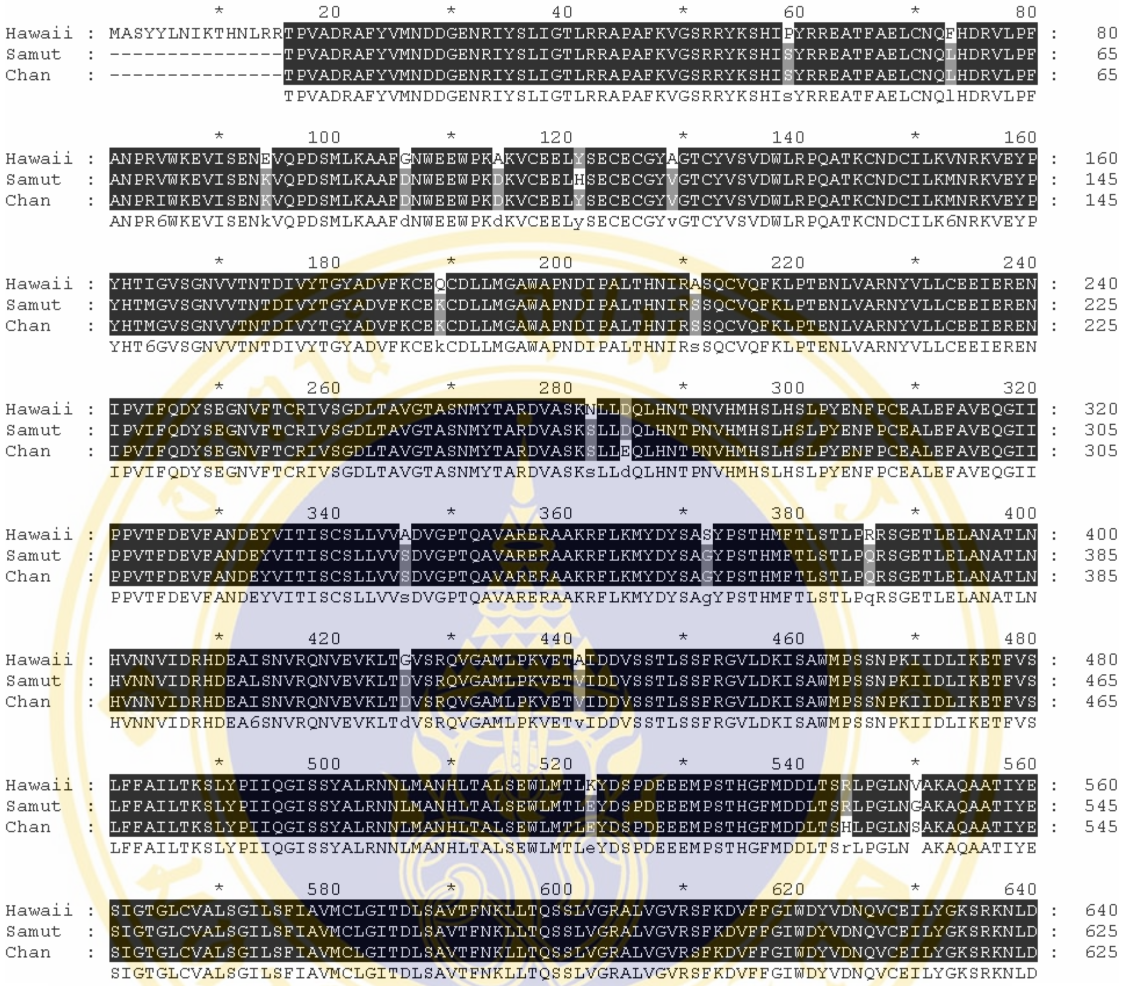
Table 4. Percent nucleotide (above diagonal) identities between the three Thai isolates and Hawaii isolate in the ORF1 gene of TSV.

	Isolate	Percent identity of nucleotide sequence			
		1	2	3	4
1	Hawaii	*	97.0	96.4	96.6
2	Samut Sakorn		*	99.2	98.4
3	Chanthaburi			*	97.8
4	Rayong				*

5.2.2 Comparisons of the deduced amino acid sequence of TSV ORF1 gene

In order to analyze the genetic variation among the Thai isolates and the reference sequence (Hawaii isolate), the deduced amino acid sequences of the ORF1 gene of the Thai isolates and the Hawaii isolate were compared. Of the 3 Thai isolates, the ORF1 gene of Samut Sakorn and Chanthaburi isolates consisted of 2,093 residues while the ORF1 gene of Rayong isolate consisted of 222 residues due to a premature termination by the codon UGA at position 669-671. Therefore, the deduced amino acid sequences for the 3 ORF1 genes of TSV were aligned and analyzed as presented in figure 11. The comparison between the amino acid sequences of the ORF1 gene of the Thai isolates, excluding Rayong isolate, and those of Hawaii isolate revealed 33 amino acid substitutions, of which amino acid changes were conserved among the two Thai isolates. Most of the substitutions were in the region close to the 5’ end rather than the central and the 3’ end region (Fig. 11). As summarized in table 5, the amino acid identities between ORF1 gene of Thai isolates and Hawaii isolate ranged from 96.7 to 97.2% whereas the identities between Samut Sakorn and Rayong isolates was higher and showed 99.3%. Nevertheless, in comparison with the reference sequence, the Samut Sakorn isolate showed the highest identity of 97.2%.

The analysis of Thai isolates and Hawaii isolate revealed only a few differences in amino acid sequences of the helicase, protease and polymerase genes. In the predicted helicase gene, the amino acid sequence of three domain motifs characteristic of helicase designed by Koonin and Dolja was conserved in three Thai isolates and Hawaii isolate (15). In the amino acid sequence of predicted protease coding region, the histidine at position 1387, the aspartic acid at position 1427 and the cystein at position 1525, and the glycine at position 1542, the histidine at position 1544 and glycine at position 1547 amino acid of residue involved in the catalytic triad and in substrate binding, as shown as dots and exclamation marks, respectively (Fig. 11), were also conserved in all isolates. In the motifs characteristic of RdRp identified for the picorna-like virus superfamily, the motif I-VI and VIII were conserved, but, the motif VII is differentiated among the 4 TSV viruses. In the VII motif, there is asparagine substitution for serine in all Thai isolates at the position 1980 amino acid on the RdRp protein.



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                  *        20         *        40         *        60         *        80
Hawaii : MASYYLNIKTHNLRRTPVADRAFYVMNDDGENRIYSLIGTLRRAPAFKVGSRRYKSHIPYRREATFAELCNQFHDRVLPF :   80
Samut  : ---------------TPVADRAFYVMNDDGENRIYSLIGTLRRAPAFKVGSRRYKSHISYRREATFAELCNQLHDRVLPF :   65
Chan   : ---------------TPVADRAFYVMNDDGENRIYSLIGTLRRAPAFKVGSRRYKSHISYRREATFAELCNQLHDRVLPF :   65
                        TPVADRAFYVMNDDGENRIYSLIGTLRRAPAFKVGSRRYKSHIsYRREATFAELCNQlHDRVLPF

                  *       100         *       120         *       140         *       160
Hawaii : ANPRVWKEVISENEVQPDSMLKAAFGNWEEWPKAKVCEELYSECECGYAGTCYVSVDWLRPQATKCNDCILKVNRKVEYP :  160
Samut  : ANPRVWKEVISENKVQPDSMLKAAFDNWEEWPKDKVCEELHSECECGYVGTCYVSVDWLRPQATKCNDCILKMNRKVEYP :  145
Chan   : ANPRIWKEVISENKVQPDSMLKAAFDNWEEWPKDKVCEELYSECECGYVGTCYVSVDWLRPQATKCNDCILKMNRKVEYP :  145
         ANPR6WKEVISENkVQPDSMLKAAFdNWEEWPKdKVCEELySECECGYvGTCYVSVDWLRPQATKCNDCILK6NRKVEYP

                  *       180         *       200         *       220         *       240
Hawaii : YHTIGVSGNVVTNTDIVYTGYADVFKCEQCDLLMGAWAPNDIPALTHNIRASQCVQFKLPTENLVARNYVLLCEEIEREN :  240
Samut  : YHTMGVSGNVVTNTDIVYTGYADVFKCEKCDLLMGAWAPNDIPALTHNIRSSQCVQFKLPTENLVARNYVLLCEEIEREN :  225
Chan   : YHTMGVSGNVVTNTDIVYTGYADVFKCEKCDLLMGAWAPNDIPALTHNIRSSQCVQFKLPTENLVARNYVLLCEEIEREN :  225
         YHT6GVSGNVVTNTDIVYTGYADVFKCEkCDLLMGAWAPNDIPALTHNIRsSQCVQFKLPTENLVARNYVLLCEEIEREN

                  *       260         *       280         *       300         *       320
Hawaii : IPVIFQDYSEGNVFTCRIVSGDLTAVGTASNMYTARDVASKNLLDQLHNTPNVHMHSLHSLPYENFPCEALEFAVEQGII :  320
Samut  : IPVIFQDYSEGNVFTCRIVSGDLTAVGTASNMYTARDVASKSLLDQLHNTPNVHMHSLHSLPYENFPCEALEFAVEQGII :  305
Chan   : IPVIFQDYSEGNVFTCRIVSGDLTAVGTASNMYTARDVASKSLLEQLHNTPNVHMHSLHSLPYENFPCEALEFAVEQGII :  305
         IPVIFQDYSEGNVFTCRIVSGDLTAVGTASNMYTARDVASKsLLdQLHNTPNVHMHSLHSLPYENFPCEALEFAVEQGII

                  *       340         *       360         *       380         *       400
Hawaii : PPVTFDEVFANDEYVITISCSLLVVADVGPTQAVARERAAKRFLKMYDYSASYPSTHMFTLSTLPRRSGETLELANATLN :  400
Samut  : PPVTFDEVFANDEYVITISCSLLVVSDVGPTQAVARERAAKRFLKMYDYSAGYPSTHMFTLSTLPQRSGETLELANATLN :  385
Chan   : PPVTFDEVFANDEYVITISCSLLVVSDVGPTQAVARERAAKRFLKMYDYSAGYPSTHMFTLSTLPQRSGETLELANATLN :  385
         PPVTFDEVFANDEYVITISCSLLVVsDVGPTQAVARERAAKRFLKMYDYSAgYPSTHMFTLSTLPqRSGETLELANATLN

                  *       420         *       440         *       460         *       480
Hawaii : HVNNVIDRHDEAISNVRQNVEVKLTGVSRQVGAMLPKVETAIDDVSSTLSSFRGVLDKISAWMPSSNPKIIDLIKETFVS :  480
Samut  : HVNNVIDRHDEALSNVRQNVEVKLTDVSRQVGAMLPKVETVIDDVSSTLSSFRGVLDKISAWMPSSNPKIIDLIKETFVS :  465
Chan   : HVNNVIDRHDEAISNVRQNVEVKLTDVSRQVGAMLPKVETVIDDVSSTLSSFRGVLDKISAWMPSSNPKIIDLIKETFVS :  465
         HVNNVIDRHDEA6SNVRQNVEVKLTdVSRQVGAMLPKVETvIDDVSSTLSSFRGVLDKISAWMPSSNPKIIDLIKETFVS

                  *       500         *       520         *       540         *       560
Hawaii : LFFAILTKSLYPIIQGISSYALRNNLMANHLTALSEWLMTLKYDSPDEEEMPSTHGFMDDLTSRLPGLNVAKAQAATIYE :  560
Samut  : LFFAILTKSLYPIIQGISSYALRNNLMANHLTALSEWLMTLEYDSPDEEEMPSTHGFMDDLTSRLPGLNGAKAQAATIYE :  545
Chan   : LFFAILTKSLYPIIQGISSYALRNNLMANHLTALSEWLMTLEYDSPDEEEMPSTHGFMDDLTSHLPGLNSAKAQAATIYE :  545
         LFFAILTKSLYPIIQGISSYALRNNLMANHLTALSEWLMTLeYDSPDEEEMPSTHGFMDDLTSrLPGLN AKAQAATIYE

                  *       580         *       600         *       620         *       640
Hawaii : SIGTGLCVALSGILSFIAVMCLGITDLSAVTFNKLLTQSSLVGRALVGVRSFKDVFFGIWDYVDNQVCEILYGKSRKNLD :  640
Samut  : SIGTGLCVALSGILSFIAVMCLGITDLSAVTFNKLLTQSSLVGRALVGVRSFKDVFFGIWDYVDNQVCEILYGKSRKNLD :  625
Chan   : SIGTGLCVALSGILSFIAVMCLGITDLSAVTFNKLLTQSSLVGRALVGVRSFKDVFFGIWDYVDNQVCEILYGKSRKNLD :  625
         SIGTGLCVALSGILSFIAVMCLGITDLSAVTFNKLLTQSSLVGRALVGVRSFKDVFFGIWDYVDNQVCEILYGKSRKNLD
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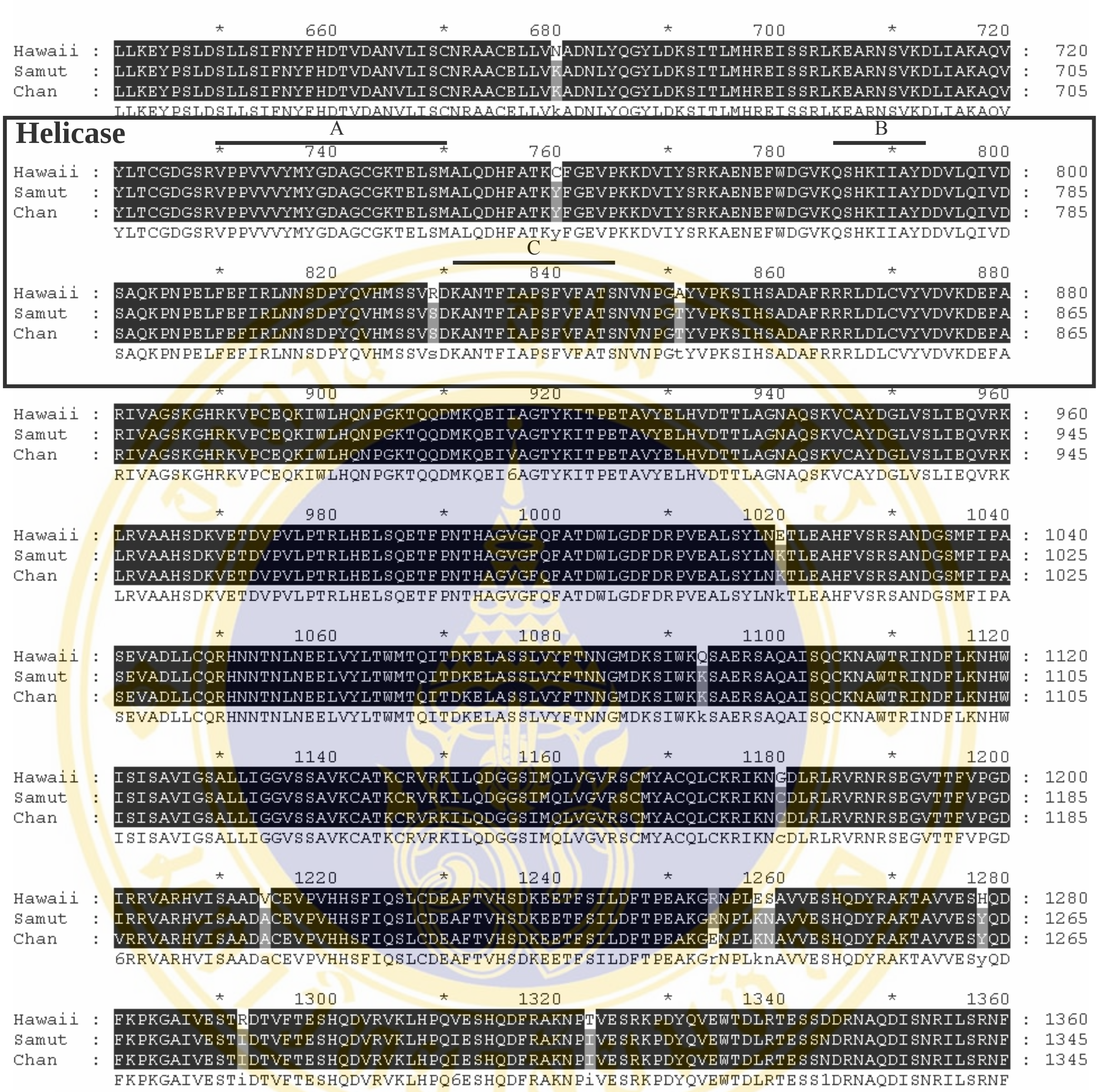
Figure 11: Multiple alignment of amino acid sequences of ORF 1 gene

The deduced amino acid sequences of the ORF 1 gene of TSV were compared between three Thai isolates and Hawaii isolate. The Hawaii sequence is shown in the top line. The followings are Samut Sakorn (Samut) and Chanthaburi (Chan) sequences. The single letter amino acid is used. Helicase domain motifs are labeled A, B and C. Putative residues involved in catalytic triad and in substrate binding for the protease domain are shown as dots and exclamation marks. Conserved motifs of the RdRp are labeled I-VIII.



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                       *       660         *       680         *       700         *       720
Hawaii  : LLKEYPSLDSLLSIFNYFHDTVDANVLISCNRAACELLVNADNLYQGYLDKSITLMHREISSRLKEARNSVKDLIAKAQV :  720
Samut   : LLKEYPSLDSLLSIFNYFHDTVDANVLISCNRAACELLVKADNLYQGYLDKSITLMHREISSRLKEARNSVKDLIAKAQV :  705
Chan    : LLKEYPSLDSLLSIFNYFHDTVDANVLISCNRAACELLVKADNLYQGYLDKSITLMHREISSRLKEARNSVKDLIAKAQV :  705
          LLKEYPSLDSLLSIFNYFHDTVDANVLISCNRAACELLVkADNLYQGYLDKSITLMHREISSRLKEARNSVKDLIAKAQV

Helicase                          A                                                B
                       *       740         *       760         *       780         *       800
Hawaii  : YLTCGDGSRVPPVVVYMYGDAGCGKTELSMALQDHFATKCFGEVPKKDVIYSRKAENEFWDGVKQSHKIIAYDDVLQIVD :  800
Samut   : YLTCGDGSRVPPVVVYMYGDAGCGKTELSMALQDHFATKYFGEVPKKDVIYSRKAENEFWDGVKQSHKIIAYDDVLQIVD :  785
Chan    : YLTCGDGSRVPPVVVYMYGDAGCGKTELSMALQDHFATKYFGEVPKKDVIYSRKAENEFWDGVKQSHKIIAYDDVLQIVD :  785
          YLTCGDGSRVPPVVVYMYGDAGCGKTELSMALQDHFATKyFGEVPKKDVIYSRKAENEFWDGVKQSHKIIAYDDVLQIVD
                                                    C
                       *       820         *       840         *       860         *       880
Hawaii  : SAQKPNPELFEFIRLNNSDPYQVHMSSVRDKANTFIAPSFVFATSNVNPGAYVPKSIHSADAFRRRLDLCVYVDVKDEFA :  880
Samut   : SAQKPNPELFEFIRLNNSDPYQVHMSSVSDKANTFIAPSFVFATSNVNPGTYVPKSIHSADAFRRRLDLCVYVDVKDEFA :  865
Chan    : SAQKPNPELFEFIRLNNSDPYQVHMSSVSDKANTFIAPSFVFATSNVNPGTYVPKSIHSADAFRRRLDLCVYVDVKDEFA :  865
          SAQKPNPELFEFIRLNNSDPYQVHMSSVsDKANTFIAPSFVFATSNVNPGtYVPKSIHSADAFRRRLDLCVYVDVKDEFA

                       *       900         *       920         *       940         *       960
Hawaii  : RIVAGSKGHRKVPCEQKIWLHQNPGKTQQDMKQEIIAGTYKITPETAVYELHVDTTLAGNAQSKVCAYDGLVSLIEQVRK :  960
Samut   : RIVAGSKGHRKVPCEQKIWLHQNPGKTQQDMKQEIVAGTYKITPETAVYELHVDTTLAGNAQSKVCAYDGLVSLIEQVRK :  945
Chan    : RIVAGSKGHRKVPCEQKIWLHQNPGKTQQDMKQEIVAGTYKITPETAVYELHVDTTLAGNAQSKVCAYDGLVSLIEQVRK :  945
          RIVAGSKGHRKVPCEQKIWLHQNPGKTQQDMKQEI6AGTYKITPETAVYELHVDTTLAGNAQSKVCAYDGLVSLIEQVRK

                       *       980         *      1000         *      1020         *      1040
Hawaii  : LRVAAHSDKVETDVPVLPTRLHELSQETFPNTHAGVGFQFATDWLGDFDRPVEALSYLNETLEAHFVSRSANDGSMFIPA : 1040
Samut   : LRVAAHSDKVETDVPVLPTRLHELSQETFPNTHAGVGFQFATDWLGDFDRPVEALSYLNKTLEAHFVSRSANDGSMFIPA : 1025
Chan    : LRVAAHSDKVETDVPVLPTRLHELSQETFPNTHAGVGFQFATDWLGDFDRPVEALSYLNKTLEAHFVSRSANDGSMFIPA : 1025
          LRVAAHSDKVETDVPVLPTRLHELSQETFPNTHAGVGFQFATDWLGDFDRPVEALSYLNkTLEAHFVSRSANDGSMFIPA

                       *      1060         *      1080         *      1100         *      1120
Hawaii  : SEVADLLCQRHNNTNLNEELVYLTWMTQITDKELASSLVYFTNNGMDKSIWKQSAERSAQAISQCKNAWTRINDFLKNHW : 1120
Samut   : SEVADLLCQRHNNTNLNEELVYLTWMTQITDKELASSLVYFTNNGMDKSIWKKSAERSAQAISQCKNAWTRINDFLKNHW : 1105
Chan    : SEVADLLCQRHNNTNLNEELVYLTWMTQITDKELASSLVYFTNNGMDKSIWKKSAERSAQAISQCKNAWTRINDFLKNHW : 1105
          SEVADLLCQRHNNTNLNEELVYLTWMTQITDKELASSLVYFTNNGMDKSIWKkSAERSAQAISQCKNAWTRINDFLKNHW

                       *      1140         *      1160         *      1180         *      1200
Hawaii  : ISISAVIGSALLIGGVSSAVKCATKCRVRKILQDGGSIMQLVGVRSCMYACQLCKRIKNGDLRLRVRNRSEGVTTFVPGD : 1200
Samut   : ISISAVIGSALLIGGVSSAVKCATKCRVRKILQDGGSIMQLVGVRSCMYACQLCKRIKNCDLRLRVRNRSEGVTTFVPGD : 1185
Chan    : ISISAVIGSALLIGGVSSAVKCATKCRVRKILQDGGSIMQLVGVRSCMYACQLCKRIKNCDLRLRVRNRSEGVTTFVPGD : 1185
          ISISAVIGSALLIGGVSSAVKCATKCRVRKILQDGGSIMQLVGVRSCMYACQLCKRIKNcDLRLRVRNRSEGVTTFVPGD

                       *      1220         *      1240         *      1260         *      1280
Hawaii  : IRRVARHVISAADVCEVPVHHSFIQSLCDEAFTVHSDKEETFSILDFTPEAKGRNPLESAVVESHQDYRAKTAVVESHQD : 1280
Samut   : IRRVARHVISAADACEVPVHHSFIQSLCDEAFTVHSDKEETFSILDFTPEAKGRNPLKNAVVESHQDYRAKTAVVESYQD : 1265
Chan    : VRRVARHVISAADACEVPVHHSFIQSLCDEAFTVHSDKEETFSILDFTPEAKGENPLKNAVVESHQDYRAKTAVVESYQD : 1265
          6RRVARHVISAADaCEVPVHHSFIQSLCDEAFTVHSDKEETFSILDFTPEAKGrNPLknAVVESHQDYRAKTAVVESyQD

                       *      1300         *      1320         *      1340         *      1360
Hawaii  : FKPKGAIVESTRDTVFTESHQDVRVKLHPQVESHQDFRAKNPTVESRKPDYQVEWTDLRTESSDDRNAQDISNRILSRNF : 1360
Samut   : FKPKGAIVESTIDTVFTESHQDVRVKLHPQIESHQDFRAKNPIVESRKPDYQVEWTDLRTESSNDRNAQDISNRILSRNF : 1345
Chan    : FKPKGAIVESTIDTVFTESHQDVRVKLHPQIESHQDFRAKNPIVESRKPDYQVEWTDLRTESSNDRNAQDISNRILSRNF : 1345
          FKPKGAIVESTiDTVFTESHQDVRVKLHPQ6ESHQDFRAKNPiVESRKPDYQVEWTDLRTESS1DRNAQDISNRILSRNF
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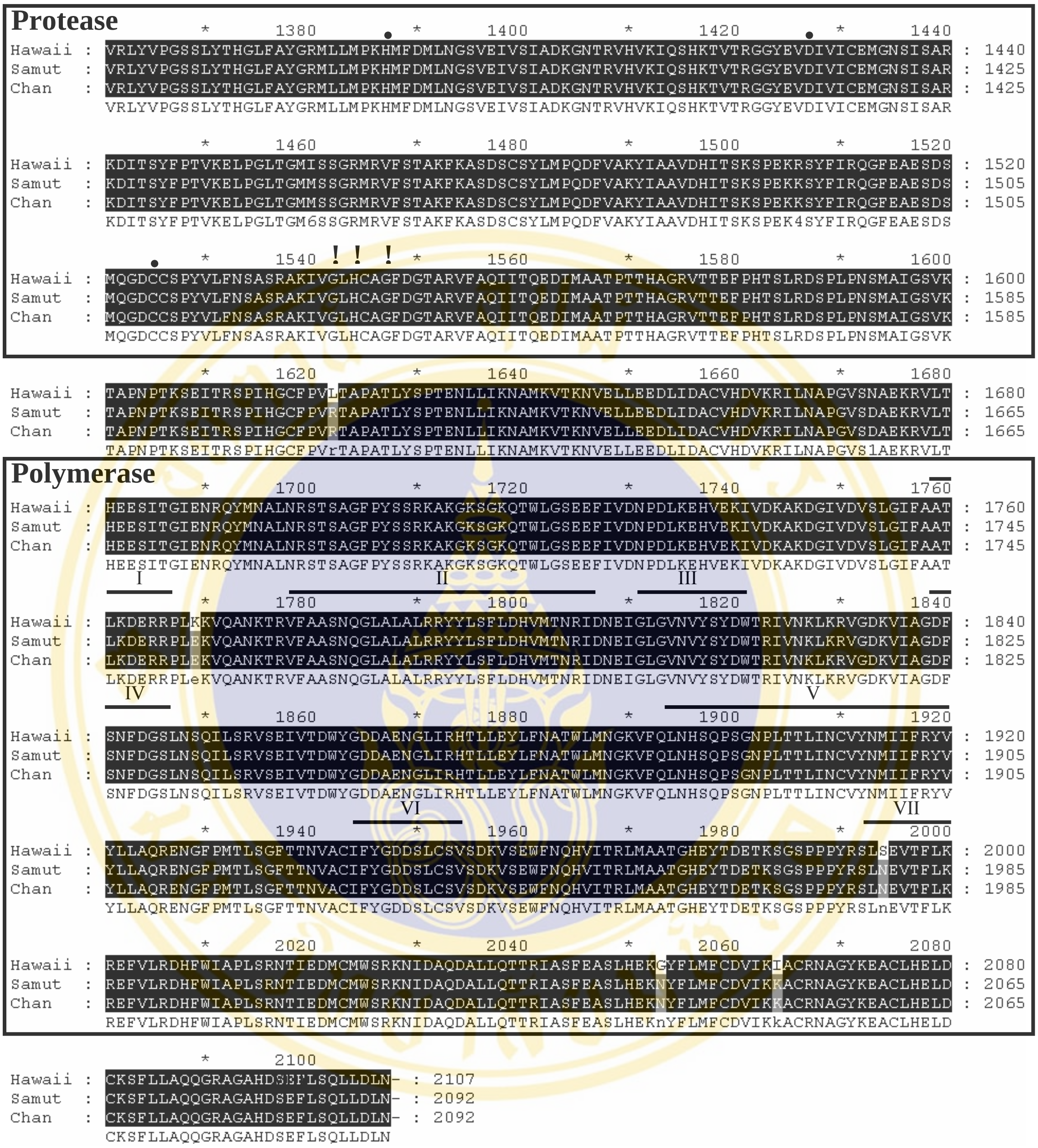
Figure 11: Multiple alignment of amino acid sequences of ORF 1 gene (Continued)



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                  *       1380         *       1400         *       1420         *       1440
Hawaii : VRLYVPGSSLYTHGLFAYGRMLLMPKHMFDMLNGSVEIVSIADKGNTRVHVKIQSHKTVTRGGYEVDIVICEMGNSISAR : 1440
Samut  : VRLYVPGSSLYTHGLFAYGRMLLMPKHMFDMLNGSVEIVSIADKGNTRVHVKIQSHKTVTRGGYEVDIVICEMGNSISAR : 1425
Chan   : VRLYVPGSSLYTHGLFAYGRMLLMPKHMFDMLNGSVEIVSIADKGNTRVHVKIQSHKTVTRGGYEVDIVICEMGNSISAR : 1425
         VRLYVPGSSLYTHGLFAYGRMLLMPKHMFDMLNGSVEIVSIADKGNTRVHVKIQSHKTVTRGGYEVDIVICEMGNSISAR

                  *       1460         *       1480         *       1500         *       1520
Hawaii : KDITSYFPTVKELPGLTGMISSGRMRVFSTAKFKASDSCSYLMPQDFVAKYIAAVDHITSKSPEKRSYFIRQGFEAESDS : 1520
Samut  : KDITSYFPTVKELPGLTGMMSSGRMRVFSTAKFKASDSCSYLMPQDFVAKYIAAVDHITSKSPEKKSYFIRQGFEAESDS : 1505
Chan   : KDITSYFPTVKELPGLTGMMSSGRMRVFSTAKFKASDSCSYLMPQDFVAKYIAAVDHITSKSPEKKSYFIRQGFEAESDS : 1505
         KDITSYFPTVKELPGLTGM6SSGRMRVFSTAKFKASDSCSYLMPQDFVAKYIAAVDHITSKSPEK4SYFIRQGFEAESDS

                  *       1540         *       1560         *       1580         *       1600
Hawaii : MQGDCCSPYVLFNSASRAKIVGLHCAGFDGTARVFAQIITQEDIMAATPTTHAGRVTTEFPHTSLRDSPLPNSMAIGSVK : 1600
Samut  : MQGDCCSPYVLFNSASRAKIVGLHCAGFDGTARVFAQIITQEDIMAATPTTHAGRVTTEFPHTSLRDSPLPNSMAIGSVK : 1585
Chan   : MQGDCCSPYVLFNSASRAKIVGLHCAGFDGTARVFAQIITQEDIMAATPTTHAGRVTTEFPHTSLRDSPLPNSMAIGSVK : 1585
         MQGDCCSPYVLFNSASRAKIVGLHCAGFDGTARVFAQIITQEDIMAATPTTHAGRVTTEFPHTSLRDSPLPNSMAIGSVK

                  *       1620         *       1640         *       1660         *       1680
Hawaii : TAPNPTKSEITRSPIHGCFPVLTAPATLYSPTENLLIKNAMKVTKNVELLEEDLIDACVHDVKRILNAPGVSNAEKRVLT : 1680
Samut  : TAPNPTKSEITRSPIHGCFPVRTAPATLYSPTENLLIKNAMKVTKNVELLEEDLIDACVHDVKRILNAPGVSDAEKRVLT : 1665
Chan   : TAPNPTKSEITRSPIHGCFPVRTAPATLYSPTENLLIKNAMKVTKNVELLEEDLIDACVHDVKRILNAPGVSDAEKRVLT : 1665
         TAPNPTKSEITRSPIHGCFPVrTAPATLYSPTENLLIKNAMKVTKNVELLEEDLIDACVHDVKRILNAPGVS1AEKRVLT

                  *       1700         *       1720         *       1740         *       1760
Hawaii : HEESITGIENRQYMNALNRSTSAGFPYSSRKAKGKSGKQTWLGSEEFIVDNPDLKEHVEKIVDKAKDGIVDVSLGIFAAT : 1760
Samut  : HEESITGIENRQYMNALNRSTSAGFPYSSRKAKGKSGKQTWLGSEEFIVDNPDLKEHVEKIVDKAKDGIVDVSLGIFAAT : 1745
Chan   : HEESITGIENRQYMNALNRSTSAGFPYSSRKAKGKSGKQTWLGSEEFIVDNPDLKEHVEKIVDKAKDGIVDVSLGIFAAT : 1745
         HEESITGIENRQYMNALNRSTSAGFPYSSRKAKGKSGKQTWLGSEEFIVDNPDLKEHVEKIVDKAKDGIVDVSLGIFAAT

                  *       1780         *       1800         *       1820         *       1840
Hawaii : LKDERRPLKKVQANKTRVFAASNQGLALALRRYYLSFLDHVMTNRIDNEIGLGVNVYSYDWTRIVNKLKRVGDKVIAGDF : 1840
Samut  : LKDERRPLEKVQANKTRVFAASNQGLALALRRYYLSFLDHVMTNRIDNEIGLGVNVYSYDWTRIVNKLKRVGDKVIAGDF : 1825
Chan   : LKDERRPLEKVQANKTRVFAASNQGLALALRRYYLSFLDHVMTNRIDNEIGLGVNVYSYDWTRIVNKLKRVGDKVIAGDF : 1825
         LKDERRPLeKVQANKTRVFAASNQGLALALRRYYLSFLDHVMTNRIDNEIGLGVNVYSYDWTRIVNKLKRVGDKVIAGDF

                  *       1860         *       1880         *       1900         *       1920
Hawaii : SNFDGSLNSQILSRVSEIVTDWYGDDAENGLIRHTLLEYLFNATWLMNGKVFQLNHSQPSGNPLTTLINCVYNMIIFRYV : 1920
Samut  : SNFDGSLNSQILSRVSEIVTDWYGDDAENGLIRHTLLEYLFNATWLMNGKVFQLNHSQPSGNPLTTLINCVYNMIIFRYV : 1905
Chan   : SNFDGSLNSQILSRVSEIVTDWYGDDAENGLIRHTLLEYLFNATWLMNGKVFQLNHSQPSGNPLTTLINCVYNMIIFRYV : 1905
         SNFDGSLNSQILSRVSEIVTDWYGDDAENGLIRHTLLEYLFNATWLMNGKVFQLNHSQPSGNPLTTLINCVYNMIIFRYV

                  *       1940         *       1960         *       1980         *       2000
Hawaii : YLLAQRENGFPMTLSGFTTNVACIFYGDDSLCSVSDKVSEWFNQHVITRLMAATGHEYTDETKSGSPPPYRSLSEVTFLK : 2000
Samut  : YLLAQRENGFPMTLSGFTTNVACIFYGDDSLCSVSDKVSEWFNQHVITRLMAATGHEYTDETKSGSPPPYRSLNEVTFLK : 1985
Chan   : YLLAQRENGFPMTLSGFTTNVACIFYGDDSLCSVSDKVSEWFNQHVITRLMAATGHEYTDETKSGSPPPYRSLNEVTFLK : 1985
         YLLAQRENGFPMTLSGFTTNVACIFYGDDSLCSVSDKVSEWFNQHVITRLMAATGHEYTDETKSGSPPPYRSLnEVTFLK

                  *       2020         *       2040         *       2060         *       2080
Hawaii : REFVLRDHFWIAPLSRNTIEDMCMWSRKNIDAQDALLQTTRIASFEASLHEKGYFLMFCDVIKIACRNAGYKEACLHELD : 2080
Samut  : REFVLRDHFWIAPLSRNTIEDMCMWSRKNIDAQDALLQTTRIASFEASLHEKNYFLMFCDVIKKACRNAGYKEACLHELD : 2065
Chan   : REFVLRDHFWIAPLSRNTIEDMCMWSRKNIDAQDALLQTTRIASFEASLHEKNYFLMFCDVIKKACRNAGYKEACLHELD : 2065
         REFVLRDHFWIAPLSRNTIEDMCMWSRKNIDAQDALLQTTRIASFEASLHEKnYFLMFCDVIKkACRNAGYKEACLHELD

                  *       2100
Hawaii : CKSFLLAQQGRAGAHDSEFLSQLLDLN- : 2107
Samut  : CKSFLLAQQGRAGAHDSEFLSQLLDLN- : 2092
Chan   : CKSFLLAQQGRAGAHDSEFLSQLLDLN- : 2092
         CKSFLLAQQGRAGAHDSEFLSQLLDLN
```

Figure 11: Multiple alignment of amino acid sequences of ORF 1 gene (Continued)

Table 5. Percent amino acid (below diagonal) identities between the three Thai isolates and the Hawaii isolate in the ORF1 gene of TSV.

	Isolate	Percent identity of amino acid sequence		
		1	2	3
1	Hawaii	*		
2	Samut Sakorn	97.2	*	
3	Chanthaburi	96.7	99.3	*

5.3 Selection of dsRNA target sequences in mRNA of nonstructural protein

The critical step in RNAi study is to select an appropriate region for dsRNA design to target mRNA of interest for degradation. In general, the nonstructural protein region is more conserved than structural protein region. Therefore, coding sequences of essential enzymes in viral replication [helicase, protease and RNA dependent RNA polymerase (RdRp)] were chosen as a target for inhibition of TSV. Based on the nucleotide sequences of ORF1 gene of Thai isolates, TSV specific primers were designed and used to amplify DNA fragments corresponding to helicase, protease and RdRp regions for conventional dsRNAs synthesis. Those three targeted region were located at 2115-2668, 3997-4730 and 5394-6077 relative to the first ATG of the ORF1 (Fig. 12). The amplified fragments of helicase, protease and polymerase (Fig. 13) were cloned into pGEM$^{®}$T-easy vector then subcloned into LITMUS 28i vector to facilitate *in vitro* transcription since it provides T7 RNA polymerase promoters in both orientations. A schematic diagram represents the construction strategies of LITMUS28i-Helicase, LITMUS28i-Protease and LITMUS28i-Polymerase is shown in Figure 14.

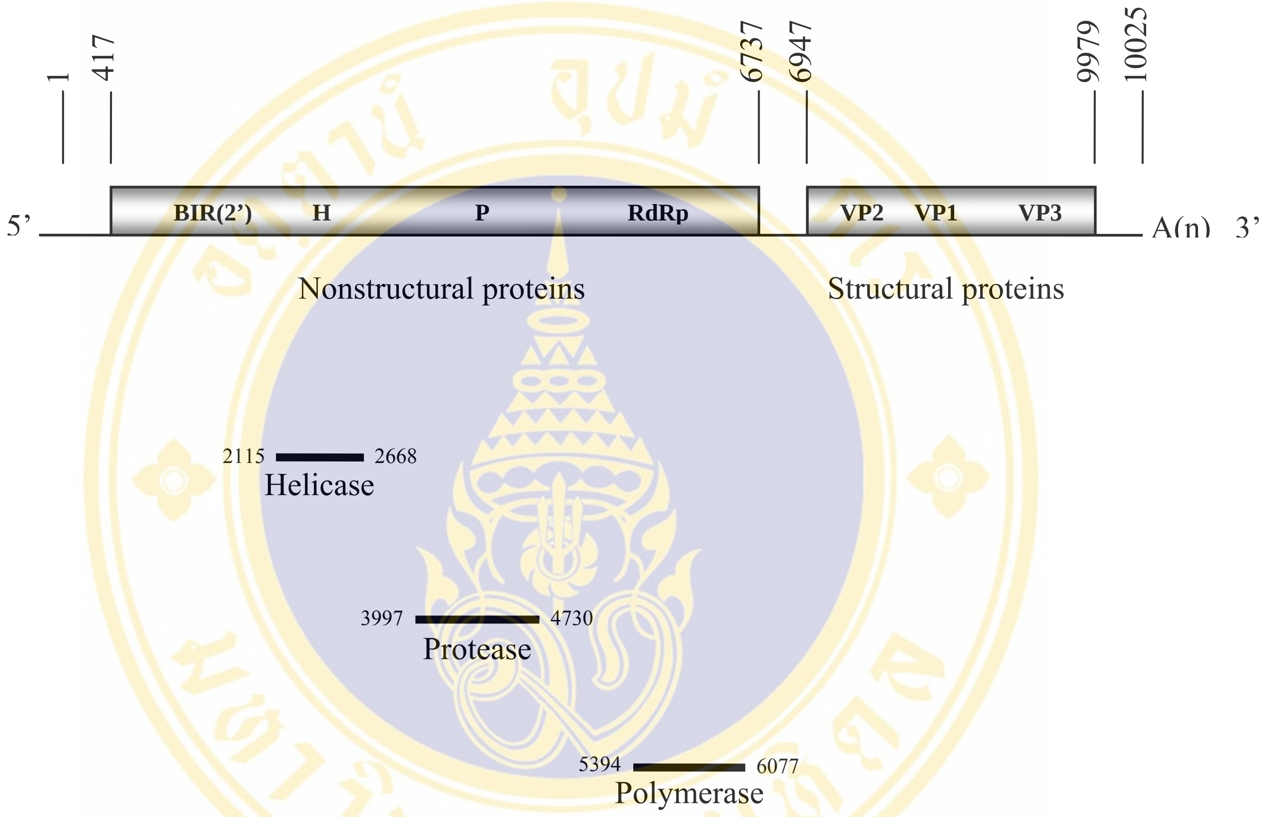


Figure 12: Locations of conventional dsRNAs target sites and structure of Taura syndrome virus genome

The RNAi target regions for conventional dsRNAs are indicated as black bars below the coding regions of ORF1 gene. Double stranded RNAs were generated corresponding to the predicted helicase (2115-2668, 554 bp); protease (3997-4730, 734 bp) and polymerase genes (5394-6077, 684 bp).

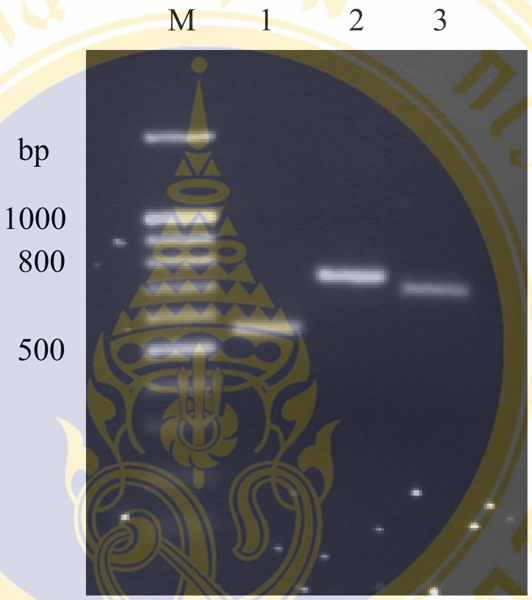


Figure 13: PCR amplification of recombinant plasmids containing putative helicase, protease and polymerase regions of TSV

The putative helicase, protease and polymerase regions of the ORF1 gene were amplified with TSV-specific primer pairs. These specific primers were synthesized base on sequence from Thai isolate.

Lane M: 100 bp DNA ladder
Lane 1: The 550 bp band of putative helicase region
Lane 2: The 730 bp band of putative protease region
Lane 3: The 680 bp band of putative polymerase region

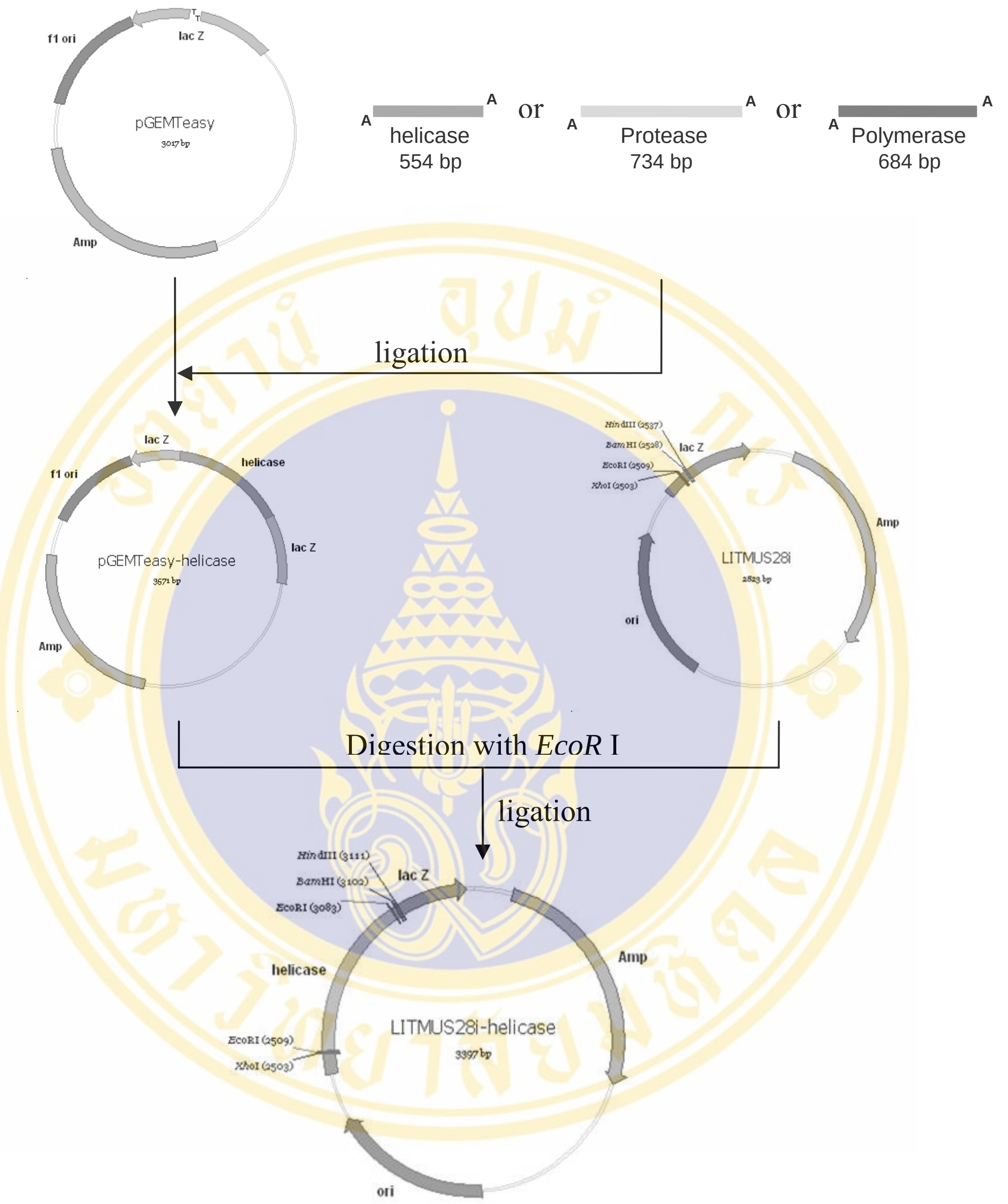


Figure 14: A schematic diagram for construction of LITMUS28i-Helicase

The purified PCR products of the helicase, protease and polymerase were ligated into pGEM®T-easy vector before subcloned into the *EcoR* I site of LITMUS 28i vector.

Besides conventional dsRNAs, stem loop dsRNAs were also generated. In this study, only stem loop for TSV-helicase and TSV-polymerase but not TSV-protease were produced. DNA fragments of helicase and polymerase in pGEM®T-easy vector in sense direction were used as template for construction of stem loop generating plasmids. The two primer pairs (TSV-stem-Hel-PstI/TSV-stem-Hel-SpeI and TSV-stem-Poly-PstI/TSV-stem-Poly-SpeI; Table 1) were synthesized to amplify additional fragments of helicase and polymerase, respectively. The PCR fragments were then cloned into *Spe*I and *Sal*I restriction site of pGEM®T-easy vector in antisense direction to generate plasmid constructs for stem loop dsRNA synthesis. A schematic diagram represents the construction strategies of pGEMTeasy-Helicase and pGEMTeasy-Polymerase is shown in Figure 15.

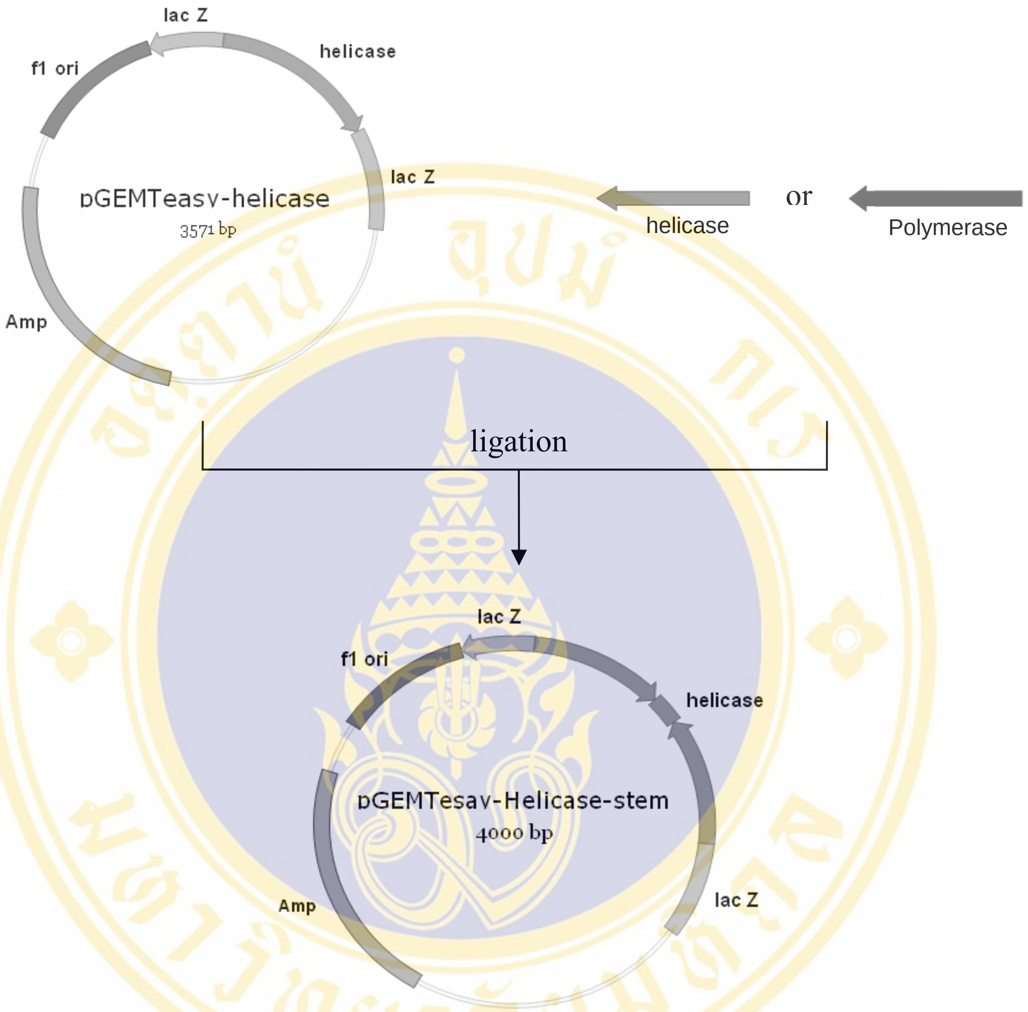


Figure 15: A schematic diagram for construction of pGEMTeasy-Helicase-stem

The PCR products of the helicase and polymerase were cloned into the *Spe* I/*Pst* I site of pGEM®T-easy vector containing the corresponding DNA fragment in antisense direction.

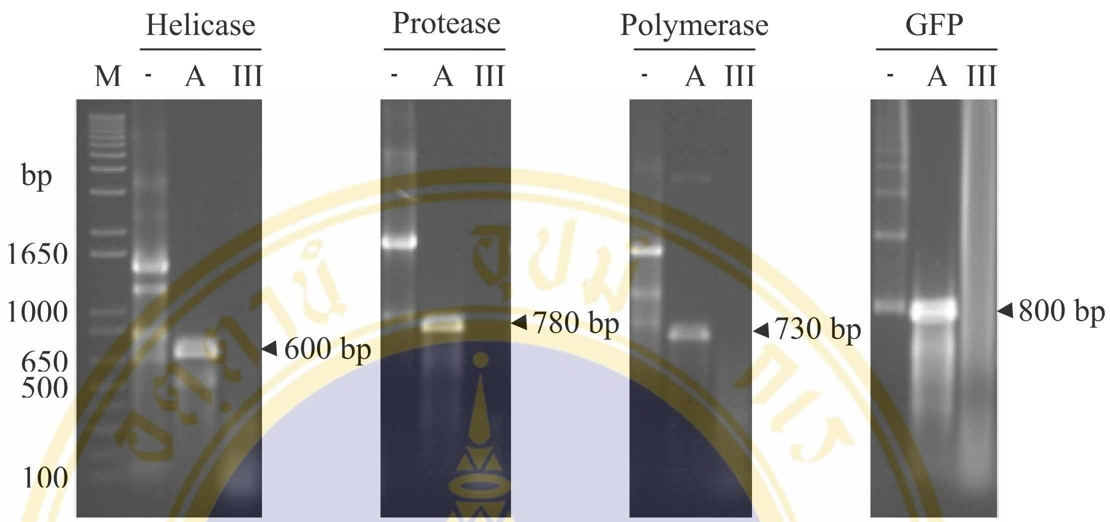
5.4 Preparation and characterization of dsRNAs

To prepare dsRNAs that could target their corresponding site of mRNA for inhibition, we used *in vitro* transcription for both conventional and stem loop dsRNAs production. To generate conventional dsRNAs corresponding to helicase, protease, polymerase and GFP, sense and antisense RNAs were *in vitro* transcribed in two separate reactions from DNA template that had T7 promoter at each end. Following transcription reaction, the resulting transcripts were mixed and annealed post transcriptionally at last as described in method.

The integrity of the dsRNAs were characterized by enzymatic digestion and analyzed by agarose gel electrophoresis (Fig. 16). The untreated dsRNA showed as multiple bands of possibly annealed dsRNA products linked together in oligomeric forms. Upon RNaseA digestion, a predominant band representing the expected size of dsRNA was clearly seen. It is worth noting that the RNase A treated dsRNA products migrated slower than double stranded DNA marker with corresponding sizes. This is consistent to those were reported by other group (71). This is attributed to its net charge density that is lower than that of B-form DNA (72). In contrast, addition of RNase III into these samples resulted in degradation of the RNA yielded very small fragment. Together these results confirmed that dsRNA was successful produced from annealing of complementary strands of *in vitro* transcribed RNA.

In case of stem loop dsRNA, the transcript that was produced in transcription reaction could be simultaneously form stem loop structure through its complementary base pairing because it contains sequence, loop sequence and reverse complementary sequence in the transcript. Result in Figure 16b showed that discrete bands of RNA in undigested RNA sample. However, upon RNase A digestion, only major populations of RNAs with slightly small sizes appeared. In contrast, digestion with RNase III resulted in degradation of the RNA to generate very small degraded product. In addition, an addition band of RNA corresponded to the single-stranded RNA in the loop region in the hairpin structure was found in each RNA used. Taken together, these results confirmed that the inverted repeat sequence on both ends of the RNA molecules could form complementary dsRNA in the stem loop structure.

(a) Conventional dsRNAs



(b) Stem loop dsRNAs

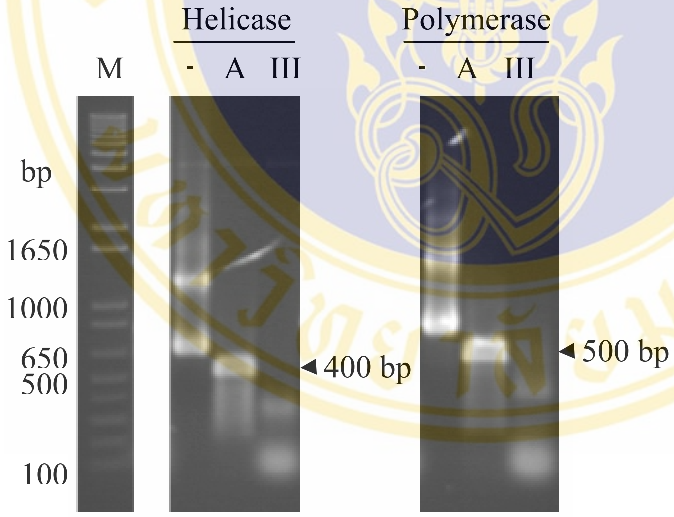


Figure 16: Characterization of conventional and stem loop dsRNAs by RNase digestion.

The conventional and stem loop dsRNAs were generated and tested for their integrity by RNase A and III digestion. Each dsRNAs were separated on 1.2% agarose gel and visualized by staining with ethidium bromide. Lane: M, 1 kb plus DNA ladder. Note that dsRNA migrates more slowly than double stranded DNA marker.

5.5 Infectivity of TSV

In order to determine the infectivity of Taura syndrome virus prepared from infected shrimp for further study involving the characterization of viral specific dsRNA to inhibit TSV replication by RNA interference mechanism, a set of experiments were conducted to carry out the experiments.

5.5.1 TSV experimental infection in *P. vannamei*

In the preliminary experiment, Taura syndrome virus prepared from TSV infected shrimp was tested whether it was infectious. Therefore, a highly susceptible target host, *P. vannamei*, was injected with TSV. From the result shown in Figure 17, upon infection, the presence of virus in the shrimps were monitored by RT-PCR. A TSV specific band of 554 bp was detected in TSV in the samples. The result showed that the RNA prepared from haemolymph and epidermis of infected shrimp gave positive results. Notably, there was plenty of TSV RNA in dead shrimp to implicate TSV infection even though it lacked clinical sign. Thus, the result of this experiment was that the TSV stock prepared from TSV infected shrimp was active and infectious. On the other hand, the result also indicated that *P. vannamei* was susceptible to TSV infection. We have observed from the experiment that the challenge of *P. vannamei* with TSV can lead to death in 5-7 days postinfection, before the appearance of any clinical signs of TSV. The haemolymph drawn from shrimp injected with PBS, which served as the control, was negative when assayed by RT-PCR.

5.5.2 TSV experimental infection in *P. monodon*

In another study, the same stock of TSV was injected into a native host, *P. monodon*, to determine whether it was susceptible to TSV infection. If it was susceptible to TSV infection, the primary lymphoid cell (Oka cells) cultures of *P. monodon*, which is available in our laboratory, will be tested for its susceptibility and could possibly used as system for RNA interference assay. Hence, we performed experiment with *P. monodon* using the same inoculum of TSV. Using RT-PCR analysis, TSV RNA could be detected in haemolymph sample of *P .vannamei*, but not in the epidermis sample (Fig. 18). As compared with *P. vannamei*, a lower level of virus was seen in haemolymph sample of *P. monodon*. The result clearly demonstrated

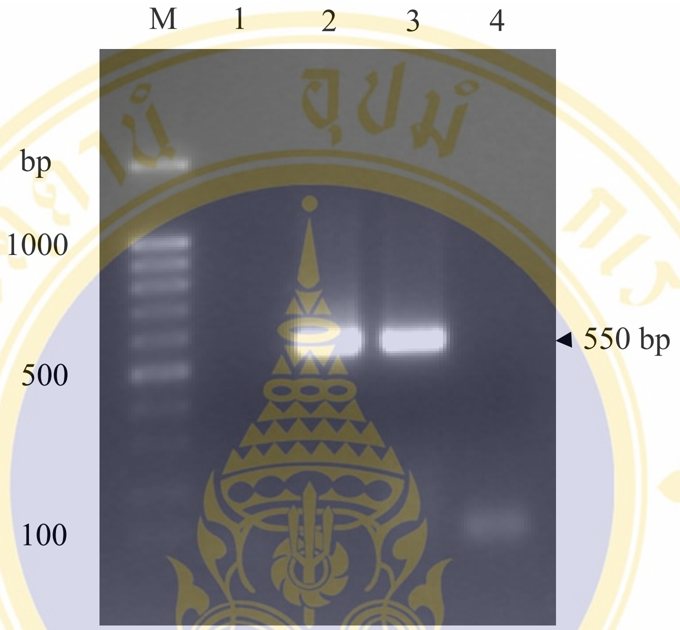


Figure 17: The experimental infection of TSV in *P. vannamei*

P. vannamei were injected with lysate of TSV infected shrimp. Five days later, total RNA was extracted from haemolymph or epidermis for TSV detection. The TSV specific fragment of 550 bp resulted from RT-PCR assay using the Fhel/Rhel primers, indicated the presence of TSV genomic RNA in the sample

Lane M: 100 bp DNA ladder
Lane 1: The haemolymph sample of shrimp injected with PBS
Lane 2: The epidermis sample of shrimp infected with TSV
Lane 3: The haemolymph sample of shrimp infected with TSV
Lane 4: Negative RT-PCR control

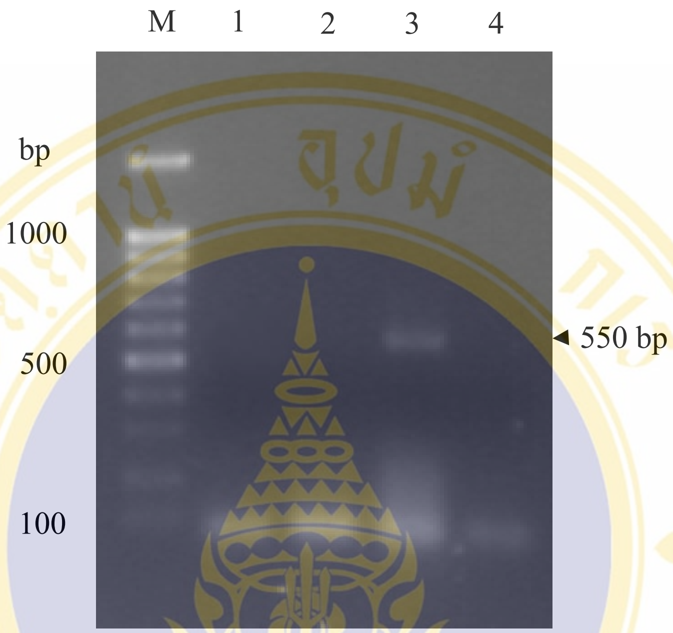


Figure 18: The experimental infection of TSV in *P. monodon*

P. monodon were injected with lysate of TSV infected shrimp. Five days later, total RNA was extracted from haemolymph or epidermis for TSV detection. The TSV specific fragment of 550 bp resulted from RT-PCR assay using the Fhel/Rhel primers, indicated the presence of TSV genomic RNA in the sample.

Lane M: 100 bp DNA ladder
Lane 1: The haemolymph sample of shrimp injected with PBS
Lane 2: The epidermis sample of shrimp infected with TSV
Lane 3: The haemolymph sample of shrimp infected with TSV
Lane 4: Negative RT-PCR control

that the TSV exhibited distinct infectivity in these two shrimp species. However, TSV was able to infect black tiger shrimp experimentally. The result also demonstrated that *P. monodon* is more resistant to TSV than *P. vannamei*. As expected, clinical signs of TSV have not been observed in this shrimp species although the virus was detectable in haemolymph sample. The haemolymph drawn from shrimp injected with PBS was negative for TSV when assayed by RT-PCR.

5.5.3 Experimental infection in Oka cells with TSV

Because the culturing system of primary lymphoid cells (Oka cells) of *P. monodon* is available in our laboratory and successfully used for shrimp viral infection, and previous study showed that a low level of TSV was observed in the haemolymh sample. Therefore, the next experiment was performed to determine the ability of TSV to replicate in Oka cell culture of *P. monodon*. The Oka cell culture was prepared and innoculated with undiluted and filtered haemolymph of shrimp infected with TSV. A triplicate experiment was performed and analyzed by RT-PCR. In addition to RT-PCR analysis, cytopathic effect or CPE is one of the parameter for the observation of viral infection although we not yet known if virus produce CPE or not. In general, CPE was observed at 48-72 h after inoculation and was characterized by the conversion into round shape or detachment of cell from the monolayer of fibroblast like cells (73).Therefore, characteristic of CPE in Oka cell culture was periodically observed under a microscope after TSV infection. As CPE reflects the quantity of viral accumulation, we considered that the severity of CPE would represent the increased amount of newly synthesized virus. As a positive control for CPE characteristic, Oka cells were innoculated with virus generating CPE such as YHV. From the figure 19, no CPE was observed in uninfected control cells at all five times intervals, but, at 48 hr after infection the CPE of cell enlargement and detachment on monolayer was first detected in YHV infected cells. As compared with the YHV infected cells, the TSV infected cells showed no sign of CPE in triplicate. The morphology of TSV infected cells was similar to uninfected cells. Although no obvious CPE was observed in TSV infected cells, we could not assume that Oka cells were not susceptible to TSV infection because TSV may not cause CPE in Oka cells.

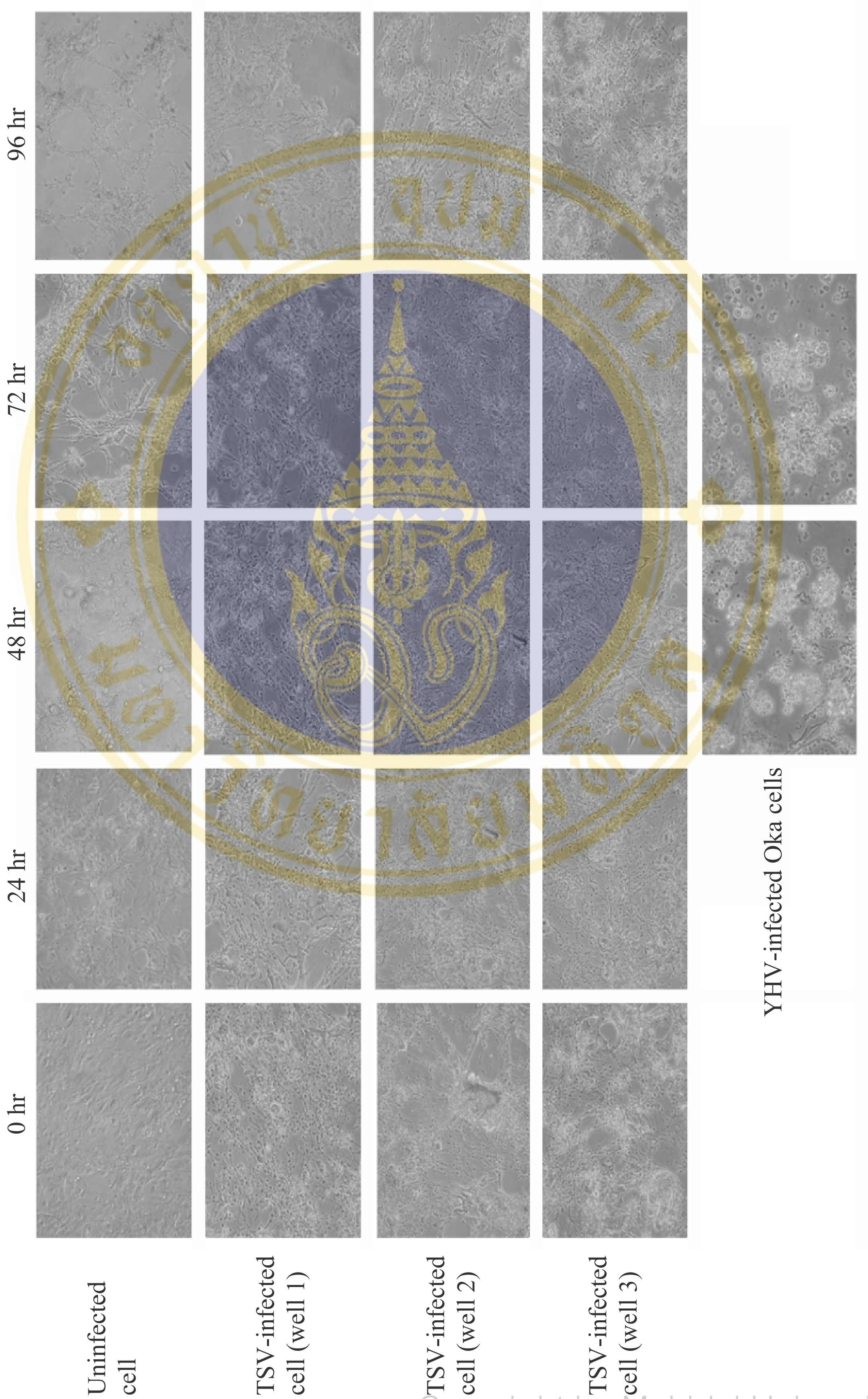

0 hr
24 hr
48 hr
72 hr
96 hr
Uninfected cell
TSV-infected cell (well 1)
TSV-infected cell (well 2)
TSV-infected cell (well 3)
YHV-infected Oka cells

Figure 19: Morphology of primary Oka cells infected with haemolymph from shrimp infected with TSV

Primary Oka cells were infected with haemolymph from shrimp infected with TSV. Change of cell morphology was observed at 0, 24, 48, 72 and 96 hours post infection. Uninfected cell was shown at the top. Triplicate of TSV-infected cell were shown in the following. The characteristic of CPE was first observed at 48 hr post infection in Oka cells infected with YHV. The images were taken at a magnification of 40X with a microscope.

To demonstrate the amplification of TSV, RT-PCR was employed to monitor the level of virus in the culture media as well as the cell lysate. From the result of cell lysate (Fig. 20), a band of TSV was detected in one of the replicate from 48 to 96 hr post inoculation. As observed, the level of virus at 48 hr post inoculation did not increase apparently above 96 hr post inoculation. However, the result shown in Figure 21, no TSV amplification was found in culture medium of Oka cells at any time points of the replicate. Taken together, we concluded that TSV, in this study, has very poor ability to infect and amplify in Oka cells.

As Oka cells did not appear to be a good system for TSV amplification, thus it is not suitable for evaluating the silencing effect of dsRNA molecules for inhibiting TSV replication by RNAi. Therefore, the live *P. vannamei* would be used for the study of the RNA interference since TSV presented in this study could infect and replicate in this shrimp species.

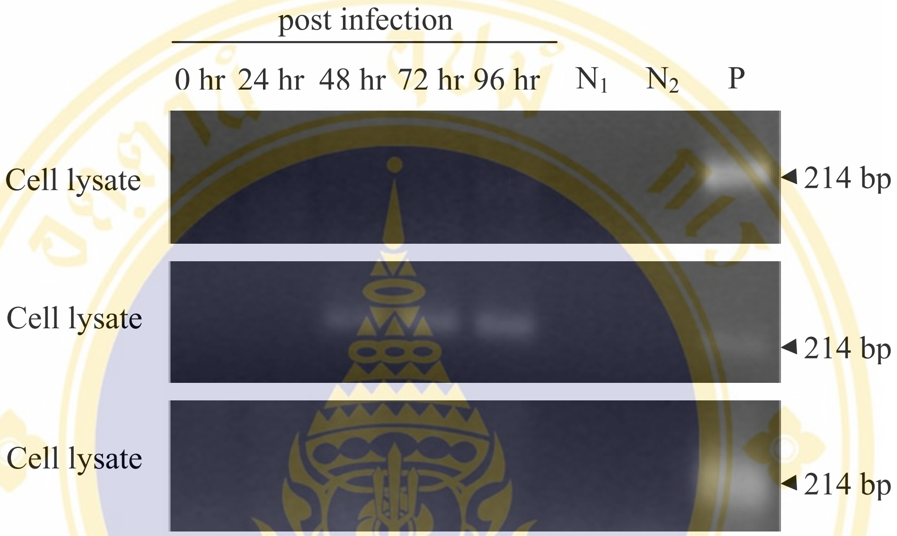


Figure 20: RT-PCR analysis for the presence of TSV in TSV-infected Oka cells

Primary Oka cells were inoculated with haemolymph from shrimp infected with TSV for 14 hours. Oka cells were then washed twice. Total RNA extracted from infected Oka cells were analyzed by RT-PCR for determination of viral replication at time point. The time course experiments were done in triplicate.

Lane N_1: Negative RT-PCR control for TSV
Lane N_2: Negative PCR control for TSV
Lane P : Positive control for TSV

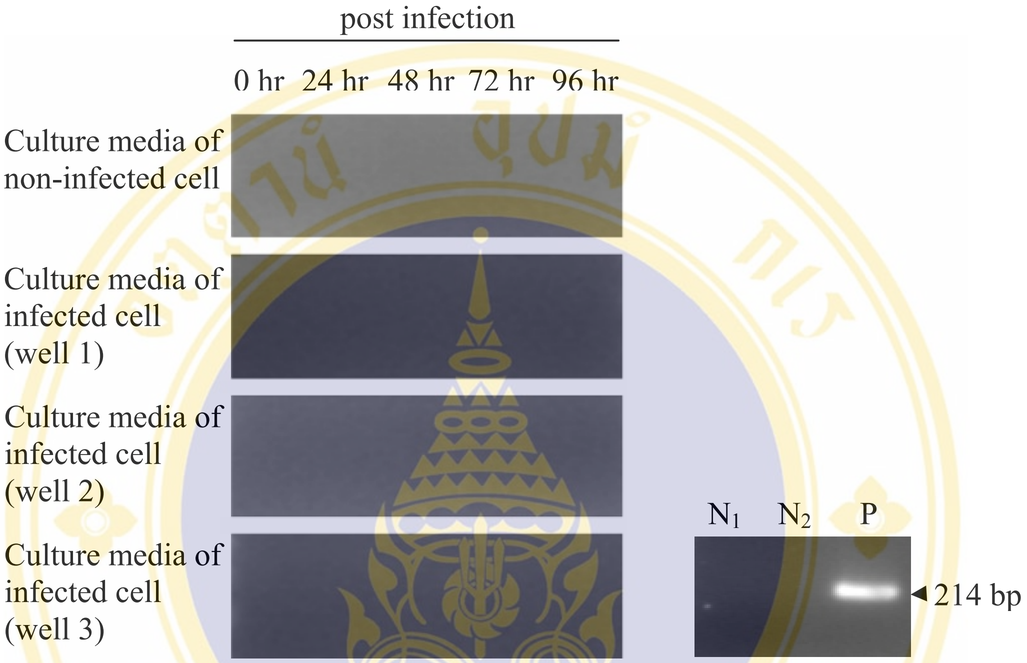


Figure 21: RT-PCR analysis for the presence of TSV in culture media of TSV-infected Oka cells

Primary Oka cells were infected with haemolymph from shrimp infected with TSV for 14 hours. Oka cells were then washed twice. Culture media were collected at 0, 24, 48, 72 and 96 hours post infection. Culture media were analyzed by RT-PCR for determination of viral replication at time point. The time course experiments were done in triplicate.

Lane N_1: Negative RT-PCR control for TSV
Lane N_2: Negative PCR control for TSV
Lane P : Positive control for TSV

5.5.4 Optimization of the dosage of virus inoculation

To estimate the quantity of virus particle for inoculation, three dilution of virus from Chanthaburi isolate were prepared as follows: 1:10, 1:50 and 1:100 dilutions. Each of dilution was injected intramuscularly into experimental shrimps. Negative control group was nonexposure shrimps. Symptoms on the infected shrimps and death were observed daily with all dilutions. However, no symptom was observed on the infected shrimps until the end of experiment at all dilution and most of shrimps can evidently remain alive despite TSV infection. The recently dead shrimps or the survivors on the final day (Day 5) of the experiment were sacrificed. Total RNA prepared from gill samples were used for analysis by RT-PCR. From the results shown in Figure 22, there was a difference in the level of viral infection caused by the different dilution. At the lowest (1:100) dilution, the 214 bp band of TSV were observed in shrimps sacrificed on Day 3 and 4 postinfection, but not in shrimp died on Day 1 and the degree of viral infection in infected shrimps was decreased over time. The highest level of virus was observed on Day 3 postinfection while the lower level of virus was seen on following day. These results indicated that the virus was incapable of producing infection at this dilution. At 1:50 dilution, all of sacrificed shrimps on Day 3 and 4 postinfection showed expected band of TSV. The level of virus observed in shrimps that were sacrificed on Day 3 postinfection was comparable to those that were sacrificed on Day 4 postinfection, suggesting that 1:50 dilution was sufficient to maintain virus infection in shrimp. Unlike 1:100 dilution, the TSV RNA was observed in shrimps died as early as Day 1 postinfection at 1:10 dilution. However, the level of virus was slightly increased in shrimps sacrificed on Day 4 postinfection when compared to shrimps sacrified on Day 3 postinfection. As expected, specific band of TSV were not detected in noninfected shrimps which served as negative control. Therefore, the reasonable inoculation dosage for TSV should be 1:50 dilution.

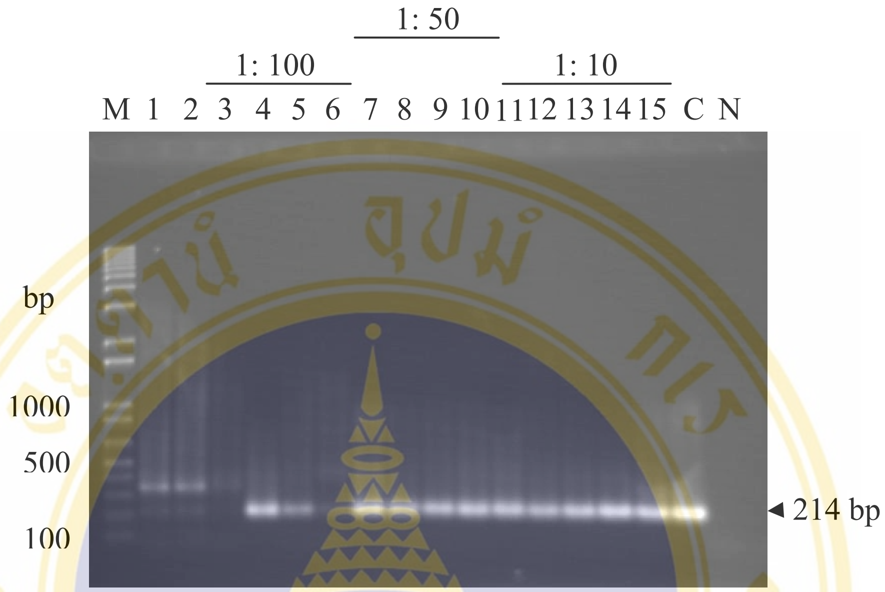


Figure 22: Infectivity of TSV after infection in *P. vannamei* with different dilution of virus

Shrimps were infected with TSV at 1:10, 1:50 and 1:100 dilutions of virus during the course of experiment. Total RNA was extracted from the gill of individual shrimp for determination of viral replication by RT-PCR assay.

Lane M: 100 bp DNA ladder
Lane1-2: uninfected shrimp
Lane 3: infected shrimp at Day 1 p.i.
Lane 4: infected shrimp at Day 3 p.i.
Lane 5: infected shrimp at Day 4 p.i.
Lane 6: infected shrimp at Day 4 p.i.
1:100 dilution
Lane 7: infected shrimp at Day 3 p.i.
Lane 8: infected shrimp at Day 3 p.i.
Lane 9: infected shrimp at Day 4 p.i.
Lane 10: infected shrimp at Day 4 p.i.
1:50 dilution
Lane 11: infected shrimp at Day 1 p.i.
Lane 12: infected shrimp at Day 3 p.i.
Lane 13: infected shrimp at Day 3 p.i.
Lane 14: infected shrimp at Day 4 p.i.
Lane 15: infected shrimp at Day 4 p.i.
1:100 dilution
Lane C : Positive control for TSV
Lane N : Negative RT-PCR control

5.6 Silencing of TSV replication in shrimp by dsRNA

Since live *P. monodon* and Oka cells were unable to use as a system for evaluating virus specific dsRNA capable of inhibiting viral replication by RNA interference assay, the live *P. vannamei* was used instead.

5.6.1 Effects of dsRNAs on the viral RNA level (RNAi experiment 1)

The dsRNAs targeted three different regions such helicase, protease and polymerase on the TSV genome were generated *in vitro* as described in method. To investigate the inhibitory effects of dsRNAs on TSV replication, 25 µg of dsRNAs specific to those regions were injected intramuscularly into shrimp, followed by TSV infection at 1:50 dilution. Equal amount of total RNA from gill tissues were subjected to RT-PCR analysis after 3 days postinfection. The level of viral RNA was monitored as an index of viral replication by RT-PCR in order to show the inhibitory effects of viral replication in live shrimp by dsRNAs. Figure 23a showed the level of viral RNA as a result of viral replication after 72 hr postinfection in TSV infected shrimps. In shrimps that were administered with TSV specific dsRNAs such conventional dsRNAs of helicase and protease (Figs. 23b-c) and stem loop dsRNAs of helicase and polymerase (Figs. 23e-f) had the reduction effect when compared with shrimps that were TSV infected shrimps (Figs. 23a).The result showed that these specific dsRNAs are able to interfere with viral replication, except for the conventional dsRNA of polymerase which was ineffective in reducing the level of virus. Additionally, the inhibitory effects of these virus specific dsRNAs were observed and shown in figure 26b-f. In comparison, we found that the conventional dsRNA of helicase was the strongest silencer, followed by conventional dsRNA of protease (Fig. 23c) and stem loop dsRNAs of helicase (Fig. 23e) and polymerase (Fig. 23f). To demonstrate the specificity of the dsRNAs targeting, the conventional dsRNA of GFP with no homology to TSV sequence was used as unrelated dsRNA. The level of viral RNA observed in shrimps administered with conventional dsRNA of GFP (Fig. 23g) was comparable to that observed in infected shrimps (Fig. 23a). With this result, we showed that these TSV specific dsRNAs, except for the conventional dsRNA of polymerase could specifically inhibit the expression of TSV mRNA and the replication of TSV. These results also indicated that conventional dsRNA of GFP had

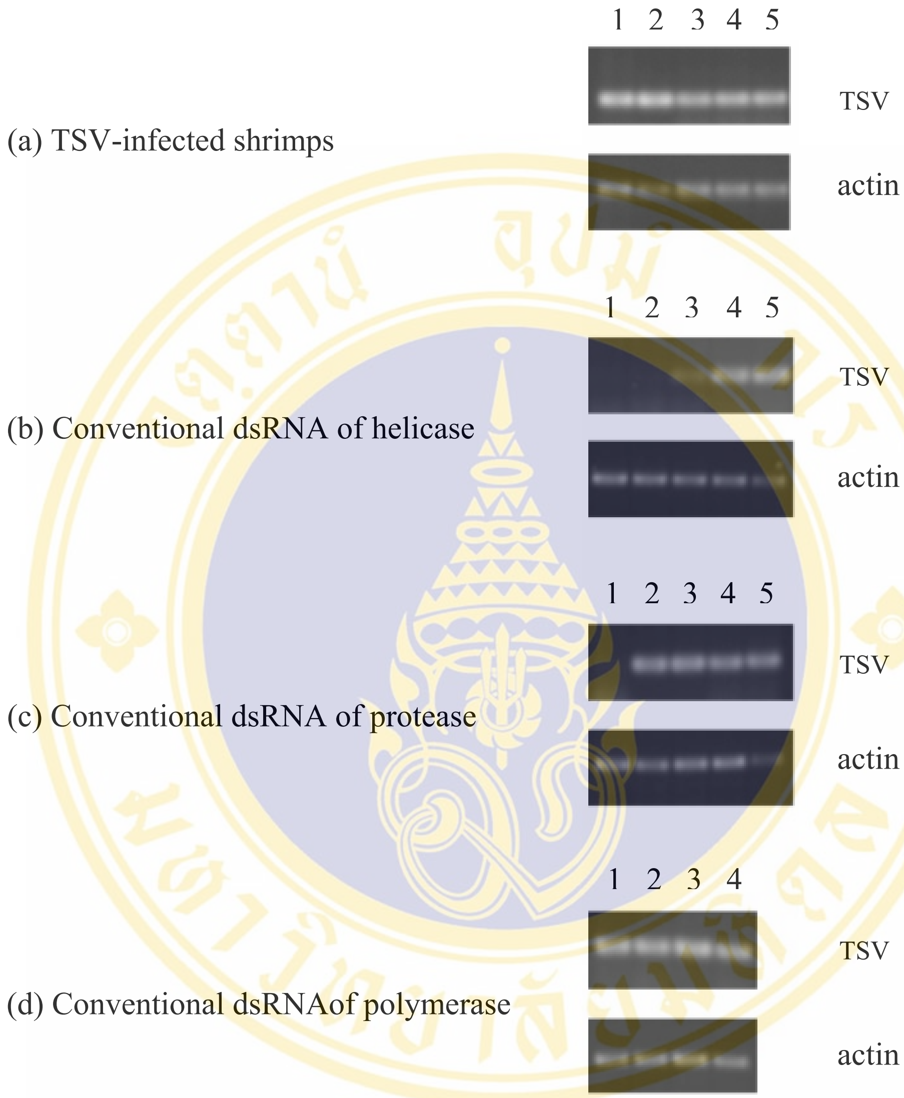
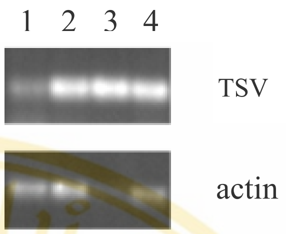


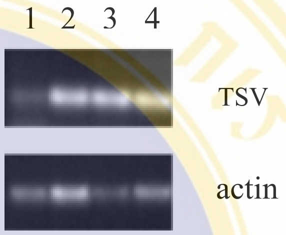
Figure 23: RNAi experiment 1; inhibition effects of dsRNAs on TSV replication in live *P. vannamei*

Shrimps were infected with TSV alone or TSV 24 hours after dsRNAs administration (25 µg) as indicated. After 3 days post infection, the viral replication was monitored in gill samples by RT-PCR. The levels of TSV in each shrimp were shown in the upper panel. β-actin shown in the lower panel served as internal control. Number represents RNA from individual shrimp.

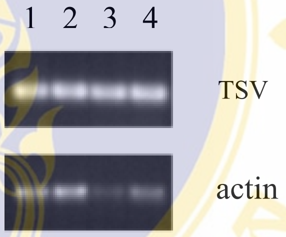
(e) Stem loop dsRNA of helicase



(f) Stem loop dsRNA of polymerase



(g) Conventional dsRNA of GFP



(h) Non-infected shrimps

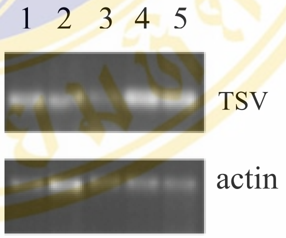


Figure 23: RNAi experiment 1; inhibition effects of dsRNAs on TSV replication in live *P. vannamei* (Continued)

no inhibitory effect on viral replication in shrimps. But unexpectedly, TSV RNA was also observed in non-infected shrimps (Fig. 23h), suggesting that shrimps might contaminate with TSV during the course of experiment or were chronic infection during cultivation. However, these data suggested that these specific dsRNAs were capable of suppressing TSV replication.

5.6.2 Effects of dsRNAs on the viral RNA level (RNAi experiment 2)

To eliminate the possibility that shrimps were contaminated with TSV during the course of experiment; the similar experiment was conducted carefully in which new material such as water tank, air tubing and air stone were replaced. Nevertheless, there was a bit difference from previous experiment in which higher dose of dsRNA (35µg) was injected into shrimps and the level of the viral RNA was assessed by 2 days postinfection using both haemolymph and gill samples. We speculated that if shrimps themselves were chronic infection with TSV, we would observe a band of virus in non-infected shrimps. Surprisingly, we still observed the viral level in some non-infected shrimps (Fig. 24h). These results clearly demonstrated that shrimps were infected with TSV during cultivation. As shown in figure 24a, all six samples of TSV infected shrimps were positive for TSV. Three out of six samples had a very low level of virus while the rest of them gave a strong amplification of TSV. Nonetheless, these results were similar to those obtained from the first experiment in which virus specific dsRNAs including conventional dsRNA of polymerase had the trend to suppress the replication of TSV in shrimps as demonstrated by the reduction in viral RNA level in shrimps administered with virus specific dsRNAs (Figs. 24b-f). Indeed, the conventional dsRNA of helicase still showed the greatest inhibitory effect on TSV replication. Also, the conventional dsRNA of protease and polymerase and the stem loop dsRNAs of helicase and polymerase could suppress the level of virus. Interestingly, unrelated dsRNA showed a little inhibitory effect on TSV replication as demonstrated by the injection of conventional dsRNA of GFP (unrelated dsRNA) resulted in the decrease in the TSV level in some shrimps. These observations found in gill tissues, but not haemolymph tissues.

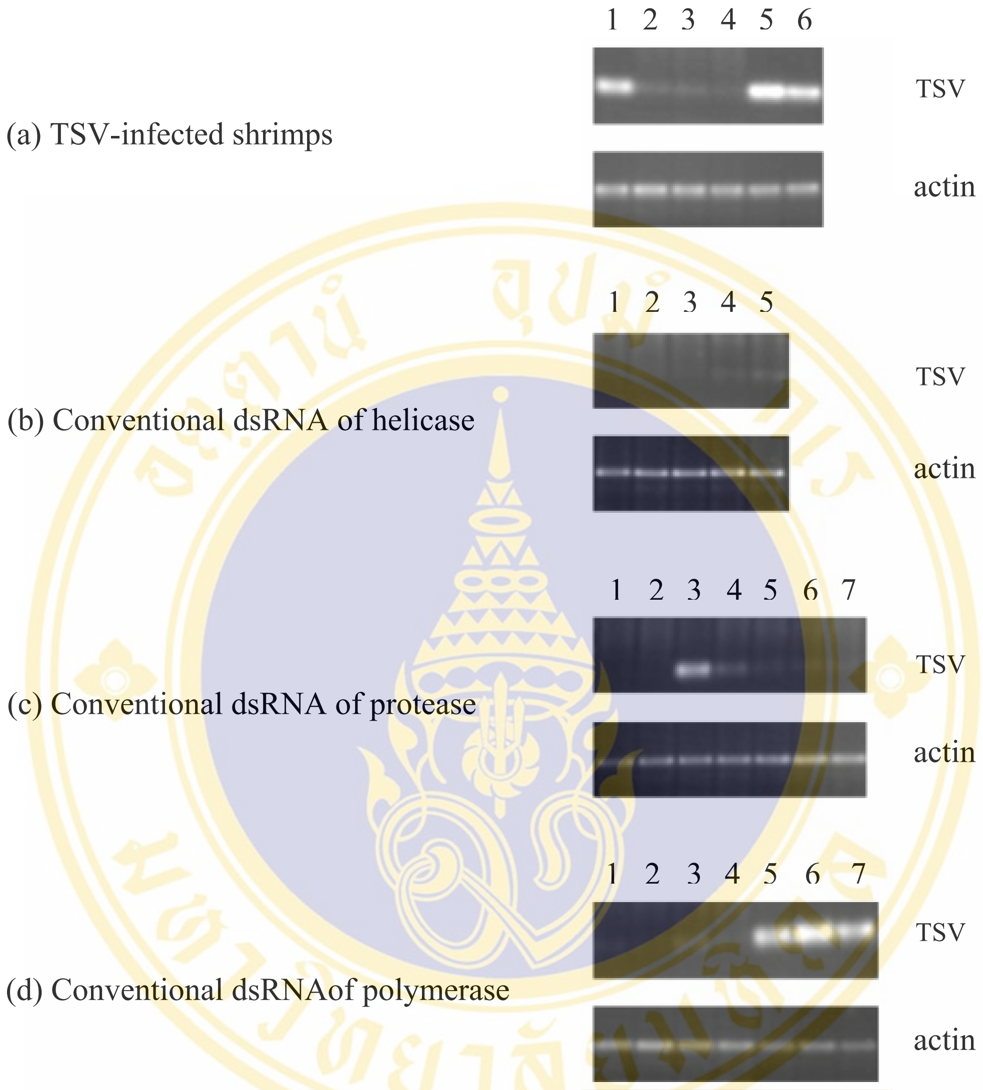
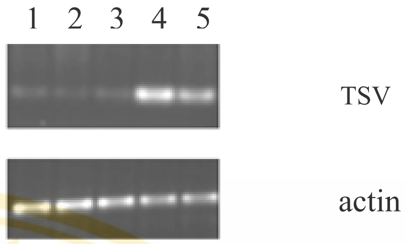
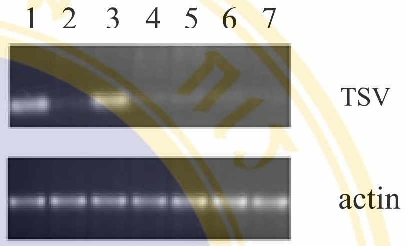


Figure 24: RNAi experiment 2; inhibition effects of dsRNAs on TSV replication in live *P. vannamei* (gill sample)

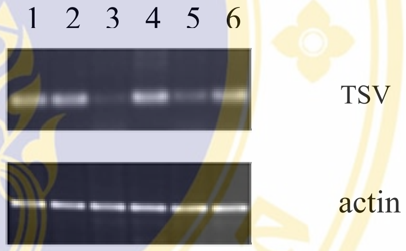
Shrimps were infected with TSV alone or TSV 24 hours after dsRNAs administration (35 μg) as indicated. After 2 days post infection, the viral replication was monitored in gill samples by RT-PCR assay. The levels of TSV in each shrimp were shown in the upper panel. β-actin shown in the lower panel served as internal control. Number represents RNA from individual shrimp.



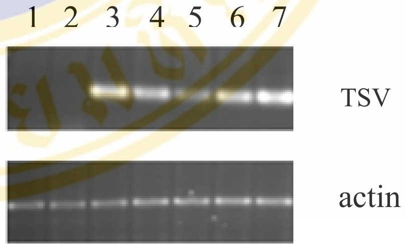
(e) Stem loop dsRNA of helicase



(f) Stem loop dsRNA of polymerase



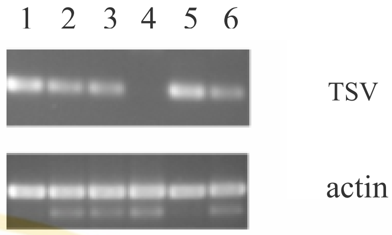
(g) Conventional dsRNA of GFP

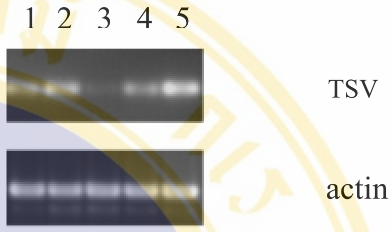


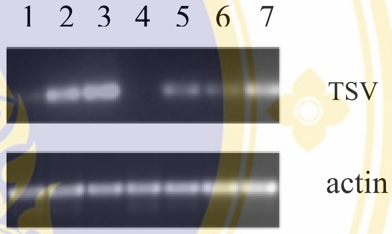
(h) Non-infected shrimps

Figure 24: RNAi experiment 2; inhibition effects of dsRNAs on TSV replication in live *P. vannamei* (gill sample) (Continued)

Due to variation in the viral level that observed in gill samples, we therefore monitored the viral level in haemolymph samples (Fig. 25). The RT-PCR obtained from haemolymph samples was different from gill samples. The levels of virus observed in haemolymph of TSV infected shrimps were higher than that of gill samples of the corresponding shrimp. Similar results were obtained in which higher level of virus was observed in the haemolymph samples. Although there was a difference in the viral suppression levels between gill samples and haemolymph samples, as shown in figure 24 and 25. We could not strongly conclude that virus specific dsRNAs had inhibitory effects on TSV replication since the level of virus observed in TSV infected shrimps varied from very low level to high level and experimental shrimps were infected with TSV before. However, all these data showed the trend of inhibitory effects by using dsRNAs targeting the helicase, protease and polymerase regions in TSV in shrimps that were infected with TSV.


(a) TSV-infected shrimps


(b) Conventional dsRNA of helicase


(c) Conventional dsRNA of protease

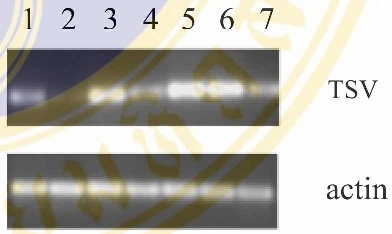
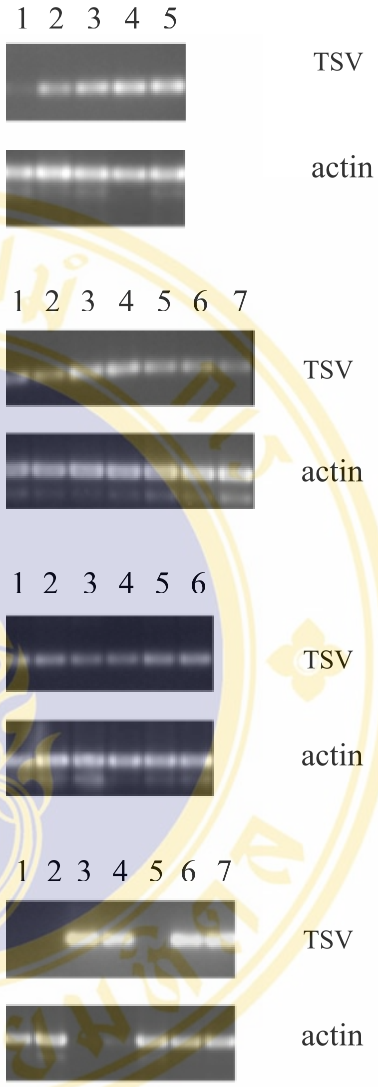

(d) Conventional dsRNAof polymerase

Figure 25: RNAi experiment 2; inhibition effects of dsRNAs on TSV replication in live *P. vannamei* (haemolymph sample)

Shrimps were infected with TSV alone or TSV 24 hours after dsRNAs administration (35 µg) as indicated. After 2 days post infection, the viral replication was monitored in haemolymph samples by RT-PCR assay. The levels of TSV in each shrimp were shown in the upper panel. β-actin shown in the lower panel served as internal control. Number represents RNA from individual shrimp.



(e) Stem loop dsRNA of helicase

(f) Stem loop dsRNA of polymerase

(g) Conventional dsRNA of GFP

(h) Non-infected shrimps

Figure 25: RNAi experiment 2; inhibition effects of dsRNAs on TSV replication in live *P. vannamei* (haemolymp sample) (Continued)

CHAPTER VI
DISCUSSION

The poor performance, slow growth rate and disease susceptibility of the major cultured shrimp species, *P. monodon*, in Thailand have led many shrimp farmers shifted to cultivation of *P. vannamei*. Since then *P. vannamei* has risen in popularity to become the second species of shrimp farmed in Thailand. However, *P. vannamei* is known to be carriers of viral diseases such as TSV and WSSV. Unfortunately, the illegal importations of cheaper, non-disease free shrimp broodstocks have led to introduction of TSV to Thailand. As a consequent, TSV outbreak occurred in early 2003. Due to the lack of its effective means for controlling and prevention, the incident of TSV seems to continue with higher impact (7). Hence, development of effective method to control disease caused by this virus as well as other viruses would help to prevent economic lost of shrimp culture industry.

In this study, three isolates of TSV were collected from infected *P. vannamei* from farms in Samut Sakorn, Chanthaburi and Rayong, respectively. Nucleotide sequences ORF1 enncoding for nonstructural polyproteins (helicase, protease and RdRp) of these TSV isolates were determined and analyzed. The resulting nucleotide sequence identity of these isolates showed that they varied slightly from each other (range from 97.8 to 99.2%). In addition, these sequences were approximately 3% difference from that of the Hawaii reference sequence reported by Mari J. *et al.* (2002). These results clearly demonstrated the close relationship of our three TSV isolates to the Hawaii reference isolate.

Of the nucleotide changes found in our study, about one-third of these changes resulted in amino acid alterations. The Samut Sakorn isolate has the total of 143 nucleotides different from Hawaii isolate, of which 43 amino acid were changed with non-synonymous substitutions among the nucleotide differences. In case of the

Chanthaburi isolate, there were 47 non-synonymous amino acid changes among the 160 nucleotide difference against the Hawaii isolate. Surprisingly, single base change at the position 669 nucleotide gives rise to a new stop codon in the BIR region in Rayong isolate. This mutated position was possibly due to copy errors introduced by the DNA polymerase during PCR amplification and cloning procedure. On the other hand, another explanation is that the Rayong isolate consisted of quasispecies populations since RNA viruses have high mutation rates and this termination mutation might represent a minor variant within the viral population. If it does so, it is interesting to note that nonsense mutation in Rayong isolate may demolish the viral replication as it causes the truncation of protein synthesis.

Careful examination of the deduced amino acid sequence in helicase, protease and RdRp proteins of these isolates compared to the reference sequence of Hawaiian isolate revealed that all invariant residues reside inside the 3 conserved motifs of helicase are identical (Figure 11). Similarly, sequence in the catalytic domains of protease particularly the residues proposed to involve in the catalytic triad and in substrate binding of protease are also identical (15). A slight change was found in RdRp domain as one residue at position 2007 (motif VII) was found mutate (serine → asparagine). This change appeared as the characteristic among the Thai isolates. However identical sequences were found among the other seven motifs (motif I-VI and VIII). Thus, the result implicates that structural or functional properties of these catalytic proteins are not different. Since TSV used for PCR amplification and sequence determination in this study were from field specimens. Thus, the sequence showed here should represent the major or dominant sequence of the TSV in each isolate. However, we cannot exclude the variation derived from quasi-species in the viral population that is quite common among RNA virus (74). The fact that pattern of nucleotide substitutions in ORF1 of the Thai isolates compared to the reference isolate disperse thoroughly in the ORF1 without the sign of hot spot for substitutions. This is quite different from sequence variation in the structural genes (ORF2) of TSV where higher degree of mutation could be observed in cluster which might provide benefit to TSV infectivity. From this scenario suggest that nucleotide sequence within ORF1 of TSV should be a good target for RNAi mediated viral suppression experiment.

In this study, two approaches for dsRNA, conventional and stem loop structure, synthesis were chosen for comparison, both of which yield similar quality of dsRNA essential to initiate RNAi pathway. While the conventional dsRNA required synthesis two complementary strands prior to proceeding annealing of the two complementary strands has been proven a general and effective method for synthesizing RNAi inducer in many organism (63, 75). Generation of dsRNA as hairpin structure with long perfect complementary stem, up to 0.5 kb, was shown possible. The advantages of this alternative approach are: 1) it requires only a single *in vitro* transcription reaction and 2) the inverted repeat on both ends of the transcript could automatically form hairpin structure without additional step. Hence, the stem loop approach seems more convenient and cost effective. Indeed, in our hand the quality of the hairpin RNA appears better quality since more prominent form of intact hairpin RNA, the precursor of dsRNA, was obtained. Thus synthesizing large quantity of this stem loop RNA for *in vivo* testing its inhibitory effect on TSV replication seems more appropriate.

In our preliminary experiments on TSV infectivity in *P. vannamei*, we demonstrated that viral inoculum prepared from crude extracts of TSV infected specimen was infectious. Although TSV multiplication and mortality was observed subsequent to infection, we were unable to observe the clinical signs of TSV infection in our experimental infected shrimps. TSV generally infects juvenile *P. vannamei* (0.1-0.5g) within two to four weeks of stocking into grow-out ponds whereas bigger shrimps e.g. subadult (12.5-18.5g) are known to be more resistant than the juvenile shrimps (76). Since we used subadult size of *P. vannamei* to test viral infectivity, this might explain why clinical signs of Taura syndrome disease were not observed.

In addition, we also investigated the susceptibility of *P. monodon* to TSV infection. The result obtained from our study implicates that *P. monodon* could be experimentally infected with TSV. However, the level of virus that detected in haemolymph sample of *P. monodon* was lower than that detected in *P. vannamei*. Nevertheless, TSV did cause neither mortality nor disease symptoms during the period of our experiment, it is possible that *P. monodon* is less susceptible to TSV infection than *P. vannamei*. This result is similar to the earlier observation by other group who demonstrated that cultured *P. monodon* originating from Indonesia was found RT-

PCR positive for TSV (3). *P. monodon* is therefore served as potential carriers of TSV. Alternatively, the absent of clinical signs of TSV infected black tiger shrimps may resulted from probably other factors involving the host, virus and environmental conditions.

Since the system for culturing primary lymphoid cell (Oka cells) of *P. monodon* has been well established in our laboratory. This primary cell is highly susceptible to YHV and shown as excellent model to investigate RNAi mediated Yellow head virus suppression mechanism (67). The clear phenotypic changes are readily observed upon YHV infection and RNAi mediated YHV inhibition. We therefore attempted to investigate whether this system can be applied for testing TSV suppression by RNAi. First, we investigated the susceptibility of this primary lymphoid cell to TSV infection. Oka cells were infected with TSV then we monitored the level of virus at various times of after inoculation as an index of viral replication in either cell or culture media by RT-PCR analysis. Only in one third of cell lysates was detected positive by TSV, unexpectedly, we were unable to show the presence of TSV in culture mediums of any of these triplicate. Base on this contradicted result it is possible that TSV detected in one of the sample may have been the result of residual extracellular viruses that remains in the cell culture plate after washing or TSV was very badly infected Oka cells so it could hardly make a copy of itself. Therefore, our result showed that TSV was very poor capable of infecting Oka cells suggesting that the primary lymphoid cells of *P. monodon* are not a good model for studying TSV inhibition.

The failure to infect TSV in Oka cells led us to use live *P. vannamei* as study model. The evaluation of silencing effect of dsRNA molecules for inhibiting viral replication by RNAi might depend on the amount of virus that use to cause persistent infection and thus also effects on the interpretation. We therefore, investigated the infectivity of TSV upon three dilutions, which might contribute to the suitable dilution that should be used to in our RNAi experiment. However, our data suggested that *P. vannamei* infected with crude lysate at 1:50 dilution is optimal. With this condition consistent TSV replication could be observed with 72 hr post inoculum.

To investigate the inhibitory effects of dsRNA molecules on TSV replication, *P. vannamei* were injected with designated dsRNA 24 hr prior to TSV challenge. The inhibitory effects of dsRNAs on TSV replication and viral mRNA transcripts were investigated by the analyzing the levels of viral RNA by RT-PCR analysis. Results from the first experiment showed the trend that virus specific dsRNAs particularly dsRNA targeting helicase can suppress TSV replication as the level of TSV was significantly reduced or to undetectable level in some shrimps. It is of interested to note that the suppressive effects of each TSV specific dsRNA are not equal. On the contrary, dsRNA targeting polymerase region did not seem to exhibit inhibitory effect. Whereas unrelated dsRNA of GFP exhibit no inhibitory effect on TSV. Our subsequence results also confirmed that conventional dsRNAs of helicase, protease and polymerase and stem loop dsRNAs of helicase and polymerase had suppressive effects on viral mRNA transcripts and viral replication even though the differences in the ability of dsRNAs interfere with TSV replication was observed. The differences in the ability of each dsRNA targeted to different regions of the same mRNA transcript suggested that target accessibility of the viral genome plays an important for RNAi effect. As not all virus specific dsRNAs were equally effective; among the dsRNAs tested, dsRNA directed against a helicase region was the most efficient while dsRNA directed against a polymerase region hardly had inhibitory effect on viral replication. These results may be due to a highly secondary structure of the polymerase, which may leave few single stranded gaps that siRNAs can access as described for other viruses such as poliovirus (77) In contrast, some regions of the helicase are accessible to the RNAi machinery. These data also suggested that the most effective site for the inhibition of TSV replication may locate within the helicase region. In the second experiment, higher amount of dsRNAs were introduced into shrimps and viral level was assessed 2 day postinfection. Although the dose dependence of the dsRNA effect was not investigated in this RNAi study, there seems increasing suppression of viral RNA level as injection of the increasing amount of dsRNA. Thus, this experiment confirmed the trend of inhibitory effects on TSV replication by using virus specific dsRNAs. The sequence independent antiviral immune response in shrimp as reported early by Rabalino *et al* (78) was not clearly observed in our study. To our knowledge, the introduction of TSV specific dsRNAs tended to reduce the viral replication in shrimps and this is likely due to the induction of RNAi-related mechanism that exists in shrimp (68). Our unexpected finding that dsRNA mediated inhibitory effect on TSV observed only in some shrimps may be due to insufficient level of dsRNA introduced into the shrimps since these dsRNA mediated suppression is known as concentration dependent phenomenon (61, 67, 79).

In fact, we examined the TSV RNA level as an index of viral replication in order to determine the dsRNA effect on TSV inhibition. TSV-specific primers were used to amplify a 214 bp fragment in the viral genome and its transcripts. The results from this primer pair were not enough to strengthen the reliability of virus specific amplification. In addition, the same primer pairs may amplify fragmented and/or incomplete TSV genome since it targets sequence close to the 5' region. Therefore, we suggest that another primer pair will be needed to amplify other TSV region or, alternatively, histopathology could be used as a confirmation method for TSV.

The most surprising result is that TSV was detected in our control group of shrimps which were not challenged with the virus. This detection was not due to carry over or cross contamination of PCR product since the amplification was observed only when reverse transcription was performed. Despite contamination control during the experiment, there is a risk of TSV transfer involved in other possible sources including water supply and viral exchange between shrimp. As regarded to the strategy used to prevent virus contamination, the most likely explanation is that *P. vannamei* used in our experiment was already infected with TSV. If this is the case, it might explain the inefficient suppression of TSV replication in our experiment since it is known that the presence of corresponding virus particularly at high level could compromise the efficiency of RNAi.

Although our results on TSV suppression by RNAi present so far are quite convincing, more experiments using better control condition is necessary to further investigate for example the use of SPF shrimp. Unlike the potential use of RNAi technology in therapeutic application in human or big animal where direct administration is possible, the direct application of protective dsRNA into shrimp is impossible. Although it is not an over expectation that RNAi would exhibit inhibitory effect on TSV or other virus in shrimp, the ultimate goal of applying this technique to control the viruses in farm is a real challenge. Several major questions remain to be address for example by which route the inhibitory dsRNA will be introduced.

CHAPTER VII

CONCLUSION

7.1 Three TSV isolates identified from infected shrimp samples were collected during the TSV outbreaks in white shrimp farming and were originated in Thailand; Samut Sakorn, Chanthaburi and Rayong.

7.2 Comparison of TSV samples isolated from naturally infected *P. vannamei* from Thailand and Hawaii reported by Mari J. *et al.* demonstrate that there were minor variations at nucleotide (97.8-99.2% identity) and amino acid (99.3% identity) levels in the cultured shrimp host in central of Thailand. The Samut Sakorn isolate has more closely related with the Hawaii isolate than other Thai isolates.

7.3 By using the RT-PCR technique, TSV has been detected in the haemolymph and gill tissues of *P. vannamei* with experimentally induced TSV infection. In contrast, TSV has only detected in the haemolymph of the most common cultured *P. monodon*, thus, these should be considered for the potential carriers of the Taura syndrome disease.

7.4 The result from the infectivity tests showed that the TSV inoculum prepared from TSV infected shrimp was viable and infectious. Nevertheless, TSV was poorly infected and multiplied in the primary cell culture prepared from lymphoid organ of *P. monodon*. Therefore, it can not be use as system for viral replication for evaluating the inhibitory effects of virus specific dsRNAs by RNAi.

7.5 In RNAi experiment, we have shown that TSV specific dsRNAs could possibly exhibit antiviral activity in TSV infected shrimps as in the case of other penaeid shrimps viruses. The TSV replication as well as viral mRNA transcripts can be suppressed by dsRNAs specific for different viral genes. DsRNA targeting helicase had the most suppressive effect on the level of TSV in shrimps.

REFERENCES

1. Aquastar Laboratories Ltd. The Role of Shrimp Culture in Economic Changes, Bangkok 1994.
2. Sullivan GO. Shrimp Market Report. FAO GlobeFish. 2005.
3. Briggs M, Simon SF, Subasingheand R, Phillips M. Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. RAP Pulication, Bangkok 2004.
4. Wyban J. White shrimp boom continues. Global Aquaculture Advocate 2002; 18-19.
5. Tu C, Huang HT, Chuang SH, Hsu JP, Kuo ST, Li NJ et al. Taura syndrome in Pacific white shrimp *Penaeus vannamei* cultured in Taiwan. Diseases of Aquatic Organisms 1999; 38: 159-161.
6. Yu CI, Song YL. Outbreaks of Taura syndrome in pacific white shrimp *Penaeus vannamei* cultured in Taiwan. Fish Pathology 2000; 35(1): 21-24.
7. Nielsen L, Sang-oum W, Cheevadhanarak S, Flegel TW. Taura syndrome virus (TSV) in Thailand and its relationship to TSV in china and the Americas. Diseases of Aquatic Organisms 2005; 63: 101-106.
8. Farfante IP. Western Atlantic shrimps of the genus *Penaeus*. Fishery Bulletin 1969; 67(3): 461-591.
9. Tiger Prawn (*Penaeus monodon*) & White legged Shrimp (*P. vannamei*). Aquaculture report. 2003.
10. Fast AW. Development of appropriate and economically viable shrimp pond grow-out technology for the United States. 1991; 197-239.
11. Lee DO, Wickins JF. Crustacean Farming. Blackwell Scientific publications, Oxford 1992.
12. Nakano H, Koube H, Umezawa S, Momoyama K, Hiraoka M, Inouye K et al. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in

Japan in 1993-epizootiological survey and infection trials. Fish Pathology 1994; 29: 135-139.

13. Chou HY, Huang CH, Chiang HC, Lo CF. Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Diseases of Aquatic Organisms 1995; 23(3): 165-173.

14. Tang KFJ, Poulos BT, Wang J, Redman RM, Lightner DV. Geographic variations among infectious hypodermal and haematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection. 2002.

15. Larramore SE, Montiel de Moprales M, Laramore CR, Scarpa J. Putative attenuated infectious hypodermal and haematopoietic necrosis (IHHN) virus: case study with *Litopenaeus vannamei*. 2002.

16. Gabriel GA, Felipe AV. Infectious disease in shrimp species with aquaculture potential. Resent Res. Devl. Microbiology 2000; 4: 333-348.

17. Hasson KW, Lightner DV, Poulos BT, Redman RM, White BL, Brock JA et al. Taura syndrome in *Penaeus vannamei*: demonstration of a viral etiology. Diseases of Aquatic Organisms 1995; 23: 115-126.

18. Lightner DV. A handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. 1996.

19. Bonami JR, Hasson KW, Mari J, Poulos BT, Lightner DV. Taura syndrome of marine penaeid shrimp: characterization of the viral agent. Journal of General Virology 1997; 78: 313-319.

20. Mari J, Bonami JR, Lightner DV. Shrimp Taura syndrome virus: genomic characterization and similarity with members of genus *Cricket paralysis-like viruses*. Journal of General Virology 2002; 83: 915-926.

21. Lighter DV, Redman RM, Hasson KW, Pantoja CR. Taura syndrome in Penaeus vannamei (Crustacea: Decapoda): Gross signs, histopathology and ultrastructure. Diseases of Aquatic Organisms 1995; 21(1): 53-59.

22. Brock JA, Gose R, Lightner DV, Hasson KW. An overview on Taura syndrome important disease of farmed *Penaeus vannamei* swimming through troubled water, Proceedings the special session on shrimp farming, Aquculture '95. 1995; 84-94.

23. Poulos BT, Kibler R, Bradley-Dunlop D, Mohney LL, Lightner DV. Production and use of antibodies for the detection of the Taura syndrome virus in penaeid shrimp. Diseases of Aquatic Organisms 1999; 37: 99-106.

24. Hasson KW, Hasson J, Aubert H, Redman RM, Lightner DV. A new RNA-friendly fixative for the preservation of penaeid shrimp samples for virological detection using cDNA genomic probes. J. Virol. Methods 1997; 66: 227-236.

25. Mari J, Bonami JR, Lightner DV. Taura syndrome of Penaeid shrimp: cloning of viral genome fragments and development of specific gene probes. Diseases of Aquatic Organisms 1998; 33: 11-17.

26. Nunan LM, Poulos BT, Lightner DV. Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura Syndrome Virus (TSV) in experimentally infected shrimp. Diseases of Aquatic Organisms 1998; 34: 87-91.

27. Hasson KW, Lightner DV, Mohney LL, Redman RM, Poulos BT, Mari J. The geographic distribution of Taura Syndrome Virus (TSV) in the Americas: determination by histology and *in situ* hybridization using TSV-specific cDNA probes. Aquaculture 1999; 171: 13-26.

28. Medzhitov R, Janeway CA. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997; 91: 295-298.

29. Arason GJ. Lectins as defence molecules in vertebrates and invertebrates. Fish & Shellfish Immunol 1996; 6: 277-289.

30. Murakami T, Niwa M, Tokunaga F, Miyata T, Iwanaga S. Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. Chemotherapy 1991; 37: 327-334.

31. Brown JH. Antibiotics: their use and abuse in aquaculture. Aquaculture 1989; 20: 34-43.

32. Agrawal N, Dasaradhi PVN, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK. RNA Interference: Biology, Mechanism, and Applications. Microbiology and Molecular Biology Reviews 2003; 67: 657-685.

33. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 2001; 15: 188-200.

34. Tuschl T, Borkhardt A. Small interfering RNAs. Mol. Intervent 2002; 2: 158-167.

35. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409: 363-366.

36. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 1999; 286: 950-952.

37. Hammond SM, Berstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 2000; 404: 293-296.

38. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RHA. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. 2001; 15: 2654-2659.

39. Nykanen A, Haley B, Zamore PD. ATP requirement and small interfering RNA structure in the RNA interference pathway. Cell 2001; 107: 309-321.

40. Fagard M, Boutet S, Morel J-B, Bellini C, Vaucheret H. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. 2000; 97: 11650-11654.

41. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 2001; 239: 1146-1150.

42. Caudy AA, Myers M, Hannon GJ, Hammond SM. Fragile X-related protein and VIG associate with RNA interference machinery. Genes Dev. 2002; 16: 2491-2496.

43. Dykxhoorn DM, Novina CD, Sharp PA. Killing the Messenger: Short RNAs that Silence Gene Expression. Nature Revs. Mol. Cell Biol. 2003; 4: 457-467.

44. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. 2001; 15: 188-200.

45 Hammond SM. Dicing and slicing The core machinery of the RNA interference pathway. FEBS letters.

46. Cogoni C, Macino G. Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature 1999; 399: 166-169.

47. Fire A. RNA triggered gene silencing. Trends Genet. 1999; 15: 358-363.

48. Hutvagner G, Zamore PD. RNAi: nature abhors a double strand. Current Opinion in Genetics and Development 2002; 12: 225-232.

49. McMannus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. Nature Reviews Genetics 2002; 10: 737-747.

50. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411: 494-498.

51. Bohmert K, Camus I, Bellini C, Bouchez D, Caboche M, Benning C. AGO1 defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J 1998; 17: 170-180.

52 Lynn K, Fernandez A, Aida M, Sedbrook J, Tasaka M, Masson P, Barton MK. The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. Development 1999; 126: 469-481.

53. Smardon A, Spoerke J, Stacey S, Klein M, Mackin N, Maine E. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Curr Biol 2000; 10: 169-178.

54. Hannon GJ. RNA Interference. Nature 2002; 418: 244-251.

55. Wassenegger M, Pelissier T. A model for RNA-mediated gene silencing in higher plants. Plant Mol. Biol. 1998; 37: 349-362.

56. Li WX, Ding SW. Viral suppressors of RNA silencing. Current Opinion in Biotechnology 2001; 12: 151-154.

57. Bitko V, Barik S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiology 2001; 1: 34-36.

58. Gitlin L, Karelsky S, Andino. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 2002; 418: 430-434.

59.Coburn GA, Cullen BR. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. Journal of Virology 2002; 76: 9225-9231.

60. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. Nature 2002; 418: 435-438.

61. Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO J 2003; 6: 602-608.

62. Wang Q, Contag CH, Ilves H, Johnston BH, Kaspar RL. Small Hairpin RNAs Efficiently Inhibit Hepatitis C IRES-Mediated Gene Expression in Human Tissue Culture Cells and a Mouse Model. Molecular Therapy 2005; 12: 562-568.

63. Sambrook J, Russell DW. Molecular Cloning a laboratory manual: volume 3. 3rd edition. Cold Spring Harbor Laboratory Press, New York 2001.

64. Sambrook J, Russell DW. Molecular Cloning a laboratory manual: volume 2. 3rd edition. Cold Spring Harbor Laboratory Press, New York 2001.

65. Kasornchandra J, Khongpradit R, Ekpanitthanpong U, Boonyaratpalin S. Progress in the developement of shrimp cell cultures in Thailand. Methods Cell Sci. 1999; 21: 231-235.

66. Assavalapsaku W, Smith DR, Panyim S. Propagation of infectious yellow head virus particles prior to cytopathic effect in primary lymphoid cell cultures of *Penaeus monodon*. Diseases of Aquatic Organisms 2003; 55(3): 253-258.

67. Tirasophon W, Roshorm Y, Panyim S. Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochemical and Biophysical Research Communications 2005; 334: 102-107.

68. Robalino J, Bartlett T, Shepard E, Prior S, Jaramillo G, Scura E et al. Double-Stranded RNA Induces Sequence-Specific Antiviral Silencing in Addition to Nonspecific Immunity in a Marine Shrimp: Convergence of

RNA Interference and Innate Immunity in the Invertebrate Antiviral Response?. Journal of Virology 2005; 79: 13561-13571.

69. Kiatpathomchai W, Jitrapakdee S, Panyimc S, Boonsaeng V. RT-PCR detection of yellow head virus (YHV) infection in *Penaeus monodon* using dried haemolymph spots. Journal of Virological Methods 2004; 119: 1-5.

70. Tsai J-M Shiau L-J, Lee H-H, Chan PWY, Lin CY. Simultaneous detection of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV)) by multiplex reverse transcription-polymerase chain reaction (RT-PCR) in Pacific white shrimp *Penaeus vannamei*. Diseases of Aquatic Organisms 2002; 50: 9-12.

71. Abels JA, Moreno-Herrero F, Heijden TVD, Dekker C, Dekker NH. Single-Molecule Measurements of the Persistence Length of Double-Stranded RNA. Biophysical Journal 2005; 88: 2737-2744.

72. Gast F-U, Sanger HL. Gel dependence of electrophoretic mobilities of double-stranded and viroid RNA and estimation of the contour length of the contour length of a viroid by gel electrophoresis. Electrophoresis 2005; 15: 1493-1498.

73. Chen SN, Wang CS. Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses. Methods in Cell Science 1999; 21: 199-206.

74. Malet I, Belnard M, Agut H, Cahour A. From RNA to quasispecies: a DNA polymerase with proofreading activity is highly recommended for accurate assessment of viral diversity. Journal of Virological Methods 2003; 109: 161-170.

75. Gott JM. RNA interference, Editing, and Modification. 1st edition. 2004.

76. Brock JA. Special topic review: Taura syndrome, a disease important to shrimp farms in the Americas. World Journal of Microbiology and Biotechnology 1997; 415-418.

77. Saulnier A, Pelletier I, Labadie K, Florence C-G. Complete Cure of Persistent Virus Infections by Antiviral siRNAs. Molecular Therapy 2005.

78. Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P et al. Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J.Virol. 2004; 78: 10442-10448.

79. Park WS, Naoko M-K, Hayafune M, Nakajima E, Matsuzaki T, Shimada F. Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Research 2002; 30(22): 4830-4835.

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