New insight into detection and evolution of Australian *Penaeus stylirostris* densovirus

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review (Approval number A1836)

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ABSTRACT

Penaeus stylirostris densovirus (*Pst*DV) has caused high mortality in *Penaeus stylirostris* and runt deformity syndrome in *Penaeus vannamei* and *Penaeus monodon*. This study aims to 1) develop a practical diagnostic approach to separate infectious *Pst*DV from endogenous viral elements, and 2) to determine the factors that underlie molecular evolution of the Australian *Pst*DV. Regarding the first, we mapped the *Pst*DV insertion patterns in the genome of Australian *P. monodon*. A detection platform based on recombinase polymerase amplification (RPA) and a lateral flow dipstick (LFD) was developed for detecting *Pst*DV based on the DNA region that is least likely to be endogenized. Under the optimal conditions, 30 min at 37 °C for RPA followed by 5 min at room temperature for LFD, the platform was 10 times more sensitive than the Saksmerphrome et al's interim 3-tube nested PCR and showed no cross-reaction with other shrimp viruses. It also reduced false positive results arising from viral inserts to ~5% compared to 76–78% by the IQ2000^{TM} nested PCR kit and the 309F/R PCR protocol currently recommended by World Organization for Animal Health for *Pst*DV detection. Together with simplicity and portability, the protocol serves as an alternative tool to PCR for screening *Pst*DV.

As for the second objective, A 1-bp deletion (1305delA) within the nuclear localization signal-encoding sequence of open reading frame 1 (ORF1; NS1) of Australian *Pst*DV was identified as a cause of 199-amino acid shortened NS1 protein and production of a second protein, C-terminal NS1. This mutation is believed to reduce virulence as it strongly modifies the characteristics of NS1, which is responsible for the majority of enzymatic activities in *Pst*DV. This finding supports a hypothesis regarding accommodation of *Pst*DV in Australian prawns in relation to viral genetics. However, a high degree of evolution (1.55×10^{-3} substitutions/site/year) for the virus, which was attributable to the viral recombination, was observed with 10 potential genomic breakpoints identified. With this finding, we suggest that awareness of the emergence of new virulent strains should be increased as a preventative measure against future outbreaks of *Pst*DV in the Australian Indo-Pacific.

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization
aa	Amino acid
*Aae*DNV	*Aedes aegypti densovirus*
*Aal*DNV	*Aedes albopictus* densovirus
AGE	Agarose gel electrophoresis
A. longinaris	*Artemesia longinaris*
Aus Grp 2	Australian *Pst*DV endemic strains (Group 2)
BEAST	Bayesian evolutionary analysis by sampling trees
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BPs	Breakpoints
BPS	Bayesian skyline plot
Bst	*Bacillus stearothermophilus*
C. angulatus	*Cyrtograpsus angulatus*
°C	Degree Celsius
CP	Capsid protein
Da	Dalton
Δc-AIC	Changes in Akaike information criteria
1305delA	Base A is deleted at the nt position 1305 of ORF1

1305insA	Base A is inserted at the nt position 1304 of ORF1
dN	Non-synonymous substitutions
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNV	Densovirus
dS	Synonymous substitution
dsDNA	Double-stranded DNA
E.coli	*Escherichia coli*
ESS	Effective sample sizes
EST	Expressed sequence taq
EVEs	Endogenous viral elements
FAM	Fluorescein amidite
FAO	Food and Agriculture Organization
FEL	Fixed Effects Likelihood
FITC	Fluorescein isothiocyanate
FN	False negative
FP	False positive
FPLV	Feline panleukopenia parvovirus
Fr1	Fragment 1 of ORF1
Fr2	Fragment 2 of ORF1
g	Gravitational force
GARD	Genetic Algorithms for Recombination Detection

GAV	Gill-associated virus
ΔG	Gibbs free energy
h	Hour
HPD	Highest posterior density
H. penicillatus	*Hemigrapsus penicillatus*
IHHNV	Infectious hypodermal and hematopoietic necrosis virus
Inc.	Incorporated
i.e.	id est
K	Lysine
kbp	Kilo base pair
kcal	Kilocalorie
kDa	Kilo Dalton
LAMP	Loop mediated DNA isothermal amplification
LB	Lysogeny broth
LFD	Lateral flow dipstick
MCMC	Markov chain Monte Carlo
MCMS	Midcrop Mortality Syndrome
min	Minute
*Mr*NV	*Macrobrachium rosenbergii* nodavirus
M. rosenbergii	*Macrobrachium rosenbergii*
µl	Microlitre

µM	Micromolar
mg	Milligram
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
MUSCLE	Multiple sequence comparison by log-expectation
MW	Molecular weight
–	Negative
NCBI	National Centre for Biotechnology Information
N_e	Effective population size
Nfo	Endonuclease IV
ng	Nanogram
NLS	Nuclear localization signal
nm	Nanometre
nt	Nucleotide
NTP	Nucleoside triphosphate
NS1	Non-structural protein 1
NS2	Non-structural protein 2
nAus Grp 1	Non-Australian *Pst*DV strains (Group 1)
OIE	Office International des Epizooties
ORF	Open reading frame
P. aztecus	*Penaeus aztecus*

P. californiensis	*Penaeus californiensis*
P. esculentus	*Penaeus esculentus*
P. japonicus	*Penaeus japonicus*
P. macrodactylus	*Palaemon macrodactylus*
*Pme*DV	*Penaeus merguiensis* densovirus
P. monodon	*Penaeus monodon*
P. setiferus	*Penaeus setiferus*
*Pst*DV	*Penaeus stylirostris* densovirus
P. stylirostris	*Penaeus stylirostris*
P. subtilise	*Penaeus subtilise*
P. vannamei	*Penaeus vannamei*
PCR	Polymerase chain reaction
pg	Picogram
PL	Postlarvae
+	Positive
%	Percentage
R	Arginine
RDS	Runt deformity syndrome
RE	Relaxed exponential clock model
REL	Random Effects Likelihood
RL	Relaxed lognormal clock model

RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
s	Second
S	Strict clock model
SSB	Single-strand DNA binding protein
ssDNA	Single-stranded DNA
SE	South East
Sau	*Staphylococcus aureus*
SLAC	Single Likelihood Ancestor Counting
$	Dollar
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
THF	Tetrahydrofuran
TN	True negative
TP	True positive
TSV	Taura syndrome virus
TYLCV	Tomato yellow leaf curl virus
U	Unit
UK	United Kingdom
USA	United State of America
UV	*Ultraviolet*

VP	Viral capsid protein
WSSV	White spot syndrome virus
YHV	Yellow head virus

LIST OF PUBLICATIONS

1. Jaroenram W, Owens L (2014). Separation of endogenous viral elements from infectious Penaeus stylirostris densovirus using recombinase polymerase amplification. Mol Cell Probe **28**: 284-287

2. Jaroenram W, Owens L (2014). Recombinase polymerase amplification combined with a lateral flow dipstick for discriminating between infectious *Penaeus stylirostris* densovirus and virus-related sequences in shrimp genome. J Virol Methods **208**:144-151

3. Jaroenram W, Chaivisuthangkura P, Owens L (2015). One base pair deletion and high rate of evolution: Keys to viral accommodation of Australian *Penaeus stylirostris* densovirus. Aquaculture **443**:40-48

CHAPTER 1

GENERAL INTRODUCTION

At present, shrimp (mostly penaeids) are viewed as a major trade fishery species due to the increase in consumer demand for these animals. This demand has driven the global shrimp industry to grow continuously at 9% a year since the 1970s (Zabbey *et al.*, 2010). According to the Food and Agriculture Organization of United Nations (FAO), global shrimp production is approximately six million metric tonnes a year, 60% of which enters the international market and is worth more than US$10 billion annually (Gillett R, 2008; http://www.fao.org/docrep/011/i0300e/i0300e00.htm). This great market value contributes extensive benefits to economies especially in Asia where most of the shrimp farming occurs. Unfortunately, an increase in the numbers of diseases induces significant losses for the shrimp industry. Between 1990 and 2005, for example, the global shrimp production losses due to diseases were approximately US$ 15 billion, 60% of which were attributed to viruses (Flegel, 2012). White spot syndrome virus (WSSV) and yellow head virus (YHV) have been marked as the most dangerous pathogens for shrimp aquaculture due to their lethal effect on *Penaeus monodon* (black tiger shrimp) and *Penaeus vannamei* (Pacific white shrimp), the major cultivated shrimp species (Flegel, 2012).

Penaeus stylirostris densovirus (*Pst*DV), commonly called infectious hypodermal and hematopoietic necrosis virus (IHHNV), is also of importance as it causes mass mortality in *Penaeus stylirostris* (blue shrimp), and Runt deformity syndrome (RDS) in *P. vannamei* and *P. monodon* (Brock *et al.*, 1983; Browdy *et al.*, 1993; Bray *et al.*, 1994; Primavera and Quinitio, 2000; Khawsak *et al.*, 2008; Rai *et al.*, 2012). It is also reported to be associated with slow growth in *P. monodon* (Rai *et al.*, 2009; Rai *et al.*, 2012). Despite several approaches to viral disease prevention such as good on-farm management practice (Xiang, 2001), the presence of *Pst*DV has been reported.

Success in circumvention of outbreaks of *Pst*DV requires efficient control measures which constitutes detection, containment and control. To achieve this, proper consideration and fulfilment of areas including the following issues are needed. 1) Requirement for more efficient diagnostic systems. Such systems are required for seed screening program and biosecurity. 2) Requirement for the understanding of the population dynamics and evolution of *Pst*DV (Robles-Sikisaka *et al.*, 2010). This issue is crucial for implementation of effective measures to control outbreaks/restrict movement of the virus, for example, from geographical hotspots for the virus. This thesis aims to address these two issues with the hope that integration of such outputs and knowledge will help contribute toward better strategies to counter *Pst*DV.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Introduction

Penaeus stylirostris densovirus (*Pst*DV) is a virus that causes mass mortality in *P. stylirostris* and Runt deformity syndrome (RDS) in *P. vannamei* and *P. monodon* (Brock *et al.*, 1983; Browdy *et al.*, 1993; Bray *et al.*, 1994; Primavera and Quinitio, 2000; Khawsak *et al.*, 2008; Rai *et al.*, 2012). Although RDS is not fatal for shrimp, the wide distribution in size it causes, reducing the value of affected crops to 50%, also devastates crop production because shrimp market value depends mainly on the body weight/size of shrimp (Bell and Lightner, 1984; Lightner, 1996a). This review provides brief information of *Pst*DV and highlights some relevant issues that need proper consideration and fulfilment in order to circumvent outbreaks of *Pst*DV.

2.2. *Pst*DV information

2.2.1. Classification and genome characterization

*Pst*DV is a non-enveloped icosahedral virus with an estimated size of 22 nm, smallest of all known penaeid shrimp viruses. Its genome contains a single-stranded linear DNA (ssDNA) of approximately 3.9 kilo base pairs (kbp), and shares a common structure and organization with the genome of mosquito brevidensoviruses (*Aedes albopictus* densovirus, *Aal*DNV, and *Aedes aegypti densovirus, Aae*DNV). Therefore, it is classified as a tentative species in the family *Parvoviridae,* genus *Brevidensovirus* (Fauquet *et al.*, 2005).

The genome of *Pst*DV contains 3 main open reading frames (ORFs) arranged from the left to the right on plus strand DNA: ORF2, ORF1; and ORF3 (Figure 2.1) (Rai *et al.*, 2011). Based on the Indian isolate reported recently (GenBank No. GQ411199) (Rai *et al.*, 2011), ORF2 comprises of 1092 bp with the sequence

overlaps with ORF1, but in a different reading frame. ORF2 starts with an ATG codon at nucleotide (nt) 591 and terminates with a TAG codon at nt 1681. If functional, this ORF would encode a protein of 363 amino acids (aa) corresponding to a molecular mass of 42.11 kDa. As speculated for brevidensoviruses, this protein may be a non-structural protein 2 (NS2), the function of which, as with other parvoviruses, is currently unknown (Shike *et al.*, 2000).

ORF1 comprises of 2001 nt, approximately 50% of the genome. It starts at nt 648 and terminates with a TAA codon at nt 2648. This ORF encodes a 666-aa protein with a molecular weight of 75.77 kDa. The predicted protein of ORF1 shares the highest homology with two conserved motifs presented in the major non-structural protein 1 (NS1) of mosquito brevidensoviruses: replication initiator domain and NTP-binding and helicase domains (Shike *et al.*, 2000; Rai *et al.*, 2011; Rai *et al.*, 2012). Thus, it is believed to encode essential NS1 proteins functioning in rolling-circle replication process.

ORF3 is the smallest (990 nt) amongst the three ORFs. It starts with ATG at nt 2590, and terminates with the TAA codon at nt 3577 (62 nt overlapped with ORF1). It shares the same reading frame (+2) with ORF2. This ORF encodes a 329-aa capsid protein corresponding to a molecular mass of 37.48 kDa.

Short palindromic sequences capable of forming DNA hairpin structures, a characteristic shared by all parvoviruses, have also been found in *Pst*DV genome extremities (i.e. 5’ and 3’ ends). They are presumed to be primers for rolling-cycle replication process (Astell, 1990; Shike *et al.*, 2000; Vega-Heredia *et al.*, 2012). Three putative potential transcriptional promoters designated as P2, P11 and P61 have also been characterized. P2 controls the transcription of ORF1 while P11 and P61 control those of ORF2 and ORF3, respectively (Dhar *et al.*, 2007).

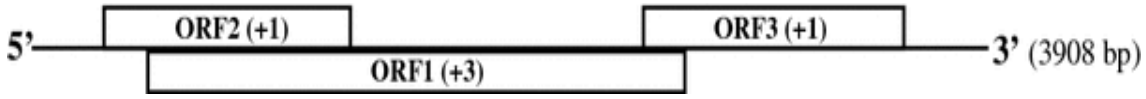


Figure 2.1: Genomic organization of coding sequences of *Pst*DV genome (Rai *et al.*, 2012). The genome is shown in plus strand. The three open reading frames designated as ORF1, ORF2 and ORF3 are indicated in boxes. ORF2 and ORF3 are in reading frame 1 (+1) and ORF1 is in reading frame 3 (+3).

2.2.2. Occurrence and geographical distribution

*Pst*DV has been reported in many regions around the world including North, South, Central America, the Caribbean and the Indo-Pacific region and is now considered cosmopolitan in distribution (Rai *et al.*, 2012). *Pst*DV was first reported in *P. stylirostris* cultured in the early 1980s in Hawaii (Lightner *et al.*, 1983a; Rai *et al.*, 2012) where it was believed to have been introduced by importation of live experimental *P. monodon* from the Philippines during the early development of shrimp aquaculture (Tang *et al.*, 2003; Flegel, 2006). Then, it was found distributed in every country of the Americas in which *P. vannamei* was cultivated (Lightner, 1996a) and in Asia and Africa (Tang and Lightner, 2006; Rai *et al.*, 2009; Saksmerprome *et al.*, 2010). In South East (SE) Asia, it is believed to be introduced from Thailand to Taiwan as both live and frozen shrimp in Taiwan are frequently imported from Thailand. This assumption is supported by the high similarity in DNA sequence (99.7%) of the virus amongst the 2 countries (Tang *et al.*, 2003). *Pst*DV has also been reported in India, and it is also believed to have come from SE Asia as these 2 geographical isolates are phylogenetically closely related (Rai *et al.*, 2009). Besides infectious *Pst*DV, non-infectious sequence, mostly referred to virus-related DNA fragments presenting in the genome of *P. monodon*, have been reported in some parts of the world such as East Africa, western Indo-Pacific region (Tang and Lightner, 2006; Tang *et al.*, 2007) and SE Asia (Saksmerprome *et al.*, 2011) (see section 2.3.1.1).

Based on its distinct genotypes in both infectious and non-infectious groups, *Pst*DV has been separated into three types (Tang and Lightner, 2006; Tang *et al.*, 2007): Type 1 from the Americas and East Asia (principally the Philippines); Type 2 from SE Asia (i.e. Thailand); Type 3A from East Africa (Mozambique but mainly Madagascar), Australia and India; and Type 3B from the western Indo-Pacific countries including Mauritius but mainly Tanzania and Mozambique, and Malaysia. The first two genotypes are infectious to penaeid shrimp while the latter two genetic variants, 3A and 3B, are not infectious even though their genomes contain all 3 ORFs similar to those labelled as infectious (Tang and Lightner, 2002a; Tang *et al.*, 2003; Tang and Lightner, 2006; Tang *et al.*, 2007). Type 3A was later been found to be integrated into the genome of *P. monodon* from East Africa, Tanzania and Mozambique (Tang and Lightner, 2006; Tang *et al.*, 2007). Although the *Pst*DV integrated sequences are reported as not infectious to host species, this has never been tested experimentally and whether or not they encode proteins remains unclear. Table 2.1 summarizes the geographical distribution of *Pst*DV to date.

Table 2.1: The geographical distribution of *Pst*DV

Continent	Country	References
America	Argentina	(Martorelli *et al.*, 2010)
	Peru	(Alfaro Aguilera *et al.*, 2010)
	Brazil	(Braz *et al.*, 2009; Coelho *et al.*, 2009)
	Venezuela	(Boada, 2008)
	Colombia	(Tang *et al.*, 2003)
	Mexico	(Unzueta-Bustamante *et al.*, 1998; Nunan *et al.*, 2000; Tang and Lightner, 2001)
	Ecuador	(Jiménez *et al.*, 1999; Nunan *et al.*, 2000)
	USA	(Kalagayan *et al.*, 1991; Nunan *et al.*, 2000; Shike *et al.*, 2000; Tang *et al.*, 2007)
	Panama	(Nunan *et al.*, 2000; Tang and Lightner, 2001)
	Belize	(Tang *et al.*, 2007)
Asia	China	(Yang *et al.*, 2007; Zhang *et al.*, 2007)
	India	(Rai *et al.*, 2009; Rai *et al.*, 2011)
	Philippines	(Nunan *et al.*, 2000; Tang *et al.*, 2003; Tang *et al.*, 2007)
	Korea	(Kim *et al.*, 2011; Kim *et al.*, 2012)
	Thailand	(Tang *et al.*, 2003; Chayaburakul *et al.*, 2004; Tang *et al.*, 2007)
	Taiwan	(Tang *et al.*, 2003)
	Brunei	(Claydon *et al.*, 2010)
Africa	Madagascar	(Tang *et al.*, 2003; Tang and Lightner, 2006)
	Mozambique	(Tang and Lightner, 2006)
	Tanzania	Tang and Lightner, 2006, Tang et al., 2003
	Mauritius	(Tang *et al.*, 2003)
Oceania	Australia	(Krabsetsve *et al.*, 2004; Saksmerprome *et al.*, 2010; Jaroenram and Owens, 2014)
	New Caledonia	(Krabsetsve *et al.*, 2004)
	Tahiti	(Owens and Hall-Mendelin, 1989)

2.2.3. Clinical signs and mode of transmission

*Pst*DV infection does not produce any specific clinical signs of disease (Lightner *et al.*, 1983b). In *P. stylirostris*, acute infected juveniles and sub-adults show reduced food consumption, becoming motionless then rolling over and slowly sinking to the pond bottom, then they die (Rai *et al.*, 2012). In *P. vannamei* and *P. monodon*, *Pst*DV typically causes chronic disease (Lightner *et al.*, 1983b) and the infection results in development and growth abnormalities (i.e. deformed rostrum, wrinkled antennal flagella, cuticular roughness, 'bubble-heads' and deformation of 6th abdominal segment and tail fan) known as RDS (Brock *et al.*, 1983; Browdy *et al.*, 1993; Bray *et al.*, 1994; Primavera and Quinitio, 2000; Khawsak *et al.*, 2008; Rai *et al.*, 2012). *Pst*DV is also associated with slow growth in *P. monodon* (Figure 2.2) (Rai *et al.*, 2009; Rai *et al.*, 2012).

Figure 2.2: Size variations observed in 50 days old *P. monodon* infected by *Pst*DV (Rai *et al.*, 2009; Rai *et al.*, 2012).

Like all other parvoviruses, *Pst*DV do not encode a DNA polymerase; its replication and multiplication processes depend on host cell machinery. It therefore needs rapidly proliferating cells of the host for its replication (Rai *et al.*, 2012). Since postlarvae (PL) and juvenile shrimps have more actively dividing cells than adult shrimp, they are more susceptible to *Pst*DV infection (Kalagayan *et al.*, 1991; Lightner, 1996b). The target organs for *Pst*DV infection include tissues of

ectodermal (cuticular epidermis, hypodermal epithelium of the fore and hind gut, nerve cord and nerve ganglia) and mesodermal (hematopoietic organs, antennal gland, tubule epithelium, gonads, lymphoid organ, connective tissue and striated muscle) origin (Lightner, 1996b; Rai *et al.*, 2012).

2.2.4. Prevalence and host ranks

*Pst*DV can spread both horizontally and vertically (Lightner *et al.*, 1983a; Motte *et al.*, 2003). Horizontal transmission occurs when individual shrimp eat dead infected shrimp or are in the water contaminated with the virus. Several studies regarding the successful horizontal transmission by ingestion of *Pst*DV-infected shrimp support this observation (Bell and Lightner, 1987; Tang and Lightner, 2006; Coelho *et al.*, 2009). Shrimp surviving *Pst*DV become carriers for life even though they show no signs of the disease (Bell and Lightner, 1987). Vertical transmission occurs due to transfer of this virus from mother to offspring. This could be through the shedding of viral particles at the time of spawning and then ingestion by larvae at first feeding, or by the transmission of virus from oocytes to larvae (Lotz, 1997). The vertical transmission of *Pst*DV through female *P. vannamei* was confirmed by nested-PCR analysis of embryos and larvae produced by *Pst*DV-infected females fertilized by *Pst*DV-free males (Motte *et al.*, 2003). Zhang and Sun also reported *Pst*DV in ovarian tissues and fertilized egg of *Penaeus chinensis* (Zhang and Sun, 1997).

The prevalence of *Pst*DV can range from low to very high but 90-100% is typical in farmed shrimp (Lightner *et al.*, 1983a; Lightner *et al.*, 1992; Martínez-Cordova, 1992; Chayaburakul *et al.*, 2004). A prevalence as high as 51.5% was reported in *P. vannamei* collected from culture areas of the northern China during 2001 and 2004 (Yang *et al.*, 2007). *Pst*DV was also reported to cause RDS and high mortalities (80%) in PL and sub-adults of *Macrobrachium rosenbergii* cultured in Taiwan but the prevalence was not presented (Hsieh *et al.*, 2006). For wild captured shrimp, the mean values of prevalence were: 100% in female *P. stylirostris* collected from the mid-region of the Gulf of California, Mexico (Morales-Covarrubias *et al.*, 1999);

51-63% in *P. vannamei* captured from the Pacific coast of Ecuador (Motte *et al.*, 2003); 18%-50% in *Penaeus californiensis* collected from the upper Gulf of California, Mexico (Pantoja *et al.*, 1999); 4.4% in *Penaeus aztecus* and *Penaeus setiferus* from the East Coast of Mexico (Guzmán-Sáenz *et al.*, 2009; Vega-Heredia *et al.*, 2012); 56% in *Artemesia longinaris* and 40% in *Palaemon macrodactylus* collected from the Bahı´a Blanca Estuary, Argentina (Martorelli *et al.*, 2010). Another survey revealed 20% prevalence in Malaysian wild female berried *M. rosenbergii* broodstock which showed no gross signs of disease (Hazreen Nita *et al.*, 2012). *Pst*DV was also detected in crabs. Low prevalence (8.3%) was reported in *Hemigrapsus penicillatus* (Hairy-clawed shore crabs) collected from the culture areas of the northern China during the year 2001-2004 (Yang *et al.*, 2007). In contrast, a high prevalence of 67% was observed with *Cyrtograpsus angulatus* collected from the Bahı´a Blanca Estuary, Argentina (Martorelli *et al.*, 2010). In Australia, a survey of 328 wild caught prawns (7 species), crabs and lobsters revealed that only *P. monodon* were positive for *Pst*DV or *Pst*DV-like amplicons (Krabsetsve and Owens, unpublished). In addition to natural infection, experimental infection with no clinical signs of disease was observed with *Penaeus subtilis* (Coelho *et al.*, 2009).

Obviously, several shrimp species and arthropods commonly found in shrimp culture areas (i.e. crabs) are susceptible to *Pst*DV. It would appear that the most dangerous scenario would be for a *Pst*DV outbreak to occur in shrimp ponds and for wild crustaceans such as crabs to have the opportunity to eat infected shrimp carcasses. They would have a high chance of becoming infected and could possibly carry the virus to an uninfected pond where the risk of transmission would be high if they were eaten by shrimp (horizontal transmission). Therefore, the practice of eliminating wild crustaceans from rearing ponds should constitute an effective control measure for *Pst*DV. Current known host species of *Pst*DV and the prevalence of the virus are listed in Table 2.2

Table 2.2: Known host species of *Pst*DV and the prevalence of the virus

Phylum	Order	Family	Species	Prevalence	References
Arthropoda	Decapoda	Penaeidae	*P. stylirostris*	100%	(Morales-Covarrubias *et al.*, 1999)
			P. vannamei	51-63%	(Motte *et al.*, 2003)
			P. monodon	25%	(Rai *et al.*, 2009)
			P. setiferus and *P. aztecus*	4.4%	(Guzmán-Sáenz *et al.*, 2009; Vega-Heredia *et al.*, 2012)
			A. longinaris	56%	(Martorelli *et al.*, 2010)
			P. californiensis	18-50%	(Pantoja *et al.*, 1999)
			P. japonicus	N/A	(Tang *et al.*, 2007)
			P. subtilis	N/A	(Coelho *et al.*, 2009)
Arthropoda	Decapoda	Palaemonidae	*M. rosenbergii*	20%	(Hazreen Nita *et al.*, 2012)
			P. macrodactylus	40%	(Martorelli *et al.*, 2010)
Arthropoda	Decapoda	Varunidae	*C. angulatus*	67%	(Martorelli *et al.*, 2010)
			H. penicillatus	8.3%	(Yang *et al.*, 2007)

2.3. Current factors hampering the success of control for *Pst*DV

2.3.1. Current problems in diagnostics: viral inserts in shrimp genome

To date, retroviruses have been the only known eukaryotic viruses that require host genomic integration for completion of their life cycle (Feschotte and Gilbert, 2012). However, several recent reports have revealed countless non-retroviral sequences that are integrated or 'endogenized' in the genomes of eukaryotes, for instance, caulimovirus (Staginnus and Richert-Pöggeler, 2006; Iskra-Caruana *et al.*, 2010) and baculovirus (Lin *et al.*, 1999) have been identified in plant genomes and insect genomes respectively for some time. The discovery of endogenous viral elements (EVEs) derived from non-retroviruses suggests that all major types of eukaryotic viruses could give rise to EVEs (Feschotte and Gilbert, 2012).

2.3.1.1. *Pst*DV endogenous viral elements in shrimp

*Pst*DV was first reported to integrate into shrimp genome by Tang and Lightner (2006). By screening *P. monodon* collected from various geographical areas using PCR with primers MG831F/R designed for the type 3A viral sequence and DNA sequencing, *P. monodon* from Madagascar (collected in 2001) and Australia (collected in 2004) revealed the viral insert sequences linked with various shrimp DNA markers including *P. monodon* clone AFE20M24 (GenBank No. AY654006.1), *P. vannamei* microsatellite TUMXLV 6.114 (GenBank No. AY46620.1), *P. monodon* clone AFE49M19.1 (GenBank No. AY654020.1), and *P. monodon* microsatellite PM2334 (GenBank No. AY500859.1). Amongst these samples, Australian *P. monodon* carried the type A viral sequence as high as 80% (16 samples out of 20). Histological examination of *P. monodon* or *P. vannamei* challenged with the Australian *P. monodon* carrying the viral inserts showed no gross pathology of *Pst*DV infection (i.e. Cowdry A inclusion bodies). Unfortunately, these shrimp species were a poor choice for the 2-week bioassay as RDS changes are chronic, not acute, and are unlikely to manifest within 2 weeks. The occurrence of endogenous *Pst*DV elements was also recently reported in Indian shrimp (Rai *et al.*, 2009). Of 177 *P. monodon* screened by the MG831F/R primers and the other three recommended by OIE [IHHNV392F/R (Tang *et al.*, 2000), IHHNV389F/R

(Tang and Lightner, 2006) and 309F/R (Tang *et al.*, 2007)], 33.7% PL and 31.7% adult shrimp possessed only type A-*Pst*DV viral sequence while 22.8% PL and 10.5% adults had both type A and infectious type. DNA sequence analysis of PCR products derived from the MG831F/R primers (832 bp) revealed linkage between the *Pst*DV sequences and various shrimp DNA microsatellites.

Recently, Krabsetsve and Owens (unpublished) screened 196 shrimp samples by PCR using 3 OIE primers (IHHNV392F/R, IHHNV389F/R and 309F/R). The samples made up of 158 shrimp (mainly *P. monodon*) collected from farms and research institutes in northern Queensland between 1993 and 2009, 3 shrimp imported from Brunei, and 35 shrimp (imported from New Caledonia, China, Malaysia and Indonesia) purchased from fish shops in northern Queensland, during 2002 and 2009 (Table B.1, Appendix B). According to the results (Table B.2, Appendix B), 124/158 samples (78.48%) of Australian shrimp, and 2/38 (5.26%) of imported shrimp (only from Brunei) show positive result by the MG831F/R primers indicating that they contain viral fragments in their genome. All samples from Malaysia (10 shrimp) neither contain infectious type nor non-infectious type of *Pst*DV. By contrast, those from China and New Caledonia were most likely to carry infectious *Pst*DV as they give positive amplicons by all the 3 ORF primer sets above. However, it is also possible that they just possessed independent inserts that cover the target regions for all the 3 primers. Further assays to prove this hypothesis are required.

2.3.1.2. Insertion pattern of *Pst*DV elements in shrimp genome

Scattered reports of *Pst*DV-related sequences inserted in shrimp (Tang and Lightner, 2006; Tang *et al.*, 2007; Rai *et al.*, 2009) opened up the question as to whether it forms only one way or in a variety of ways (Saksmerprome *et al.*, 2011) To answer the question, Saksmerprome et al. (2011) screened 99 shrimp (*P. monodon*) using PCR employing 7 overlapping primer pairs designed to cover 90% of the *Pst*DV genome (GenBank No. AF273215). All shrimp were collected from a shrimp hatchery in Thailand. PCR failure with some pairs indicated sequence gaps that

revealed a random pattern of putative viral inserts in the genomes of individual shrimp. Genome walking with one putative insert showed that the insert was associated with the host microsatellites (thrombospondin-like expressed sequence taq, EST of *P. monodon*: CF805583). The microsatellites reported differed from those previously identified for the other *Pst*DV inserts in *P. monodon* (Tang and Lightner, 2006; Tang *et al.*, 2007). This finding together with no pattern in the number and length of inserts support the hypothesis that the insertion of viral DNA may occur autonomously in a random manner and mostly likely occurs in repetitive or transposon-like elements of the host chromosome (Flegel, 2009). *Pst*DV-related sequences have also been found in the genome of *Cherax quadricarinatus* (Australian redclaw crayfish) (Rusaini *et al.*, 2013).

A recent search for homologs of parvoviral proteins in publicly available eukaryotic genome databases followed by experimental verification and phylogenetic analysis revealed that several parvovirus-related DNA sequences were presented in the germ lines of diverse animal species, including mammals, birds, fishes, arthropods, and flatworms. Some of them were expressed, suggesting that these viral DNA fragments are also functional in the host genomes (Liu *et al.*, 2011).

As for *Pst*DV, rather than causing mortality to *P. monodon*, it survives persistently and becomes endogenized (see above). Although the reasons behind this phenomenon remains unclear, accumulating evidence regarding the EVEs suggests that the *Pst*DV endogenized sequences, may be involved in viral control mediated by a host RNA interference mechanism (Flegel, 2009). However, this hypothesis needs further studies to prove. The multiple hosts of endogenous *Pst*DV are similar to those of other non-retroviruses including parvoviruses. This phenomenon supports the assumption stating that "almost any major type of eukaryotic virus may be endogenized, sometime in multiple hosts independently and over wide evolutionally periods" (Feschotte and Gilbert, 2012).

2.3.1.3. PCR detection and false positive results arising from viral inserts

As almost all parts of the *Pst*DV genome have been found integrated, many PCR primers have inevitably given false positive results and this includes the 309F/R primers (Tang *et al.,* 2007) which are currently being recommended by OIE. Tthe widely used IQ2000^{TM} detection kit (Intelligene, Taiwan) also shows a high prevalence of 76% for shrimp collected from Thailand (Saksmerprome *et al.*, 2011), and a low prevalence with 5 % to 8% for shrimp sampled from Africa and Australia (http://www.iq2000kit.com/news_detail.php?gid=1&nid=1). Since the nested primers for the IQ2000 ^{TM} kit are designed in the same region of the 309F/R primers, the same level of false positive results with the samples tested would probably occur (Saksmerprome *et al.*, 2011).

False positives caused by the inserts occurred with the OIE primers and the IQ2000^{TM} kit could lead to a negative impact on international shrimp trade and disease quarantine management. Thus, more reliable and specific detection protocols are required.

2.3.1.4. Recombinase polymerase amplification and lateral flow dipstick assay: alternative approaches for *Pst*DV detection

Recombinase polymerase amplification (RPA) is a novel approach for rapid and specific DNA amplification. Unlike standard PCR, RPA amplifies DNA under single temperature range (37–42 °C) (Piepenburg *et al.,* 2006). Therefore, sophisticated thermal cyclers are not required. Unlike other DNA isothermal technologies including loop mediated DNA isothermal amplification (LAMP) which use 4–6 primers working with *Bacillus stearothermophilus* (*Bst)* DNA polymerase to synthesize various sizes of DNA amplicons comprising concatenated inverted repeats of target DNA (Notomi *et al.*, 2000), RPA employs phage-derived recombinase working with single-strand DNA binding protein (SSB) to direct *two primers* to a homologous target sequence in a DNA template. This is coupled to a *Staphylococcus aureus*-derived DNA polymerase (*Sau* DNA polymerase) possessing

strand displacement activity to generate double-stranded DNA (dsDNA) in a similar way to PCR (Figure. 2.3) (Shibata *et al.*, 1979; Yonesaki *et al.*, 1985; Formosa and Alberts, 1986; Piepenburg *et al.*, 2006). The RPA protocol reduces complications associated with reaction preparation found in PCR and LAMP. To illustrate this point, the RPA reaction system is provided in a stabilized dried format which permits transportation and limited storage without refrigeration (http://www.twistdx.co.uk/our_technology/). In addition, the reaction can be set up at room temperature, with no requirement for cooling. Together with the requirement of only a simple heating block for maintaining the reaction, RPA provides qualitative DNA testing without the need of a laboratory (Jaroenram and Owens, 2014). RPA products can be detected by acrylamide gel electrophoresis (AGE) or in real-time (Euler *et al.*, 2012; Boyle *et al.*, 2013; Euler *et al.*, 2013). Alternatively, they can be visualized simply by a lateral flow dipstick (LFD) assay (Milenia Biotec, Giessen, Germany).

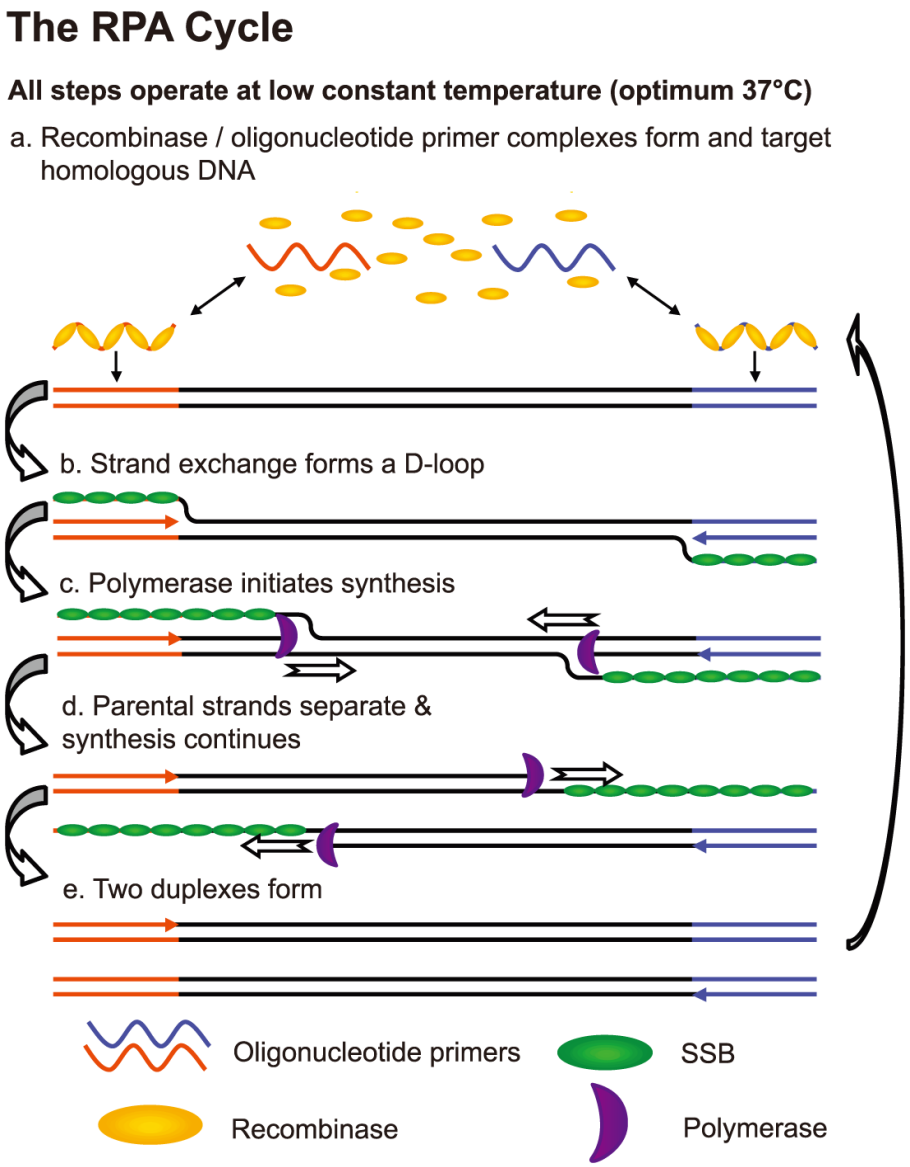


Figure 2.3: Mechanism of recombinase polymerase amplification (RPA). The RPA mechanism is driven by the three core proteins (recombinase, single-strand DNA binding protein (SSB) and strand-displacing polymerase) working under isothermal temperature with no requirement for an initial chemical or thermal melting step. This diagram was created by TwistDx Ltd (http://www.twistdx.co.uk/our_technology/) and is licensed under a Creative Commons Attribution 3.0 United States License.

The lateral flow dipstick (LFD) assay is a technique intended to detect the presence or absence of a target analyte in samples using the principle of hybridization and chromatography in membrane strips without the need for specialized and costly equipment (Milenia Biotec, Giessen, Germany). The assay can be applied to detect any DNA of interest, and the read-out is visualised by eye (Jaroenram *et al.*, 2009). For the Milenia® HybriDetect LFD which was used in this study, it is a universal test dipstick designed to develop qualitative test systems for several analytes. However, to use this dipstick format, the user needs to develop an analyte-specific solution, which contains a first detector (e.g. antibody, specific probe) labelled with fluorescein isothiocyanate (FITC), and a second one (e.g. antibody, primers) labelled with biotin. The working principle of the Milenia® HybriDetect LFD is depicted in Figure 2.4. Briefly, a ready-to-use strip precoated with nanogold particles, anti-FITC antibody and biotin ligand is prepared (Step 1). The sample to be determined (e.g. protein, gene amplicons) is mixed with the developed analyte-specific solution (Step 2). The dipstick is dipped into the solution, and the result is read after a 5-min incubation (Step 3-4). In these steps, the complexed analyte carrying FITC and biotin binds first to the gold-labeled FITC-specific antibodies in the sample application area of the dipstick. By capillary force, the gold complexes diffuse over the membrane. Only the analyte-captured gold particles will bind when they overflow the immobilized biotin-ligand molecules at the test band and generate a visible red-purple colour. Unbound gold particles further migrate then to be fixed at the control band by species-specific antibodies resulting in a read as a control line. This technique can be carried out within 10 min, about 5-10 times faster than gel electrophoresis (including gel preparation). Its additional advantage is confirmation of DNA identity by hybridization. This feature could help eliminating the false positive results arising from nonspecific amplification (Jaroenram *et al.*, 2009). Altogether, combining LFD with RPA would further increase the simplicity and efficiency of detection protocols.

To date, the combined protocol has been reported to detect only two pathogens: *Plasmodium falciparum* (Kersting *et al.*, 2014) and methicillin-resistant *Staphylococcus aureus* (Piepenburg *et al.*, 2006). Thus, exploring the possibility of

using the combined protocol to detect other pathogens including *Pst*DV is interesting.

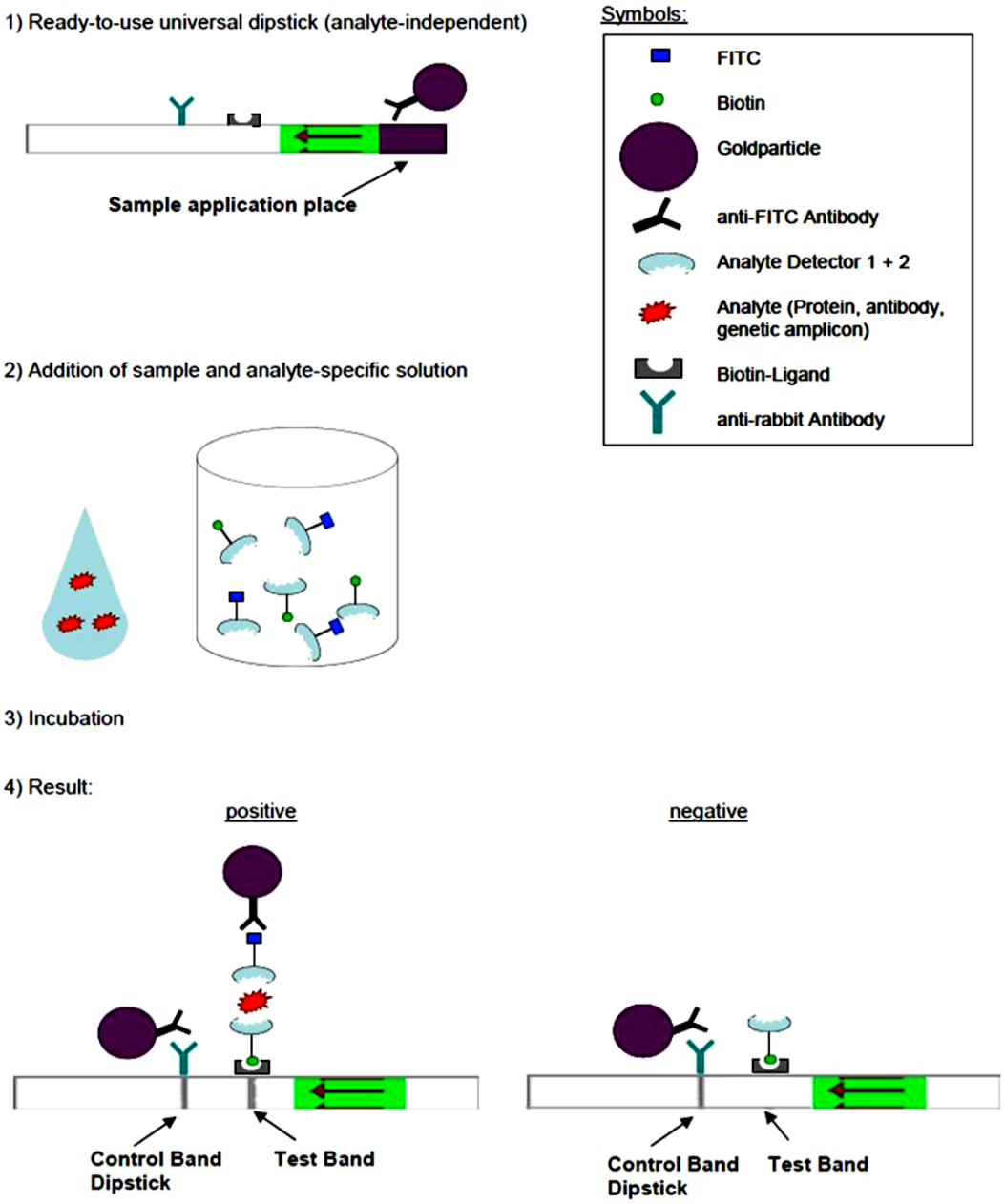


Figure 2.4: The working principle of the Milenia® HybriDetect LFD.

2.3.2. Current problems in control of *Pst*DV outbreaks: Lack of understanding of the diversity and population dynamics of *Pst*DV

Understanding the genetic diversity of *Pst*DV is significantly important as it helps to gain insights into evolution and population dynamics, and consequently may help contribute toward better strategies to counter the virus.

In 2003, a test to evaluate the level of genetic diversity of *Pst*DV genome was conducted using 14 isolates collected from penaeid shrimp in SE Asia (Philippines, Thailand and Taiwan), the Americas (Hawaii, Mexico, Colombia, Ecuador and Panama) and Africa (Tanzania, Madagascar and Mauritius) during the year 1996-2001 (Tang *et al.*, 2003). Based on the sequence analysis of a 2.9-kb fragment of *Pst*DV DNA covering ORF1, ORF2 and ORF3, the Hawaii isolate showed a very high nt identity to the Philippine (99.8%) and the Thai isolates (96.2%). This observation supports the assumption that the *Pst*DV circulating in the USA had originated from Philippines (Lightner, 1996a; Tang *et al.*, 2003). In contrast, the Hawaii isolate showed great divergence to the Tanzanian (8.2%) and the Madagascan isolates (14.1%). The notably high sequence divergences observed with the Tanzania and the Madagascar isolates might result from the virus-related sequences which were later found in the genome of *P. monodon* from these two countries (Tang and Lightner, 2006) (see section 2.3.1.1).

At roughly the same time, a similar study (in terms of methodology and analysis) was conducted by the same research group (Tang and Lightner, 2002b). By using 14 *Pst*DV isolates collected from cultured shrimp stocks in only the Americas (USA; Hawaii and Texas, Mexico, Colombia, Ecuador and Panama) between 1982 and 1997, less than 0.5% nt sequence divergence was observed (i.e. 99.6 - 100% similarity) (Tang and Lightner, 2002b). Although 14 out of 25 nt substitutions observed resulted in aa changes, there was no significant association between clinical outcomes and any particular aa substitutions. According to the authors, the results therein lead to the suggestion that the high stability of the *Pst*DV genome among the 14 isolates over the period examined is due to “the development of a

more balanced host-pathogen relationship". This suggestion was supported by the observation that no high mortalities of cultivated shrimp caused by *Pst*DV have been recorded on the Pacific coast of USA and Mexico after the epizootics of 1990s (Morales-Covarrubias *et al.*, 1999). They stated that "*L. stylirostris* has developed resistance to IHHNV and/or the virus has reached an equilibrium with the host in terms of genes related to virulence" (Morales-Covarrubias *et al.*, 1999; Tang and Lightner, 2002b; Robles-Sikisaka *et al.*, 2010).

However, the Tang and Lightner (2002b) conclusion was based on only 14 samples from the one genetic lineage. Additionally, quantitative measurement of a current degree of genetic variation in terms of the underlying evolutionary processes i.e. mutation, genetic drift, gene flow was not conducted. Recently, Robles-Sikisaka *et al*. (2010) have presented contrasting results to the Tang and Lightner's study (2002b). By comparing the capsid protein (CP) gene sequence of 89 penaeid shrimp (*P. stylirostris, P. vannamei* and *Farfantepenaeus californiensis*) collected from locations in the north-western Pacific coast of Mexico between 2004 and 2005 with 14 published sequences of *Pst*DV CP gene (*P. stylirostris* and *P. monodon* from different geographic locations) using Bayesian coalescent approaches, an unexpected high mean rate of nt substitution ($1.39x10^{-4}$ substitutions/site/year) was discovered. This was comparable to nt substitution rate of RNA viruses. The degree of nt diversity was also high, almost 5 times greater than those reported previously (Tang *et al.*, 2003), and not uniform across geographic regions. The presence of several quickly evolving lineages in the north-western Pacific coast of Mexico might be viewed as geographic ''hotspots'' of *Pst*DV diversity that could lead to the emergence of virulent strains following changes in the host or environmental conditions (Robles-Sikisaka *et al.*, 2010).

In Australia, *Pst*DV has been reported officially since 1991 (Owens *et al.*, 1992) despite this country being geographically isolated with strict quarantine policies to control/avoid the importation of live shrimp from overseas. Comparison of the PCR fragment derived from the Australian *Pst*DV strain isolated from *P. monodon* from a

farm in northern Queensland in 1993 (GenBank No. AY590120) with other geographical isolates revealed that the PCR fragment shared the highest nt similarity (96.2%) to the Madagascar isolate, and it shared the highest nt divergence to the Hawaiian and New Caledonian strains (90.1% and 90.3%, respectively (Krabsetsve *et al.*, 2004). In contrast to these results, comparison of the maximum possible portion of PCR fragments (2814 bp) generated from the Australian *Pst*DV (GenBank No. GQ475529) isolated from the same area but in 2008 with 8 GenBank records of *Pst*DV isolates reported from Asia revealed 94–95% identity in nt sequence and 96 to 97% identity in aa sequence (Saksmerprome *et al.*, 2010). This result indicates that sequence of this isolate (GenBank No. GQ475529) has high identity to those of *Pst*DV from nearby areas in SE Asia. Based on the contradictory reports by the two research groups, it may be able to conclude that there are two different *Pst*DV lineages in Australia: one has a geological time frame and one has a contemporary time frame. This is based on a high nt variation between the Australian and Hawaii isolates reported by Krabsetsve *et al*. (2004) which is geologically endemic in Australian *P. monodon*, and the other is a new SE Asian strain introduction which has been recently reported by Saksmerprome *et al* (2010). It unlikely the endemic strain was an endogenous viral element (EVE) as its whole genome was later sequenced and submitted to GenBank database with an accession No. EU675312.

As the two *Pst*DV genetic variants mentioned above have been circulating in the same region (northern Queensland), it led to the question as to whether or not their genetic compositions reciprocally affect each other's evolution. If they do, do they lead to viral recombination and/or emergence of new virulent strains? Answering these questions is important as it can pave the way to uncover the underlying evolution and population dynamics of *Pst*DV in Australia. This knowledge is essential for implementing the monitoring and control measures to limit the spread of the pathogen (i.e. restrict shrimp movement or transportation), particularly in Australia and Indo-Pacific region where the marine environment and fauna are shared. Understanding the impact that evolutionary processes have on the patterns of genetic variation within and among *Pst*DV populations, and understanding the

consequences of these patterns of genetic variation for various evolutionary processes is also important and need to be studied.

2.4. Concluding remarks

The shrimp aquaculture industry is a major business carried out worldwide. Unfortunately, it is under the serious threat of viral diseases including that caused by *Pst*DV. *Pst*DV causes mass mortality in *P. stylirostris*, and RDS-associated stunted growth in *P. vannamei* and *P. monodon*, resulting in a huge devastation in each cycle of production. Despite several approaches to viral disease prevention, outbreaks of *Pst*DV have occurred. Success in circumvention of *Pst*DV outbreaks requires proper consideration and fulfillment of several areas which include, but not limited to 1) Requirement for more reliable diagnostic approaches for seed screening and biosecurity. 2) Requirement for the understanding of *Pst*DV evolution and population dynamics for effective implementation of monitoring and control measures. This study aims to address two issues with the hope that the knowledge will contribute toward better strategies to counter *Pst*DV.

CHAPTER 3

GENERAL MATERIALS AND METHODS

The methods and materials described in this chapter were common and used in more than one experimental chapter of the thesis. Those used only in one experiment were described in the chapter to which they belong.

3.1. Preparation of total DNA extract

Total DNA extracts were prepared from *Pst*DV-infected shrimp using Isolate II Genomic DNA Kit (Bioline, London, UK) according to the manufacturer's protocol with minor modification. Briefly, approximately 25 mg of samples (e.g. pleopods or gills) was homogenised in 180 µl Lysis Buffer GL mixed with 25 µl Proteinase K solution. After incubation at room temperature for 3 h, the homogenate was added with 200 µl of Lysis Buffer G3 then incubated at 70 °C for 10 min followed by adding 210 µl of 95% ethanol and mixing vigorously. Then, the homogenate-ethanol mixture was transferred into reservoir of a combined *spin column–collection tube assembly* and centrifuged at 11,000 × *g* for 1 min. After the column was emptied, it was washed twice with 500 µl of wash buffer and dried by spinning at 11,000 × *g* for 2 min to remove ethanol residue. The spin column was assembled to a new microcentrifuge tube then was added with 100 µl of 70° C preheated elution buffer. After 2 min incubation at room temperature, the column was centrifugation at 11,000 × *g* for 1 min, and then the flow-through containing total DNA was stored at -20° C until use. To measure the quality and quantity of DNA extract, 2 µl of the extract was spectrophotometrically analysed at 260 nm and 280 nm using Nanophotometer (Implen GmbH, Germany). The concentration of the DNA was adjusted to 50 ng/µl by nuclease-free water, and the DNA solution was subject to further experiments as appropriate.

3.2. Preparation of total RNA extract

Total RNA extracts used in this study were extracted from shrimp infected with viruses of interest (e.g. gill-associated virus, GAV; Taura syndrome virus, TSV; *Macrobrachium rosenbergii* nodavirus, *Mr*NV) using SV total RNA isolation system (Promega, Madison, USA) with minor modifications. Briefly, approximately 50 mg of samples (either pleopods or gills) were homogenised in 175 µl RNA lysis buffer followed by addition of 350 µl RNA dilution buffer. After centrifugation for 10 min at 12,000 × *g*, the supernatant was transferred to a 1.5-ml microcentrifuge tube containing 200 µl of 95% ethanol. The mixture was mixed vigorously, and transferred into reservoir of a combined *spin column–collection tube assembly* which was subsequently centrifuged at 12,000 × *g* for 1 min. The column was then added with freshly prepared DNase incubation mix (40 µl of yellow core buffer, 5 µl of 90 mM $MgCl_2$ and 5 µl of DNase I), and incubated for 15 min at room temperature followed by the addition of 200 µl of DNase stop solution, and 1-min centrifugation at 12,000 × *g*, respectively. After the column was emptied, it was washed twice with 500 µl RNA wash solution then dried by spinning at 12,000 × *g* for 2 min to remove ethanol residue. The spin column was assembled to a new microcentrifuge tube, and was added with 100 µl of nuclease free water. After 1-min centrifugation at 12,000 × *g*, the flow-through containing total RNA was quantitatively and qualitatively analysed in the same manner of DNA, then stored at -20˚C until used.

3.3. PCR amplification and gel electrophoresis

Unless otherwise stated, a standard PCR protocol for amplifying any target DNA described in this thesis was performed as follows: 1) Prepare PCR mixture containing 1× MyFi PCR buffer, 2U MyFi^{TM} Taq DNA Polymerase (Bioline, London, UK), 0.4 µM each forward and reverse primer, , the specified amount of DNA template and DNase-free water to a final concentration of 25 µl. 2) Amplify target DNA under the thermal profile consisting of 3-min initial denaturation at 94°C, followed by 39 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with

a final extension at 72°C for 5 min. 3) Visualize PCR products by 1% TAE agarose gel electrophoresis (AGE).

3.4. Cloning and sequencing

To perform DNA cloning and sequencing, after DNA sequences were amplified and analysed by AGE, the PCR amplicons were extracted and purified from agarose gels using Wizard® SV Gel and PCR Clean-Up system and ligated directly into plasmid pGEM®-T Easy Vector (Promega, Madison, USA) according to the manufacturer's protocol. The recombinant plasmid was cloned into JM109 *Escherichia coli* competent cells (Promega, Madison, USA), and further amplified for 16 h in Lysogeny broth (LB) (2 ml) containing 100 µg/ml of ampicillin following the manufacturer's protocol. The plasmid was extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, USA). Sequencing of both strands of DNA plasmids (5-6 clones/PCR fragment) was performed by Macrogen Inc. (South Korea) using T7 and SP6 universal primers. The obtained sequences were subject to further analysis as appropriate.

CHAPTER 4

PROFILING OF ENDOGENOUS VIRAL ELEMENTS IN THE GENOME OF AUSTRALIAN *PENAEUS MONODON*

4.1. Introduction

The main objective of this study was to develop a novel molecular approach for detecting *Pst*DV. In general, selecting target DNA sequences is a key aspect for developing any DNA detection system, as it is central to determining the specificity of a diagnostic assay. It is known that the selection process can be done simply by detecting appropriate DNA sequences which truly represent the living organism of interest. However, this concept is exceptionally difficult for *Pst*DV due to the presence of its non-infectious forms, i.e. *Pst*DV-related sequences, which are part of shrimp genome (endogenous viral elements, EVEs). *Pst*DV-related sequences caused false positive results in many PCR detection protocols, including the commonly accepted and widely used IQ2000^{TM} PCR detection kit (Intelligene, Taiwan) (See Chapter 2.3.1.3).

A recent study by Saksmerprome *et al*. (2011) revealed that all parts of the *Pst*DV genome (except for hairpin ends which were not examined) were found to be endogenised, suggesting that they may lead to false positive results for infectious *Pst*DV using not only the IQ2000^{TM} kit but also any other DNA-based detection protocols reported thus far. It also revealed that the capsid gene of *Pst*DV at the nt position 3031–3625 (hereafter call the "3031-3625" region) (Mexican isolate, GenBank No. AF273215) was least likely to produce inserts (only ~4% of false positive rate was detected). However, the mechanism underling this phenomenon remains unclear. Moreover, this finding was based on *P. monodon* collected only from Thailand. This information stimulates the question as to whether or not such an insertion pattern is common and consistent across different geographical lines of the shrimp. If it is, the "3031-3625" region would be an alternative, promising target

DNA sequence for designing a more specific detection protocol for *Pst*DV. To answer the question, the experiments herein were conducted.

4.2. Materials and methods

4.2.1. Shrimp samples and DNA temple preparation

Shrimp samples (*P. monodon*) were obtained from 2 farms in northern Queensland in October 2012. These farms showed no sign of *Pst*DV infection i.e. RDS associated slow growth syndrome. All prawns were progeny from wild caught broodstock; 75% from the eastern coast of Queensland and 25% from Northern Territory (personal communication with the farms). Most ponds have the progeny of 2-4 female broodstock to fill them. They were frozen and shipped to College of Public Health, Medical and Veterinary Sciences, James Cook University. A total of 130 individual shrimp: 80 from four ponds of farm A (26, 25, 25 and four prawns/pond), and 50 from two ponds of farm B (25 prawns each) were randomly selected for DNA extraction. Total DNA was prepared according to the protocol mentioned in Chapter 3.1. Two microliters of the template (50 ng/µl) were used in the following experiments.

4.2.2. PCR detection of *Pst*DV-related sequences in shrimp genome

All of the 130 shrimp samples were screened by PCR assays (See Chapter 3.3) employing 8 overlapping primer pairs designed to cover the whole genome of *Pst*DV (minus the hairpin ends). Pairs 1-7 and 8 (ihhnA_3168/ihhnA_R3759) were adopted from two studies by Saksmerprome *et al*., 2011 and 2012, respectively

4.3. Results

The results of *Pst*DV insert screening are summarized in Table 4 (see Table C.1-C.3, Appendix C, for detail of each farm and background of shrimp examined). Based on the criteria, adopted from Saksmerprome et al. (2011), that shrimp with positive results for amplicons using all 8 primer pairs were considered to be positive for infectious *Pst*DV (the whole genome was amplified), whereas those showing incomplete results (for only some primer pairs, reflecting "gaps") were considered as false test positive (the whole viral genome could not be amplified), probably arising from viral inserts, 13 of the 130 samples (all from Farm A) produced amplicons for all of 8 primer pairs, suggesting that they contained infectious *Pst*DV. These samples were further subject to the evolutionary study in Chapter 6. The remaining 117 samples gave negative results for one or more of the primer pairs (Table 4), suggesting that the shrimp were not infected with *Pst*DV but carried 1–7 partial fragments of the *Pst*DV DNA, probably inserted into their genomic DNA. Of the 117 samples, region 2 was found to insert in most of samples (111 shrimp), accounting for 94.9% or approximately 5% greater than region 1, while the rest were found in < 10 samples (< 6%). Interestingly, region 7 (the "3031–3625" region) was detected in only one sample (0.85%) suggesting that it is least likely to be endogenized. Further analysis was carried out on 20 arbitrarily selected samples possessing gaps in the *Pst*DV genome using PCR with the MG831F/R primers specific for *Pst*DV DNA-linked shrimp genomic DNA (Tang and Lightner, 2006). Eight samples showed positive test results, indicating the presence of EVEs in their genome, while the rest gave negative test results, which can be explained as *Pst*DV-related sequences may be associated with other regions on shrimp genomes which the primers cannot anneal to (data not shown). However, further analysis of these samples was not conducted as the aim of this study is to identify the sequence that least likely to produce inserts only.

Table 4: Positive (+) and negative (−) PCR results obtained with DNA templates from 130 *P. monodon* using 8 overlapping primer sets that targeted the whole genome of *Pst*DV. In column 1, the number in brackets indicates the number of shrimp with the same pattern of positive and negative results. For example, in "Pattern 8", 3 samples have an identical pattern of positive and negative results.

Isolate (#)	Primer pairs used and positions relative to Mexican *Pst*DV isolate (AF273215)							
	1	2	3	4	5	6	7	8
	158-723	702-1260	1059-1507	1451-2027	2002-2569	2544-3100	3031-3625	3168-3759
Infective *Pst*DV (total 13 samples)								
Pattern 1 (13)	+	+	+	+	+	+	+	+
Seven inserts (1 gap) (total 1 sample)								
Pattern 2 (1)	+	+	-	+	+	+	+	+
Six inserts (2 gaps) (total 4 samples)								
Pattern 3 (4)	+	+	+	+	+	+	-	-
Four inserts (4 gaps) (total 2 samples)								
Pattern 4 (1)	+	+	-	-	-	+	-	+
Pattern 5 (1)	+	+	-	+	-	+	-	-
Three inserts (5 gaps) (total 6 samples)								
Pattern 6 (1)	+	-	-	-	-	+	-	+
Pattern 7 (1)	+	+	+	-	-	-	-	-
Pattern 8 (3)	+	+	-	-	-	-	-	+
Pattern 9 (1)	+	+	-	+	-	-	-	-
Two inserts (6 gaps) (total 91 samples)								
Pattern 10 (91)	+	+	-	-	-	-	-	-
One insert (7 gaps) (total 9 samples)								
Pattern 11 (8)	-	+	-	-	-	-	-	-
Pattern 12 (1)	+	-	-	-	-	-	-	-
No insert (total 4 samples)								
Pattern 13 (4)	-	-	-	-	-	-	-	-
% Total positive results by each primer pair	118/130 = 90.8	124/130 = 95.4	18/130 = 13.6	20/130 = 15.4	18/130 = 13.9	21/130 = 16.2	14/130 = 10.8	19/130 = 14.6
% Total inserts by each primer pair	105/117 = 89.7	111/117 = 94.9	5/117 = 4.3	7/117 = 6.0	5/117 = 4.3	8/117 = 6.8	1/117 = 0.9	6/117 = 5.1

4.4. Discussion

Scattered reports of viral inserts in the genome of shrimp (De la Vega, 2006; Tang and Lightner, 2006; Huang *et al.*, 2008), crayfish (Rusaini *et al.*, 2013) and insect (Lin *et al.*, 1999; Crochu *et al.*, 2004; Koyama *et al.*, 2010) genomes led to the hypothesis that random, autonomous insertion of such sequences occurs in these organisms and leads to specific, heritable immunity (Flegel, 2009). To test a prediction of that hypothesis regarding random insertion of viral sequences into the shrimp genome, Saksmerprome *et al* (2011) examined Thai *P. monodon* for random genomic insertions of *Pst*DV. By PCR analysis using multiple overlapping primer pairs to cover the whole *Pst*DV genome, PCR failure with some pairs indicated sequence gaps that revealed a random pattern of putative viral inserts in the genomes of individual shrimp occurred in a variety of ways (i.e. insertion). Using the same PCR detection methodology with 130 *P. monodon* collected from farms in northern Queensland, Australia, in 2012 (this study), only 13 samples were successfully amplified by all primers, suggesting that a random pattern of gaps was present in all the remaining samples (Table 4).

It is unlikely that the high frequency of putative gaps that we found resulted from mismatches between primers and target templates as the primers used accommodate the Australian *Pst*DV (GenBank No. GQ475529 and EU675312). It was also very unlikely that these putative gaps were from DNA degradation, since it should occur randomly and be probabilistically distributed evenly over the whole genome in any template DNA from a viral population; it would be unlikely for degradation to occur selectively in specific regions of the *Pst*DV genome in different individual shrimp samples. Based on the arguments above and on the accumulating evidence regarding EVEs in more recent publications (Tang and Lightner, 2006; Tang *et al.*, 2007; Flegel, 2009; Saksmerprome *et al.*, 2011), together with the consistency of the PCR results for the 8 *Pst*DV genome regions for 13 of the shrimp, the simplest explanation for random failures with the other 117 specimens was that they indicated gaps or absences of portions of the *Pst*DV genome.

Clearly seen in Table 4, region 7 (the "3031–3625" region) was detected in only one sample (0.85%) suggesting that it is least likely to be endogenised. This finding is in accordance with a recent study on the profiling of *Pst*DV insertion examined in Thai *P. monodon* (Saksmerprome *et al.*, 2011). It indicates the common characteristics of the insertion pattern across different geographical isolates of the shrimp, and offers the opportunities for developing a more specific approach for *Pst*DV detection. To illustrate this point, the design of primers or DNA probes for a PCR detection method focused on this area would be least likely to give a false test positive result for the samples that we examined i.e., 1/117 (0.9%) instead of 105/117 (~90%) and 111/117 (~95%) for regions 1 and 2, respectively. In addition, using a multiplexed method focused on regions 3 and 7 would theoretically eliminate false positives. However, this concept was based on the insertion profile found in *P. monodon.* To apply it to other species of shrimp (i.e. *P. vannamei*) or other species of animals (i.e. crab and crayfish), which may possess different patterns of EVEs, constructing "gap maps", such as that shown in Table 4, may be needed to aid the selection of the most suitable target DNA sequence that represents only the infectious *Pst*DV. Another way to eliminate false positive test results arising from EVEs is to detect the whole genome of the virus by using, but not limited to, the 8-overlapping PCR primer method described herein. However, the complexity and cost of 8 separate PCR reactions is not attractive. Thus, having simple and robust methods suitable for routine diagnosis such as LAMP (Jaroenram *et al.*, 2009; Arunrut *et al.*, 2011; Jaroenram *et al.*, 2012) and RPA (Piepenburg *et al.*, 2006; Euler *et al.*, 2012; Boyle *et al.*, 2013) would be useful.

In conclusion, we have explored and mapped the insertion patterns of *Pst*DV-related sequences inserted in the genome of Australian *P. monodon*, and we have found a particular region in the *Pst*DV capsid gene that is least likely to be endogenised (the "3031–3625" region). We speculated that the capsid gene is largely cytoplasmic and has little opportunity to interact with the shrimp genome and therefore integrates less often. Further researches to elucidate the insertion mechanism would be particularly informative. The "3031–3625" region was used as a target sequence to develop a novel *Pst*DV detection platform described in the next chapter.

CHAPTER 5

RECOMBINASE POLYMERASE AMPLIFICATION COMBINED WITH A LATERAL FLOW DIPSTICK FOR DETECTION OF INFECTIOUS *PENAEUS STYLIROSTRIS* DENSOVIRUS

5.1. Introduction

It is known that current PCR protocols including the OIE method detect non-infectious *Pst*DV-inserts found in *P. monodon* (Saksmerprome *et al.*, 2011) (See Chapter 2.3.1.3). The implications of false positives by using the method leads to a negative impact on international shrimp trade and disease quarantine management. Thus, more reliable detection platforms are required. Referring to the previous Chapter, the sequence on the *Pst*DV capsid gene at the nt position 3031–3625 (Genbank No. AF273215) is least likely to produce inserts (only 0.85% of false positive rate was detected). This region offers opportunities for developing a more specific approach for *Pst*DV detection. In addition to specific detection systems, having simple and robust methods suitable for routine and on-site diagnosis is also crucial to provide appropriate control measures for the virus, especially for an early response to an outbreak.

RPA-LFD is an alternative to PCR for disease diagnosis (Piepenburg *et al.*, 2006; Kersting *et al.*, 2014) (see Chapter 2.3.1.4). To the best of our knowledge, the RPA-LFD has not been used to detect any shrimp pathogen. Together with the serious issue of viral insertions mentioned previously (Chapter 2.3.1.3), the approach was examined here to determine whether it would detect *Pst*DV rapidly and reduce efficiently false positive results arising from viral inserts. The approach focuses the *Pst*DV capsid gene described above. Its working principle is illustrated in Figure 5.1.

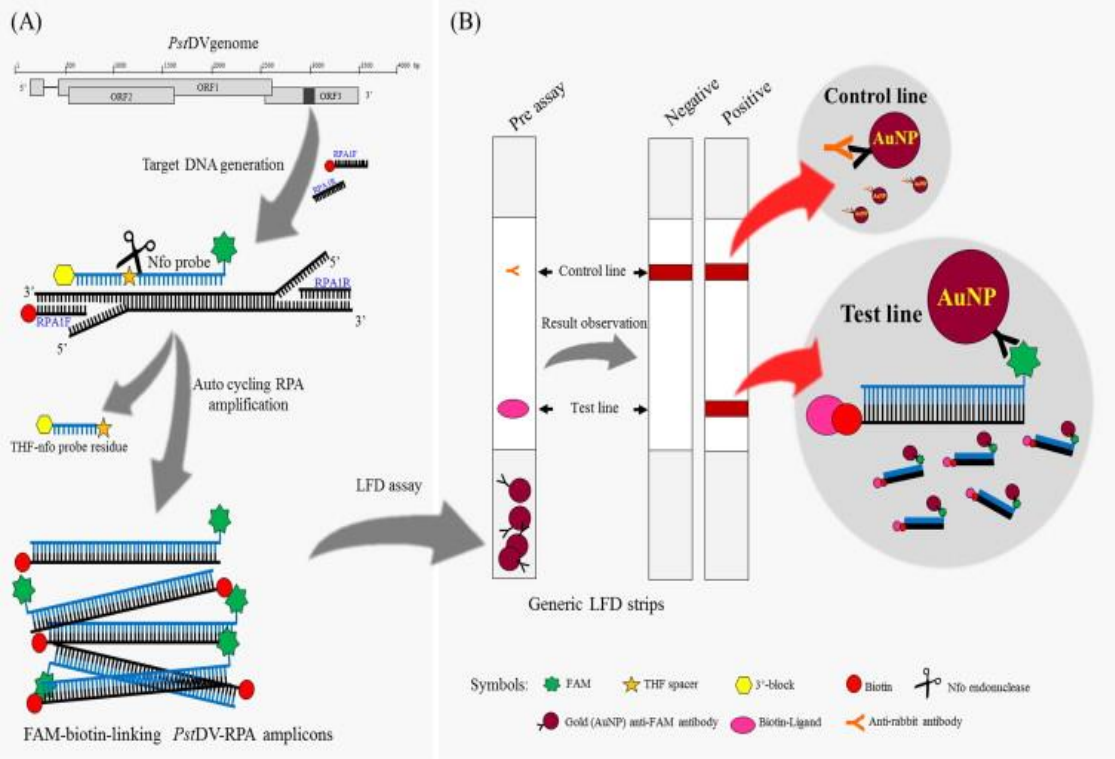


Figure 5.1: Schematic representation of RPA–LFD principle for the detection of *Pst*DV. The strategy was inspired by Piepenburg et al. (2006).

Our RPA-LFD protocol was illustrated (Figure 5.1) in a stepwise approach: (A) amplifying fluorescein amidite (FAM)-biotin-linking *Pst*DV-derived DNA amplicons. In the presence of target DNA template (nt position 3094–3214 on *Pst*DV ORF3, dark gray filled box), recombinase driven primers (Biotin labeled RPA1F/RPA1R) first generate templates for the annealing of the nfo probe (see Section 5.2.4). In the resulting double strand context, nfo endonuclease presented in the TwistAmp nfo kit (TwistDX, Cambridge, UK) recognizes the double-strand hybridization complex and cuts the *tetrahydrofuran* (THF) residue, allowing the probe to transform into an active reverse primer which subsequently works with the biotinylated primer in generating RPA products under the autocycling strand displacement activity of Sau DNA polymerase. The DNA amplicons produced effectively co-join the two antigenic residues (FAM and biotin) in one DNA molecule. (B) Detecting the RPA amplicons by LFD assay. In this step, the resulting amplicons are mixed with the appropriate buffer (see Section 5.2.6). Upon dipping

the generic LFD strips (Milenia Biotec, Giessen, Germany) into the mixture, the RPA amplicons are complexed with invisible gold-labeled anti-FAM antibodies coated on the sample pad, then travel in a buffer stream to be trapped at the test line by biotin-ligands, resulting in an appearance of red-pink color indicative of a positive result. Non-captured gold particles move through the test line to be fixed at the control line by anti-rabbit antibodies, and then produce colour serving as a flow control for the strip. In the absence of *Pst*DV target amplicons, colour will appear at a control line only.

5.2. Materials and methods

5.2.1. Samples infected with *Pst*DV

Frozen *P. vannamei* imported from China in 2012 were purchased from a local supermarket in northern Queensland, Australia. The muscles collected from 10 individual arbitrarily selected shrimp were screened for the presence of *Pst*DV using the interim 3-tube nested PCR protocol designed to detect the whole genome of *Pst*DV (Saksmerprome *et al.*, 2011). Samples with positive results were considered to be positive for infectious *Pst*DV (the entire genome was amplified). They were subjected to the following experiments.

5.2.2. Total DNA temple preparation

Total DNA was extracted from muscles of *Pst*DV-infected shrimp following the protocol described in Chapter 3.1. Two microliters (50 ng/µl) of the DNA template were used in RPA assays and recombinant plasmid construction.

5.2.3. Recombinant plasmid construction

The DNA sequences located between the nt positions 2544 and 3759 (1216 bp) of the *Pst*DV genome (GenBank No. AF273215) were amplified by PCR using ihhnA_F2544/ihhnA_R3759 (Saksmerprome *et al.*, 2010). The recombinant plasmid

containing the DNA fragment of interest was constructed, and purified according to the standard protocol described in Chapter 3.4. The plasmid was diluted in 10-fold serial dilutions to prepare stocks containing 5×10^9–0.5 copies/μl. Two microliters of the template was used in optimized RPA–LFD reaction.

5.2.4. RPA primer and probe design

According to the appendix in the TwistAmp™ reaction kit manuals (http://www.twistdx.co.uk/images/uploads/docs/Appendix.pdf), predicting the amplification performance of a given oligonucleotide primer based purely on its sequence is not yet possible for RPA, possibly due to a non-fully understood mechanism of RPA. This is unlike PCR or LAMP. Therefore, it is recommended to carry out a simple assay development process including the design and the screening of a series of candidate primers and selection of a preferred primer pair. However, we omitted this step as our RPA primers met our requirements satisfactorily. The primers were manually designed following the TwistAmp™ reaction kit manuals with additional criteria to PCR primer design as follows: (1) Primers typically are 30–35 nt long. Those shorter than 30 nt (i.e. PCR primers) or longer than 45 nt (i.e. LAMP inner primers) do not work well. (2) Long tracks of guanines at the 5′ end should be avoided, possibly because they are detrimental for the formation of recombinase filaments. (3) Guanines and cytosines at the 3′ end are preferred as they provide a more stable 'clamped' target for the polymerase. (4) Gibbs free energy (ΔG) should be lower than −4 kcal/mol for both 5′ and 3′ ends of the primers. Following these guidelines, the forward primer RPA1F with a 5′-biotine label (5′-biotin-CAACAGCAGCAAACTATGAAGACCCAATCC-3′), and the normal reverse primer RPA1R (5′-ATTTCTCCGCCATACCAATTTCTAGTGTCA-3′) for the detection of *Pst*DV were designed from the conserved sequence (nt 3094–3214) within the "3031–3625" region of *Pst*DV capsid gene (Mexican isolate, GenBank No. AF273215) (see Table 4 and Section 4.4). A DNA probe used for the LFD assay (hereafter called nfo probe) was designed from the sequence between RPA primers. The probe consists of an upstream stretch (30 nt) carrying a 5′-FAM antigenic label, which is connected via a THF spacer to an adjacent downstream oligonucleotide (15 nt) carrying a C3-spacer (polymerase extension blocking group) at its 3′ end (5′-

FAM-ACAATTTGGATTCATGGAACAAATGCGAAC-THF-GGTGACAGAAAAGCCTATAC-C3-spacer-3′). The primers and the probe were synthesized by Bio Basic, Canada.

5.2.5. RPA conditions and optimization

RPA was performed in a 50-µl volume using a TwistAmp nfo kit (TwistDX, Cambridge, UK), 0.42 µM each RPA primer, 0.1 µM nfo probe, 14 mM magnesium acetate, 1× rehydration buffer and DNase-free water. All reagents, except for DNA template and magnesium acetate, were prepared in a master mix, which was distributed into each 0.2-ml reaction tube containing a dried enzyme pellet. Magnesium acetate was pipetted into the tube lids. Subsequently, 2 µl DNA template was added to the tubes. The lids were closed, the magnesium acetate was centrifuged into the tubes to initiate the RPA mechanism, and then the tubes were incubated at the given conditions immediately.

To determine the optimum amplification temperature, the RPA reactions were carried out on a heating block set at 35, 37 and 40 °C for 45 min using 100 copies and 10 copies of plasmid DNA as temples followed by heat inactivation at 93 °C for 3 min. A reaction mixture without DNA template was included as a negative control. The products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) then analyzed by 2% AGE. To define the optimum time for amplification, the reactions were conducted at the optimal temperature for 20, 30, 45 and 60 min using various amounts of plasmid DNA as templates followed by product purification and AGE analysis as mentioned above.

5.2.6. Lateral flow dipstick (LFD) assay

To detect RPA amplicons by LFD, 2 µl of RPA product was added to 120 µl of the assay buffer (1X phosphate buffered saline with 0.1%Tween 20) in a new tube. Then, the LFD strip (Milenia Biotec, Giessen, Germany) was dipped into the

mixture for 2 min to visualize the test result. The entire LFD assay was done at room temperature.

5.2.7. Molecular specificity of RPA–LFD

The molecular specificity of RPA primers and probe was examined under the optimal conditions using 100 ng of total DNA prepared from shrimp infected with *Pst*DV, *Penaeus merguiensis* densovirus (*Pme*DV) and WSSV, 100 ng of total RNA extracted from shrimp infected with GAV, TSV, *Mr*NV, and 100 ng of DNA extracted *Pst*DV-free shrimp as templates. The resulting products were analyzed by LFD and AGE.

The ability of the LFD assay and AGE to discriminate *Pst*DV-RPA products from non-related DNA was also explored. RPA reactions containing various amounts of *Pst*DV-DNA (1 ng, 100 pg, 10 pg and 1 pg) were carried out at non-optimised conditions (35 °C for 1½ h) to ensure that non-specific amplicons were produced. Reactions with *Pst*DV-free DNA and with no DNA were also included as negative controls. The results by LFD and AGE were compared.

5.2.8. Molecular sensitivity of PRA by LFD and AGE

Ten fold serial dilutions of total DNA extracted from *Pst*DV-infected shrimp (10 ng, 1 ng, 100 pg and 10 pg) and plasmid DNA (10^5, 10^4, 10^3, 10^2 and 10 copies) were used as templates for RPA. The resulting DNA products analyzed by LFD and by AGE were compared.

5.2.9. Molecular sensitivity of one-step PCR and nested PCR by AGE

Unless otherwise stated, only two commonly accepted, standard PCR protocols for detection of infectious *Pst*DV were used here. One was the one-step method

employing 309F/R primers (Tang *et al.*, 2007) which was certified by OIE in the Manual of Diagnostic Tests for Aquatic Animals 2010 (http://www.oie.int/international-standard-setting/aquatic-manual/access-online/) which states, "Primer set 309F/R amplifies only a segment from IHHNV types 1 and 2 (the infectious forms of IHHNV), but not types 3A and 3B, which are non-infectious and part of the *P. monodon* genome." The other was the interim 3-tube nested PCR by Saksmerprome et al. (2011) who claim that, "This method employed 3 overlapping primer sets designed to cover 89.5% of the IHHNV genome such that positive result for the all three sets from a shrimp specimen would indicate the presence of infectious IHHNV". To determine the detection sensitivity, the same set of total DNA dilutions tested by RPA–LFD was amplified by the two PCR protocols mentioned above. The results by AGE were compared to those by RPA–LFD.

5.2.10. Reliability of RPA–LFD by statistical sensitivity and specificity analysis

Screening shrimp with unknown *Pst*DV status by RPA–LFD and the interim 3-tube nested PCR (reference method) was conducted using 96 frozen samples obtained from shrimp farms in northern Queensland, Australia, and 21 samples arbitrarily selected from imported shrimp purchased from local fish shops in Townsville, Australia, between 2002 and 2013 (117 samples in total). The reason to use the interim 3-tube nested PCR as a reference is because it detects the entire genome of *Pst*DV such that positive result from a shrimp specimen would indicate the presence of infectious *Pst*DV. Table 5.1 summarizes the test results by both assays relative to background of the shrimp tested (i.e. origin, year of collection, and species of shrimp). The results were used to evaluate the diagnostic performance of the RPA–LFD protocol following equations given in Table 5.2.

5.3. Results

5.3.1. Determination of the RPA–LFD conditions

The optimal temperature for RPA reaction as determined using 100 copies and 10 copies of plasmid DNA as templates revealed no differences in amplification at 35, 37 or 40 °C (Figure 5.2A). Thus, 37 °C was selected arbitrarily as the standard assay temperature. For RPA reactions performed at 37 °C for 20, 30, 45 and 60 min, DNA quantity at 30 min was similar to that at 45 and 90 min but higher than that at 20 min (Figure 5.2B). A reaction time of 30 min was thus selected as optimal.

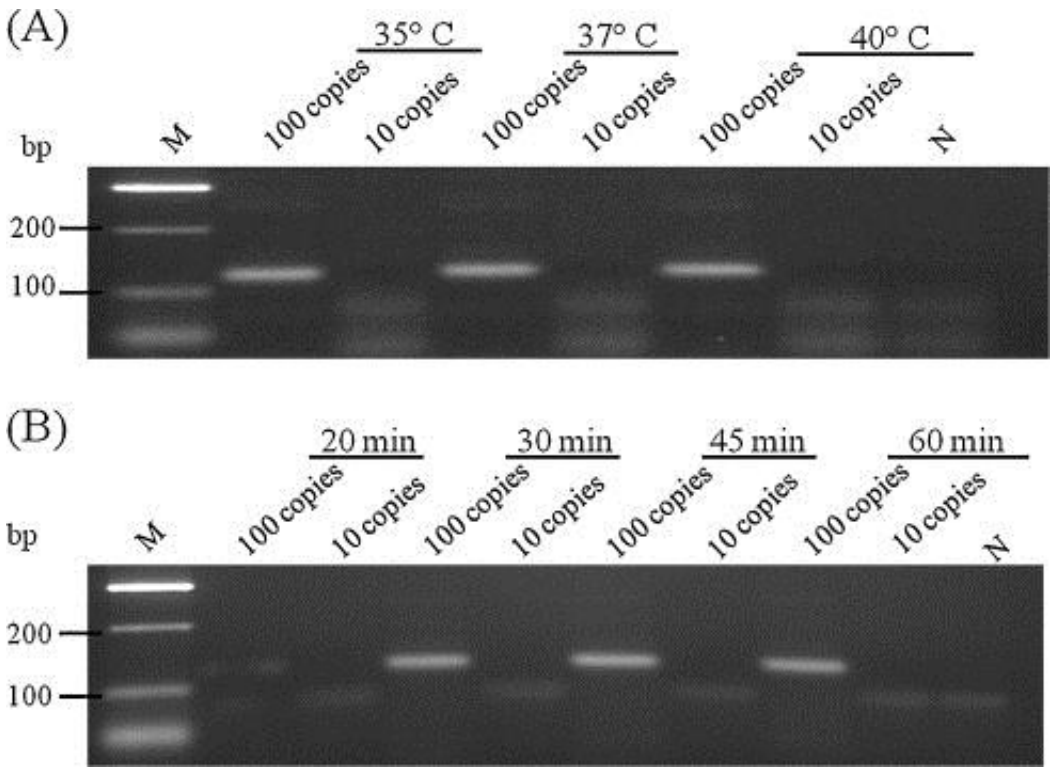


Figure 5.2: Optimization of RPA reaction using 10-fold serially diluted *Pst*DV-recombinant plasmid DNA as template. (A) Optimization of reaction temperature. (B) Optimization of reaction time. Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

5.3.2. Molecular specificity of RPA–LFD

For RPA primer and probe specificity testing at the optimal conditions, reactions were performed using DNA or RNA from other important viruses of shrimp. Clearly, no cross-reactions were found by LFD (Figure 5.3). The result was similar to that by AGE (data not shown) indicating that the primers and the probe were specific for *Pst*DV detection. The ability of the LFD assay to discriminate its target DNA from non-related DNA was also examined. AGE analysis of RPA products yielded under non-optimal conditions identified ladder-like DNA patterns for both *Pst*DV DNA (1 ng–1 pg) and *Pst*DV-free DNA (lane N1) (Figure 5.4A). In contrast, the LFD assay gave strong positive results only for its target DNA as determined by a 121-bp expected size of DNA on AGE (1 ng and 100 pg). With all other non-specific amplicons, the LFD strips gave red-purple color at only the control line (Figure 5.4B).

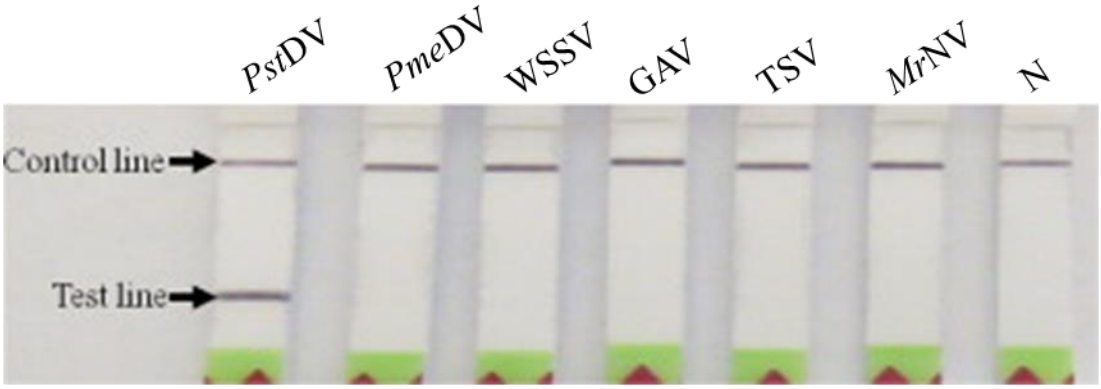


Figure 5.3: Molecular specificity of RPA–LFD carried out using shrimp viruses as template. N: total DNA extracted *Pst*DV-free shrimp (negative control).

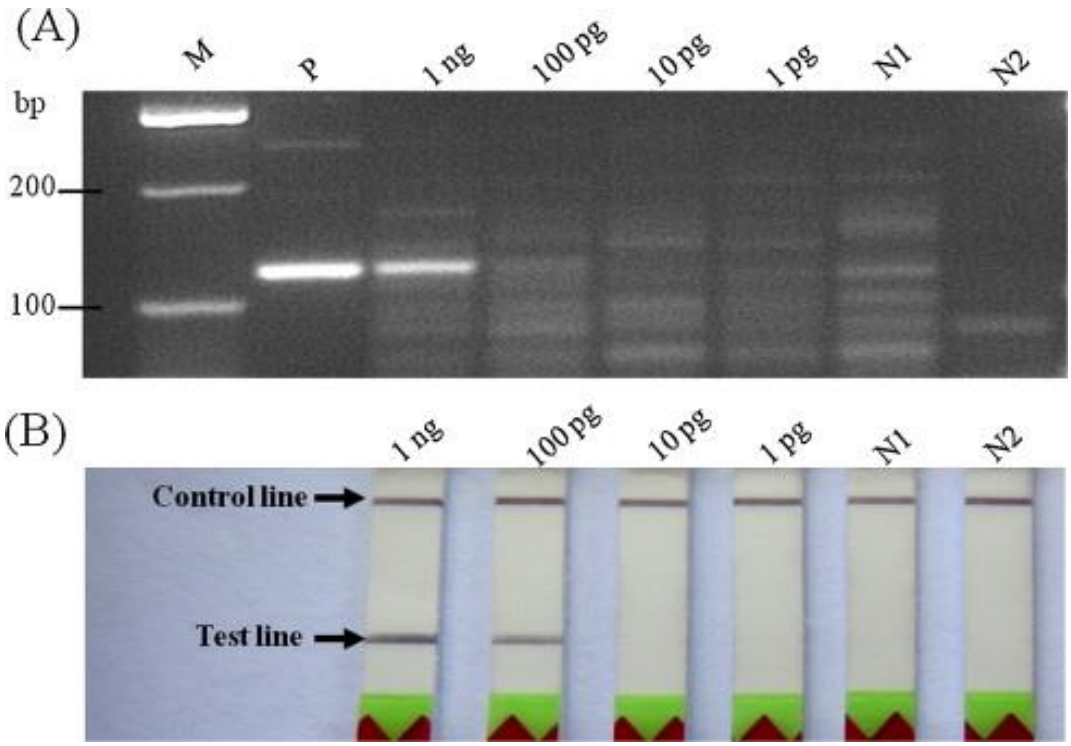


Figure 5.4: Comparison between the LFD assay and AGE for detecting *Pst*DV-RPA products. (A) AGE results for RPA amplicons amplified under non-optimal conditions using various amounts of DNA as template. M: molecular marker, P: positive control (100 ng DNA extracted from *Pst*DV-infected shrimp), 1 ng, 100 pg, 10 pg and 1 pg: dilution series of DNA prepared from *Pst*DV-infected shrimp, N1: negative control sample (100 ng of DNA extracted from *Pst*DV-free shrimp from Brunei), N2: no-template control (DNase-free water). (B) LFD assay results for the same RPA amplicons as in (A) except the positive control reaction.

5.3.3. Comparative molecular sensitivity of the RPA/AGE, RPA/LFD, PCR/AGE and nested RT-PCR/AGE

RPA–LFD using 10-fold serial DNA dilutions (total DNA extracts) identified a detection limit of 100 pg shrimp total DNA (Figure 5.5A). This was comparable to the detection limit of RPA-AGE (Figure 5.5B) and 10 times greater than that of one-step PCR (Figure 5.7A) and nested PCR (Figure 5.7B) (See figure caption for interpretation of results). Using the recombinant plasmids as templates, the RPA–LFD revealed a detection limit with strong positive signal at 100 copies (Figure

5.6A) while the AGE gave a faint band (Figure 5.6B). This result indicated that LFD detection could confirm the RPA products when the detection by AGE was not clear.

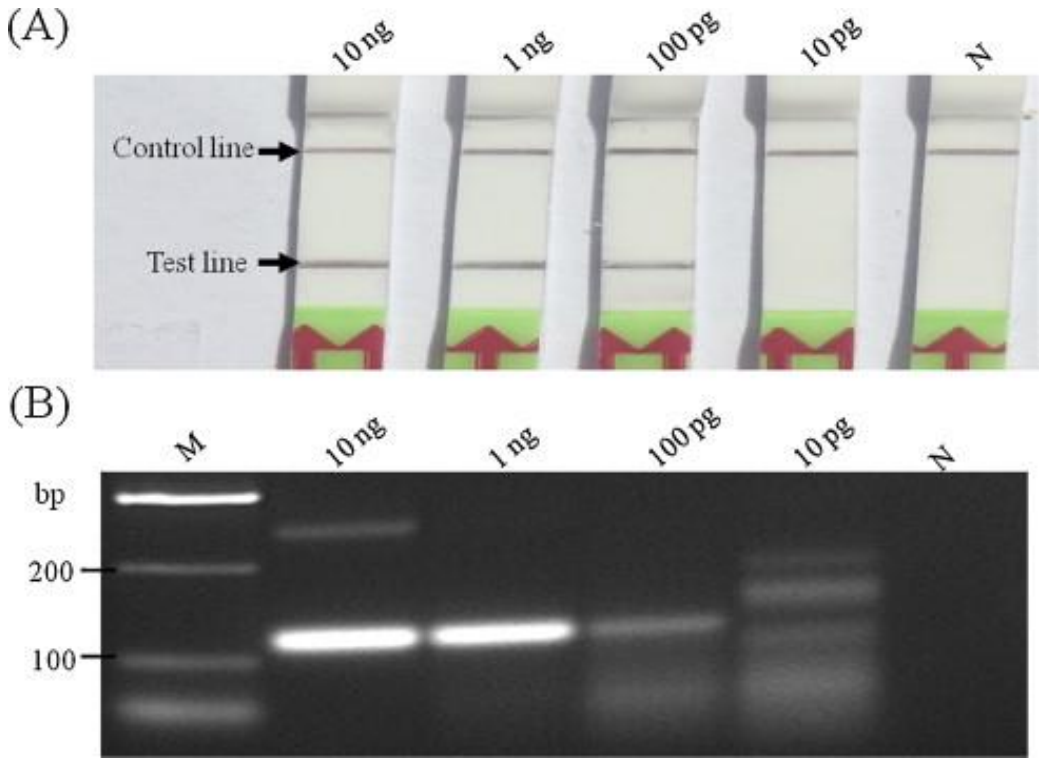


Figure 5.5: Molecular sensitivity test results of RPA using 10-fold serially diluted total DNA extracted from *Pst*DV-infected shrimp as template. (A) Results by LFD. (B) Results by AGE for the same RPA amplicons as in (A). Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

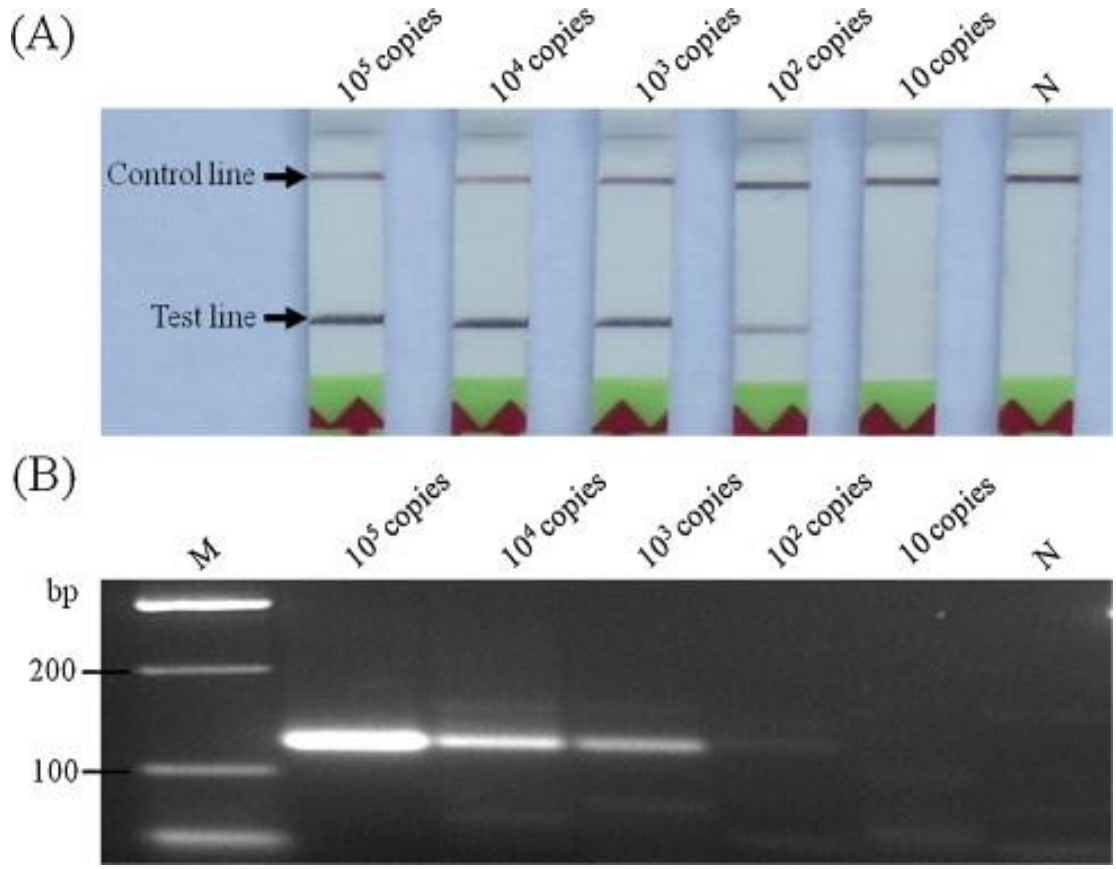


Figure 5.6: Molecular sensitivity test results of RPA using 10-fold serially diluted plasmid DNA as templates. (A) Results by LFD. (B) Results by AGE for the same RPA products as in (A). Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

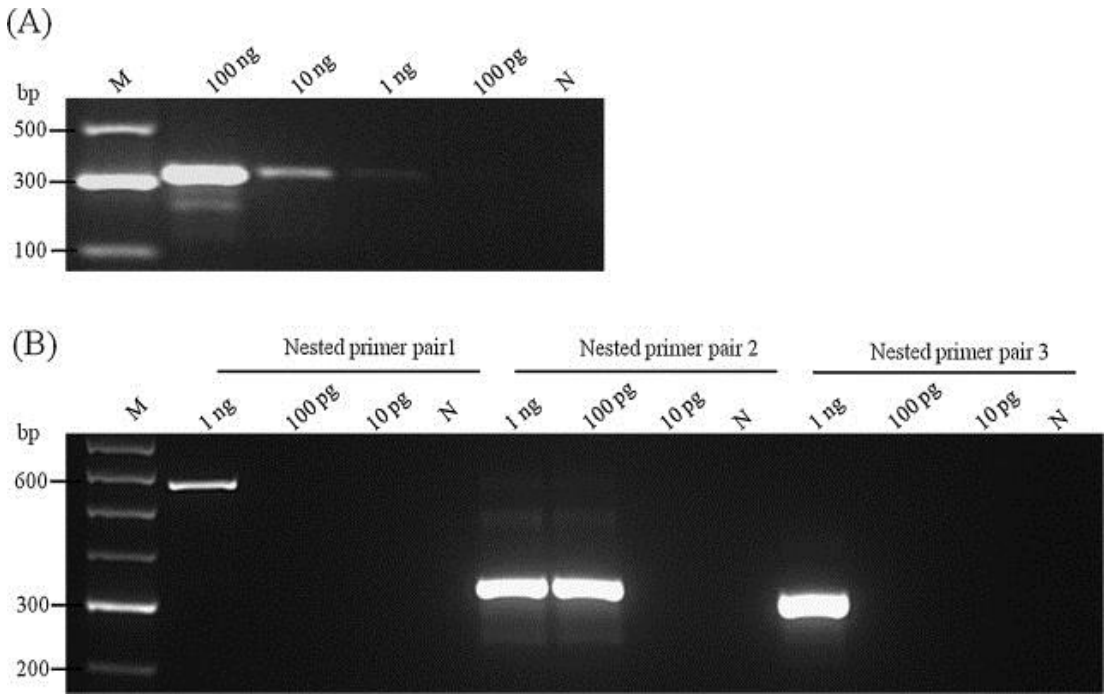


Figure 5.7: Molecular sensitivity of PCR and nested-PCR carried out using various amounts of *Pst*DV-DNA as template. (A) One-step PCR using the 309F/R primer method. (B) Interim 3-tube nested PCR employing three overlapping primer pairs covering the whole *Pst*DV genome. To determine the detection limit of this protocol, all the 3 primer sets must be taken into consideration. As the lowest detection limit of the primer pair 1 and pair 3 was 1 ng, the detection sensitivity of the whole system was therefore given to 1 ng. Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

5.3.4. Reliability of RPA–LFD by statistical sensitivity and specificity analysis

The number of shrimp, and *Pst*DV status of shrimp as determined by the reference assay (an interim 3-tube nested PCR), and the RPA-LFD protocol (Table 5.1) were used to evaluate the diagnostic performance of the RPA-LFD assay. The results were shown in Table 5.2, among 117 samples with unknown *Pst*DV status, 32 were identified as positive and 85 identified as negative for *Pst*DV by the reference method. For the 32 PCR–positive samples, RPA–LFD agreed 100% with the PCR results, indicating a statistical sensitivity of 100% (i.e. the ability of the RPA–LFD to identify correctly the actual *Pst*DV-positive shrimp tested is 100%). Among the 85 PCR-negative samples, 80 were negative with RPA–LFD, indicating a statistical

specificity of 94.12% (i.e. the ability of the RPA–LFD to identify correctly the actual *Pst*DV-negative samples tested is 94.12%). Re-testing was made with the five samples showing false positive by RPA–LFD and the similar results were obtained (data not shown). Assuming the reference assay is correct, overall, the accuracy of the RPA–LFD protocol to produce test result is 95.73%.

Table 5.1: Geographical origin, year of collection, species and number of shrimp, and *Pst*DV status of shrimp as determined by the reference assay (an interim 3-tube nested PCR), and the RPA-LFD protocol. The data provided here were used to evaluate the diagnostic performance of the RPA-LFD assay shown in Table 5.2.

				*Pst*DV status	
Origin	Year	Species	Number of sample	Positive by: reference /RPA-LFD	Negative by: reference /RPA-LFD
New Caledonia	2002	*P. stylirostris*	2	2/2	0/0
Brunei	2009	*P. monodon*	1	0/0	1/1
Malaysia	2012	*P. vannamei*	6	6/6	0/0
China	2005	*P. vannamei*	2	2/2	0/0
China	2012	*P. vannamei*	10	10/10	0/0
Australia	2012	*P. monodon*	96	12/12	84/79

Table 5.2: Diagnostic performance of the RPA-LFD protocol

	***Pst*DV status by reference assay**	
RPA-LFD	**Positive**	**Negative**
Positive	32 (TP)[a]	5 (FP)
Negative	0 (FN)	80 (TN)
%	100 (sensitivity)	94.12 (specificity)
%	95.73 (accuracy of RPA-LFD test result**)**	

[a]TP, true positive; FP, false positive; FN, false negative; TN, true negative.

Sensitivity = [TP/(TP + FN)]*100, Specificity = [TN/(TN + FP)]*100

Accuracy = [(TP+TN)/(TP+TN+FN+FP)]*100

5.4. Discussion

The detection platform described employs RPA for sensitive and specific amplification of a target nucleic acid fragment followed by a LFD assay for rapid visualization of the amplified DNA (Figure 5.1).

Temperature optimization (Sections 5.3.1) indicated that the assay was robust to temperature changes and 37 °C was chosen for further work as being between two equally effective temperatures (35 °C and 37 °C) (Figure 5.2A). Time analysis indicated that no improvement in yield occurred after 30 min (Figure 5.2B) so this was used in all analyses. The total analysis time including AGE was approximately 1 ½ h (excluding gel preparation). This time compares favourably to one-step PCR times of 2-3 h and nested PCR times of 4-5 h. However, at a very low concentration of target DNA, the RPA assay yielded nonspecific products occasionally (Figure 5.5B at 10 pg). This phenomenon may have arisen from unexpected amplification or primer-dependent artifact as suggested by Piepenburg et al. (2006).

In order to increase the specificity of test results and further speed up the total time for the RPA assay, we explored the possibility of using LFD format rather electrophoresis for detection of RPA amplicons. It was obvious that the LFD assay possesses high selectivity against mismatched DNA target sequences (Figure 5.4B). The high degree of specificity demonstrated by this assay was attributed to a use of gold-labeled anti-FAM antibodies to capture RPA product specifically. Clearly, the LFD assay could confirm the identity of RPA amplicons. As a result, it increased the overall specificity of the RPA–LFD over RPA combined with AGE and decreased time from 2 ½ h (30 min for RPA and 2 h for AGE including gel preparation) to approximately 35 min.

Detection of RPA products by LFD is simple, can be achieved within 1–2 min at room temperature. Visual detection of test results by this method abrogates the need

for post-amplification hybridization step for DNA products and probes required for LFD methods previously combined with LAMP for shrimp virus detection (Kiatpathomchai *et al.*, 2008; Jaroenram *et al.*, 2009; Puthawibool *et al.*, 2009; Nimitphak *et al.*, 2010; Arunrut *et al.*, 2011).

In conclusion, combining RPA amplification with an LFD technique resulted in a total assay time of approximately 35 min, about a quarter that of RPA-AGE and 6–8 times faster than the interim 3-tube nested PCR-AGE detection of *Pst*DV. The combined method also provided 10 times greater sensitivity than the nested PCR mentioned. High sensitivity and specificity, the relatively short detection time, and amplicon confirmation by hybridization are key advantages of this protocol. Although the protocol cannot eliminate false positive results (by viral inserts) completely, reducing those to ~5% (Table 5.2) compared to 76–78% by the IQ2000™ kit and the 309F/R PCR protocol reported previously (Saksmerprome *et al.*, 2011) presents RPA–LFD as an alternative to PCR for primary screening of *Pst*DV. As the protocol requires no other equipment than a simple heating block, it makes qualitative DNA testing potentially accessible in a non-laboratory, point-of-care environment. In addition, it serves as a model platform that could be adapted easily to detect other pathogens or genetic markers of interest using basic laboratory equipment.

CHAPTER 6

VIRAL ACCOMMODATION, EVOLUTION AND PHYLODYNAMICS OF AUSTRALIAN *PENAEUS STYLIROSTRIS* DENSOVIRUS

6.1. Introduction

Implementation of monitoring and control measures for viral epizootic outbreaks requires an understanding of the factors that underlie molecular evolution and population dynamics (Robles-Sikisaka *et al.*, 2010). This is also true for *Pst*DV circulating in Australia. Evidence for *Pst*DV existing in Australian penaeids has been accruing. Owens (1987) demonstrated histological and epidemiological evidence for the presence of *Pst*DV in prawns in northern Australia (Owens, 1987). In 1991, the presence of this virus, as indicated by histopathological evidence and high mortality, approaching 100%, in hybrid prawns (*Penaeus esculentus* x *P. monodon*) of northern Queensland was officially reported (Owens *et al.*, 1992). Genomes of *Pst*DV were subsequently sequenced from these hybrid prawns and from other Australian *Penaeus* sampled in the following 14 years (1992-2005), and submitted to GenBank database directly by Kjersti Krabsetsve and her colleagues (GenBank No. KM593908, EU675312, KM593909, KM593910, KM593911 and KM593912). In 2006, Tang and Lightner reported the presence of *Pst*DV-related sequences in the genome of *P. monodon* broodstock originating from Australia during 2004 (Tang and Lightner, 2006). These endogenous viral elements (EVEs) were assigned as a non-infectious form of *Pst*DV (GenBank No. EU675312). Recently, an infectious type of the virus was rediscovered in this region in 2008 (Saksmerprome *et al.*, 2010). Interestingly, high mortalities and obvious gross pathology i.e. RDS caused by *Pst*DV in prawn have not been recorded in this country since the first official report in 1991 despite the virus existing here, as demonstrated by positive results of PCR tests (Krabsetsve *et al.*, 2004; Saksmerprome *et al.*, 2010; Jaroenram and Owens, 2014). Our hypothesis is that prawns have developed resistance to *Pst*DV and/or the virus has mutated to reach equilibrium with the host in terms of virulence genes. To test this hypothesis, in this Chapter, we sequenced and analysed the genome of Australian *Pst*DV, and

determined its genetic recombination and molecular evolution in relation to other geographical isolates of *Pst*DV.

6.2. Material and methods

6.2.1. Shrimp samples and DNA temple preparation

*Pst*DV-related sequences inserted in the genome of *P. monodon* (EVEs) can cause false positive results for infectious *Pst*DV using any single primer pair based PCR protocol (Tang and Lightner, 2006; Saksmerprome *et al.*, 2011; Jaroenram and Owens, 2014). To ensure that our results were not derived from EVEs, we took *Pst*DV-infected *P. monodon* reported in from Chapter 4 (Jaroenram and Owens, 2014). Briefly for the Chapter 4, 130 prawns with no signs of *Pst*DV infection i.e. RDS-associated stunt growth were sampled from 2 farms in northern Queensland in 2012. They were used to examine the insertion pattern *Pst*DV-related sequences using multiple overlapping primers pairs designed to detect the whole *Pst*DV genome. Thirteen samples were positive for all the primers (i.e. whole genome was amplified) indicating that they carried infectious *Pst*DV. Among these, 9 showing strong and specific PCR bands were chosen for further studies in this chapter. The positive shrimp were divided arbitrarily into 3 groups. Gills or pleopods from each group were pooled for DNA extraction following the protocol described in Chapter 3.1.

6.2.2. PCR amplification, cloning and genome sequencing of Australian *Pst*DV

Each DNA pool was subject to a standard PCR amplification (see Chapter 3.3) using multiple overlapping primers (Table 6.1). The recombinant plasmids containing PCR fragments of interest were constructed and purified. Sequencing of both strands of DNA plasmids (5-6 clones/PCR fragment/DNA pool) was performed (see Chapter 3.4).

Table 6.1: Primers used to sequence the complete genome of *Pst*DV in this study.

Primer pair	Designation	Sequence (5′-3′)	Nucleotide start position	Product size (bp)	Reference
1	ihhnAF158	ATGGAAGATACGAACAACCAC	158	566	(Saksmerprome *et al.*, 2010)
	ihhnAR723	GGACCTGGGGTGAGAAGGCT	723		(Saksmerprome *et al.*, 2010)
2	F570	CGACGAAGAATGGACAGAAA	507	437	Present study
	R1006	GGAATCTGATGTGTCACTGATGT	1006		Present study
3	ihhnAF702	CAAGCCTTCTCACCCCAGG	702	877	(Saksmerprome *et al.*, 2010)
	R1578	ATGGCGTGGCCAAGAC	1578		(Molthathong *et al.*, 2013)
4	ihhnAF1451	GTTACCTTTGCTGCCAGAGC	1451	905	(Saksmerprome *et al.*, 2010)
	mihhnAR2355	GGTGGTACCCAGTAGTCTATATC[a]	2355		Modified from (Saksmerprome *et al.*, 2010)
5	ihhnAF2002	AGCTTGGATAATCATCGTAGCAG	2002	568	(Saksmerprome *et al.*, 2010)
	ihhnAR2569	GGGCTTGCTCTTGTTGAATCGG	2569		(Saksmerprome *et al.*, 2010)
6	F2436	CAGCCAGTACGACATCAACCCT	2436	703	Present study
	R3138	CTCCTGTTCGCATTTGTTCCAT	3138		Present study
7	mihhnAF3031	CTAAGGAAGCCGACGTAACA[a]	3031	727	Modified from (Saksmerprome *et al.*, 2010)
	ihhnAR3759	GACTCTAAATGACTGACTGACG	3759		(Saksmerprome *et al.*, 2010)

[a]Underlined nt replaces an original base A to make the primer accommodate the Australian isolate (GenBank No. EU675312)

6.2.3. Bioinformatical analysis of *Pst*DV genome

The overlapping sequences (above) were analysed using Sequencer 4.9, and the presence of potential ORFs was determined using the AlignX tool in the Vector NTI program (Invitrogen, California, USA). The predicted amino acids and the molecular weights of any potential proteins in an ORF were determined using proteomic tools at ExPASy (http://us.expasy.org). The genome sequence of Australian *Pst*DV reported in this article (hereafter called AUS-*Pst*DV2012) has been submitted into GenBank database with the reference number KM593913 (Appendix D). Comparative analyses of the genome and the encoded ORFs of this *Pst*DV isolate were performed using a BLAST search against the National Centre for Biotechnology Information (NCBI) database.

6.2.4. Point mutation analysis

To prove that any point mutation found in ORF1 of the Australian *Pst*DV (GenBank No. EU675312) was real and inheritable, ORF1 was re-amplified from individual *Pst*DV-positive shrimp obtained as above and then sequenced. The sequences were compared against the reported *Pst*DV sequences (Table 6.2) using Vector NTI.

6.2.5. Phylogenetic analysis

Our entire DNA sequence was aligned the sequences of 19 worldwide distributed *Pst*DV representatives (Table 6.2) using MUSCLE 3.7 (Edgar, 2004). After refinement using Gblocks 0.91b (Castresana, 2000; Talavera and Castresana, 2007), all sequences with a standardized length of 2988 bp (except EU675312 and our sequence having 2987 bp in length due to a single base deletion, see below), covering ORF1 (NS1 herein), ORF2 (NS2), and ORF3 (VP) were used to construct a phylogenetic tree under 1000 bootstrapping replicates using the maximum likelihood method implemented in the PhyML 3.0 (Guindon *et al.*, 2010). Graphical representation and editing of the phylogenetic tree were performed with TreeDyn 198.3 (Chevenet *et al.*, 2006).

6.2.6. Recombination and selection analysis

Prior to analysis, the 20 *Pst*DV sequences (Table 6.2) were first aligned, 2 sequences containing gaps and/or incomplete ORFs (GenBank No. GQ411199 and DQ228358), were excluded from analysis as they were not recognized by the programmes described herein. The remaining sequences were divided into 2 groups according to their origin: 1) outside Australia (AF218266, EF633688, AF273215, AY362548, KF214742, JX840067, AY355308, JN377975, AY102034, AY362547), and 2) inside Australia (KM593908, KM593909, KM593910, KM593911, KM593912, GQ475529, EU675312 and this study). The host origins of the Australian endemic strains were given in Appendix E. ORFs of each group were analysed for evidence of genetic recombination breakpoints (BPs) using the Genetic Algorithms for Recombination Detection (GARD) undertaken under 4 nt substitution bias models (F81, TrN93, HKY85 and REV) (Pond *et al.*, 2006). Site-specific signatures of positive and negative selections acting on each sequence were also evaluated using three different codon-based maximum likelihood methods: Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL) and Random Effects Likelihood (REL) (Pond and Frost, 2005). Briefly, the first method estimates the non-synonymous substitutions (dN) and synonymous substitution (dS) values from reconstructed ancestral sequences and compares them against the expected corresponding values. The second method estimates dN and dS directly from the data at each codon site, while the last method estimates these parameters across sites according to a predefined distribution. Signatures of natural selection can then be inferred using the ratio of dN and dS, with ratio values less than 1 consistent with negative selection acting on aa-altering mutations, and values greater than 1 suggesting positive selection to increase the number of replacements.

6.2.7. Inference of the nucleotide substitution rate and the demographic history

The rate of nt substitution was estimated using the computer software package called Bayesian evolutionary analysis by sampling trees (BEAST) version 2.1.3 (Bouckaert *et al.*, 2014). The program uses a Bayesian Markov chain Monte Carlo (MCMC) approach to evaluate evolutionary hypotheses without conditioning to a tree

topology, and it has been used to estimate the rate of evolution, divergence times and temporal changes in effective population size (Cordellier and Pfenninger, 2008; Duffy and Holmes, 2008; Fontanella *et al.*, 2008). The substitution rate per site per year for *Pst*DVs circulating in Australia was estimated from an independent data set that included a total of 8 dated *Pst*DV DNA sequences: one was from this study (Table 6.2, row 3) and the rest were taken from GenBank database (Table 6.2, row 1 and 7-12). The sampling time interval of these sequences was 22 years (1991 to 2012). After trimming incomplete ends that were unavailable for some isolates, all sequences with a standardized length of 2988 bp (except those from 1993 and 2012, this study, having 2987 bp in length due to the 1305delA), covering ORF1 (NS1 herein), ORF2 (NS2), and ORF3 (VP), were aligned using ClustalX 2.1 (Larkin *et al.*, 2007). The aligned output file was further analysed using BEAST 2.1.3. Evolutionary model parameters incorporated in this analysis were as follows: 1) the nt substitution and the site heterogeneity models were specified as HKY + G (4 categories) as suggested by jModeltest 0.1.1 (Posada, 2008), 2) the coalescent tree prior was set to a constant population size as suggested elsewhere (Kim *et al.*, 2012). The MCMC chains (1.7×10^8 generations, with the sampling frequency of 1,000) were performed separately under 3 molecular clock assumptions: strict clock; relaxed clock exponential; and relaxed clock log normal. The output files were analysed by Tracer 1.4 (Rambaut and Drummond, 2007) to verify chain convergence and recognized when the effective sample sizes (ESS) exceeded 200. The best-fit model was determined by comparing the marginal likelihood estimate value of each model using the harmonic smoothed mean estimator implemented in Tracer 1.4. The nt substitution rate of *Pst*DV was determined using the best-fit model. Parallel estimation for the 20 dated cosmopolitan *Pst*DV (Table 6.2) was undertaken and compared.

Population history of Australian endemic *Pst*DV was investigated using Bayesian skyline plot (BSP), which estimates changes in effective population size (N_e) through time (T) from a sample of gene sequences (Strimmer and Pybus, 2001; Drummond *et al.*, 2005); as *Pst*DV is a virus, we interpreted this parameter as the effective number of infections in units of generations/year as suggested elsewhere

(Robles-Sikisaka *et al.*, 2010). Given that the *Pst*DV generation time is unknown, we did not attempt to translate this parameter into the actual number of infections. The analysis was performed under the best-fit clock model with additional parameters including a constant skyline plot, and a uniform prior population size with an upper bound of 10^7. The MCMC algorithm was run for 2.5×10^7 generations, with sampling every 1000 generations. Visualization of parameters and BSP plots were examined in Tracer 1.4.

6.3. Results

6.3.1. Retrospective analysis of data from 1993

In the thesis work of Krabsetsve, 2005 (unpublished), a number of *P. monodon* collected from farms in northern Queensland, Australia, in 1993 were screened for the presence of *Pst*DV using PCR employing multiple overlapping primers designed to cover the whole genome sequence of the virus. The objective was to obtain the complete sequence of many Australian *Pst*DV isolates for epidemiological analysis. The complete genomic DNA of the virus was obtained and submitted to GenBank database with a given reference number EU675312. We preliminarily analysed the genome sequences of this viral strain, and we found a base A deletion at the nt position 1305 (1305delA) in ORF 1 which is relative to position 1949 in its genome. As this unexpected mutation is not found in other geographical isolates (see below for detail of analysis), it opens up the question as to whether the1305delA mutation is real, i.e. not a result from technical issues such as sequencing error. If it is real, is it consistently inherited over time? To answer these questions, subsequently experiments were conducted.

6.3.2. Whole genome analysis of Australian *Pst*DV

According to the sequence analysis, the genome of our *Pst*DV isolate (AUS-*Pst*DV2012) is 3604 nt in length and has a base composition of A (36.93%), T (20.48%), G (19.26%) and C (23.34%). The G/C and A/T content of the genome

was calculated to be 42.59% and 57.41%, respectively. Unlike any other infectious *Pst*DV including the Hawaii isolate (AF218266), our isolate has ORF1 fragmented into 2 smaller ORFs, and for the purpose of analysis, they are designated as ORF1 fragment 1 (ORF1 Fr1) and ORF1 fragment 2 (ORF1 Fr2). Their sizes are 1404 bp and 654 bp, respectively) (Figure 1A). DNA sequences of these fragments overlapped and they are in different reading frames (+1 and +3, respectively), indicating the presence of frame-shift deletion. If functional, the ORF1 Fr1 (nt 442-1845) would encode a non-structural fragment 1 (NS1 Fr1) protein of 467 amino acids (aa) in length, which corresponds to a molecular weight (MW) of 52.83 kDa. This NS1 Fr1 protein contains the highly conserved replication initiator motifs, located between the aa position 257-315, which are common to all other infectious *Pst*DV. ORF1 Fr2 (nt 1788-2441) would encode a NS1 fragment 2 (NS1 Fr2) protein of 217 aa in length, which corresponds to a MW of 24.75 kDa. This aa sequence contains 3 highly conserved NTP-binding and helicase domains, located between aa position 35-128 relative to the aa position 480-578 of the Hawaii isolate (GenBank No. AF218266). Similar to other infectious *Pst*DV, ORF2 (nt 386-1477, 1092 bp in length), our isolate encodes a 363-aa non-structural protein 2 (NS2) with a MW of 42.15 kDa, while the ORF3 (nt 2383-3372, 990 bp in length) encodes a 329-aa viral capsid protein (VP) with a MW of 37.43 kDa. These 2 ORFs are in reading frames +2 and +1, respectively. Figure 6.1 depicts the overall genome organization of our isolate in comparison with that of the Hawaiian strain.

We compared our entire sequence with matching regions of *Pst*DV sequence records at GenBank (Table 6.2) and found that our sequence (Table 6.2, row 3) had 92.5% and 93.9% identity to sequences of *Pst*DV previously reported from Australia (EU675312) and Madagascar (DQ228358), respectively (lineage I). Interestingly, it shared slightly lower identity (~92%) with several other sequences originating from the Americas and some parts of Asia (lineage III). Focusing on each ORF, ORF1 Fr1 showed the highest degree of similarity (~95%) to *Pst*DVs in lineage I. ORF1 Fr2 and ORF2 were most similar to those in lineage IV, while ORF3 showed comparable identity to all lineages (~93%). These observations were in accordance with that of aa comparison (Table 6.2).

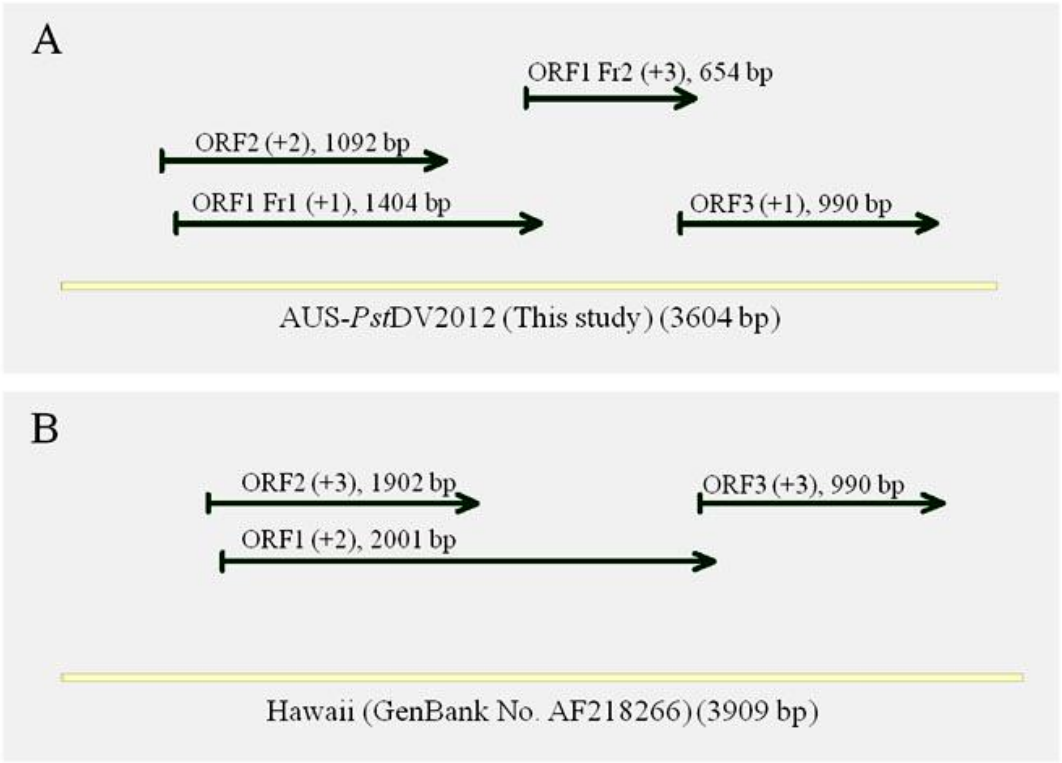


Figure 6.1: (A) Genome organization of Australian *Pst*DV obtained from this study. (B) Genome organization of infectious *Pst*DV representative taken from GenBank database. Fr1 and Fr2 are referred to as fragment 1 and 2 of ORF1, respectively. Plus sign followed by the numbers in parentheses indicates the reading frame of that ORF.

Table 6.2: Comparison of nucleotide and predicted amino acid sequences of the AUS-*Pst*DV2012 (lineage II) and other sequenced *Pst*DVs from different geographical regions. The similarity was estimated based on the full length sequences of respective ORFs or partial sequences available. For the purpose of illustration in the Table, Fr1 and Fr2 are referred to as ORF1 fragment 1 and 2, respectively. For convenience, they are shown sequentially but actually overlap.

GenBank no.	Host species	Origin/Year of isolation	Lineage[a]	Genome identity (%)	Nucleotide similarity (%)				Amino acid similarity (%)			
					ORF1 fragment		ORF2	ORF3	NS1 fragment		NS2	VP1
					Fr1	Fr2			Fr1	Fr2		
EU675312	*P. monodon*	Australia/1993	I	92.5	94.7	92.2	92.8	93.2	100	95.4	89.5	96.7
DQ228358	*P. monodon*	Madagascar/2000	I	93.9	94.5	92.1	92.9	93.3	89.7	94.9	89.6	97.0
AUS-*Pst*DV2012	*P. monodon*	Australia/2012	II	–	–	–	–	–	–	–	–	–
AY102034	*P. monodon*	Thailand/2000	III	90.3	91.8	91.7	94.9	91.1	86.9	98.2	92.5	94.5
AY362547	*P. monodon*	Thailand/2003	III	91.0	92.2	91.7	95.2	91.6	87.4	98.2	93.7	95.1
GQ411199	*P. monodon*	India/2007	III	88.9	91.7	90.8	94.7	90.1	86.4	84.8	92.0	93.6
KM593908	*P. esculentus*/*P. monodon* hybrid	Australia/1991	III	93.6	92.6	94.2	96.0	93.7	89.7	98.6	94.2	96.1
KM593909	*P. monodon*	Australia/1995	III	92.2	92.6	93.6	95.9	93.1	89.2	98.6	94.7	95.4
KM593910	*P. monodon*	Australia/1996	III	92.1	92.4	93.6	95.7	92.8	88.3	98.6	94.2	95.4
KM593911	*P. monodon*	Australia/2004	III	92.9	92.1	92.7	95.5	93.1	87.8	98.6	93.6	95.7
KM593912	*P. monodon*	Australia/2005	III	93.1	92.4	93.0	95.6	93.1	88.5	98.6	94.2	95.7
GQ475529	*P. monodon*	Australia/2008	III	90.3	91.6	87.8	95.0	91.3	87.6	97.2	92.5	95.7
AF218266	*P. stylirostris*	Hawaii/1986	IV	92.4	92.1	94.0	95.8	93.4	89.2	98.6	93.9	96.4
EF633688	*P. monodon*	China/2007	IV	92.3	92.1	93.7	95.6	93.4	88.7	98.6	93.7	96.1
AF273215	*P. stylirostris*	Mexico/1998	IV	92.2	92.1	93.9	95.7	93.2	89.0	98.2	93.9	96.1
AY362548	*P. vannamei*	Ecuador/2003	IV	92.2	92.1	94.2	95.8	92.9	89.2	98.6	93.9	95.7
KF214742	*P. vannamei*	China/2011	IV	92.0	91.5	93.7	94.4	92.6	87.6	97.2	92.3	95.4
JX840067	*P. monodon*	Viet Nam/2012	IV	91.0	91.8	93.9	95.3	92.9	88.3	97.7	93.1	95.1
AY355308	*P. monodon*	Taiwan/2003	IV	92.4	92.1	94.2	95.7	93.5	89.0	98.6	93.7	96.4
JN377975	*P. vannamei*	Korea/2010	IV	92.0	91.8	94.2	95.4	92.8	89.4	98.6	93.4	95.4

The similarity was estimated based on the full length sequences of respective ORFs or partial sequences available. For the purpose of illustration in the Table, Fr1 and Fr2 are referred to as ORF1 fragment 1 and 2, respectively. For convenience, they are shown sequentially but actually overlap.

[a] Lineages were clustered based on the phylogenetic analysis in this study.

6.3.3. Mutation analysis

From Figure 6.2A (top), A at the nt position 1305 is missing (1305delA) in ORF1 of the Australian *Pst*DV from 1993 (EU675312) and 2012 (this study). To verify this finding, *Pst*DV-ORF1 was re-amplified from 9 individual infected shrimp then sequenced. Clearly seen in Figure 2A (bottom), the ORF1 from all samples were homozygous for the 1305delA mutation when compared to other infectious isolates listed in Figure 6.2B. Examples of sequence chromatograms of the 1305delA containing *Pst*DV-ORF1 are shown in Figure 6.2C, demonstrating that the 1305delA is not a technical error.

Further inspection revealed that the 1305delA occurs at the nuclear localization signal (NLS) encoding sequence which is conserved among different geographical isolated *Pst*DV genomes (Owens, 2013). ORF1 has many potential NLSs with embedded Chelsky sequences (4 aa, three of which are basic, starting dibasic e.g., K-K/R-x-K/R where K, R, x represent arginine, lysine and any aa, respectively). One NLS starts at aa position 423 (KKK-16aa-KRK). As pointed out by Owens (2013), our investigation confirms that the 1305delA causes an aa frame-shift in the second half of NLS for both Australian *Pst*DV isolates reported in 1993 (Table 6.3, row 1) and 2012, the present study, (Table 6.3, row 2). This out-of-frame region destroyed the KRK sequence at the C terminal. When a base A was artificially added into the 1305delA position (i.e. 1304insA) of the above isolate, (Table 6.3, row 3), it put the reading back in-frame, turning ORF1 Fr1 into a complete putative ORF1 which could generate the NLS with functional characteristics (22 aa in length with KKK and KRK towards the N terminal and C terminal, respectively) of infectious *Pst*DV-derived NLSs (Table 6.3, row 4-11). Also of considerable interest is that this frame shift straddles the site of the OIE diagnostic IHHNV309 primers (Tang and Lightner, 2006) that are reported to separate infectious *Pst*DV from endogenous viral element *Pst*DV of Australia and Madagascar.

Figure 6.2: Comparison of the 1305delA-containing ORF1 (NS1) sequences obtained from various sources. (A) The Australian *Pst*DV isolated from *P. monodon* cultured in 1993 (GenBank No. EU675312) and 2012 (AUS-*Pst*DV2012) (top), and nine individual *Pst*DV-infected shrimp from which the AUS-*Pst*DV2012 originated (bottom). (B) Infectious *Pst*DV sequences from the Genbank database. (C) Examples of sequence chromatograms corresponding to *Pst*DV sample isolated from shrimp No. 1 – 5 listed in (A, bottom).

Table 6.3: The penaeid host, geographical location and the likely NLS of *Pst*DV in NS1 protein. Bolded aa are potential NLS joined by 16 aa. Superscripted number in front of the listed NLS sequences indicates the starting position of NLS in NS1 protein sequence.

Row	GenBank No.	Host species	Origin/year of isolation	Pattern of putative NLS (KKK – 16 aa – KRK)
1	EU675312	*P. monodon*	Australia/1993	^{423}**KKK**FSQSQLSLITIYTIYQKGN[a]
2	This study	*P. monodon*	Australia/2012 with 1305delA	^{423}**KKK**FSQSQLSLITIYTIYQKGN[b]
3	This study	*P. monodon*	Australia/2012 with 1304insA	^{423}**KKK**FSQSQLSLITNLHNIS**KRK**[c]
4	AF218266	*P. stylirostris*	Hawaii/1986	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
5	AY362548	*P. vannamei*	Ecuador/2003	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
6	AF273215	*P. stylirostris*	Mexico/1998	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
7	AY355307	*P. vannamei*	Taiwan/2001	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
8	GQ411199	*P. vannamei*	Korea/2010	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
9	EF633688	*P.vannamei*	China/2007	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
10	AY362547	*P. monodon*	Thailand/2003	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]
11	GQ475529	*P. monodon*	Australia/2008	^{423}**KKK**SNQSQLSLITNLQNIS**KRK**[a]

[a]Adopted from Owens' study (2013) (Owens, 2013)
[b]NLS sequence of the AUS-*Pst*DV2012 with 1305delA. It shows 54.6% identity to NLS of infectious isolates (row4-11).
[c]NLS sequence of the AUS-*Pst*DV2012 with an 'A' artificially inserted after nt 1304 in ORF1. It shows 86.4% identity to NLS of infectious isolates (row 4-11).

6.3.4. Phylogenetic tree analysis

The relationship of our *Pst*DV sequence was examined in a phylogenetic analysis with the other 19 cosmopolitan *Pst*DV representatives (Table 6.2). The cluster tree (Figure 6.3) suggested that the *Pst*DVs were divided into four lineages: I, II, III and IV with 100% bootstrap value support, indicating potential genetic differences between lineages. Our isolate was clustered in lineage II which was newly formed here.

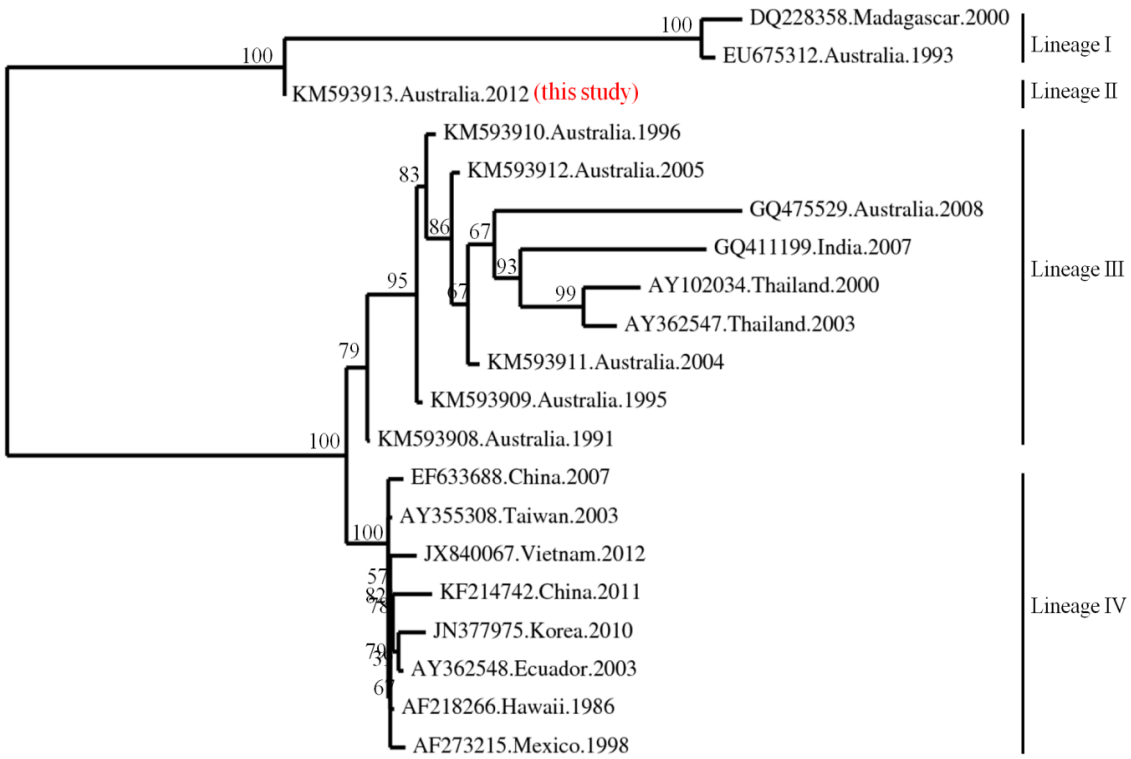


Figure 6.3: Phylogenetic relationships of the Australian *Pst*DV isolate and other geographical isolates as analysed using the maximum likelihood method implemented in the PhyML 3.0. The numbers shown at branch point indicate 1000 bootstrap values which determined the confidence indices within the tree.

6.3.5. Recombination and selection analysis

When GARD was performed, 10 recombination breakpoints (BPs) were detected in only the Australian *Pst*DVs (see Section 6.2.6, Group 2 dataset), 4 in ORF1 Fr1 (nt 139, 475, 846 and 1026) (Figure 6.4A), 1 in ORF1 Fr2 (nt 93) (Figure 6.4B), 2 in ORF2 (nt 523 and 903) (Figure 6.4C) and 3 in ORF3 (nt 258, 357 and 690) (Figure 6.4D). The detail of BPs associated with their placement support, and a brief description on how to interpret the results for the respective ORFs above is given in insets and caption of Figure 6.4

For SLAC, FEL and REL analysis (Table 6.4), non-Australian isolates (Group 1) contained 23 positive selected codons, detected only by REL (2 in ORF1 and 21 in ORF3), and 74 negatively selected codons (33 in ORF1, 10 in ORF2 and 31 in ORF3) with 3 recognized by all methods (ORF1 only). Australian isolates (Group 2) presented 7 positively selected codons, detected by REL only (4 in ORF1 Fr1 and 3 in ORF2), and 213 negatively selected codons with 6 found by consensus of all methods. The overall mean dN/dS ratio as estimated by SLAC for ORF1, 2 and 3 of Group 1 dataset was 0.259, 0.459 and 0.147, respectively. The dN/dS ratio for the respective ORF1 Fr1 and Fr2, ORF2, and ORF3 of the Group 2 dataset was 0.442, 0.032, 0.438 and 0.076. The dN/dS ratio values less than 1 for both groups are consistent with negative selection acting on aa-altering mutations. The numbers and locations with details of all possible selected codons for both groups are in Table F.1 and F.2 of Appendix F.

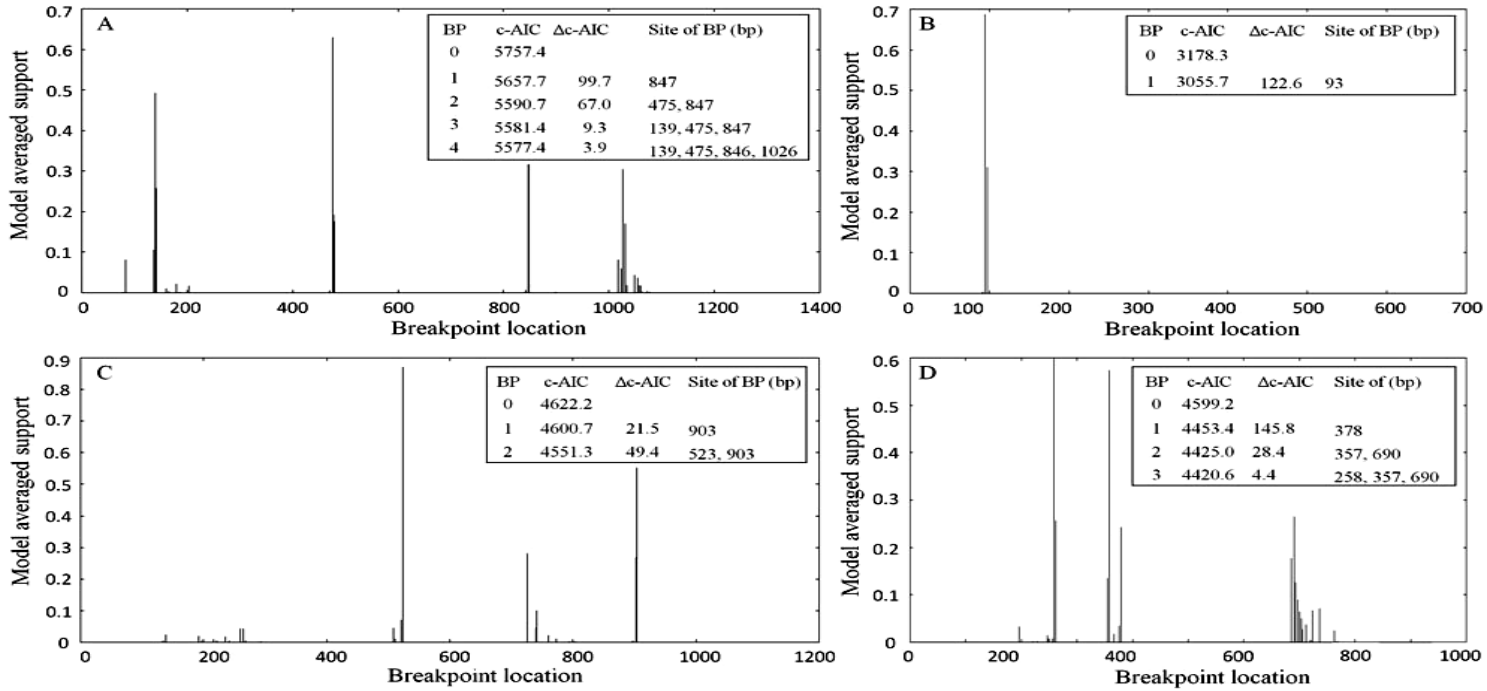


Figure 6.4: Recombination breakpoint (BP) placement support of: (A) ORF1 Fr1, (B) ORF1 Fr2, (C) ORF2 and (D) ORF3. Support probability for inferred BPs is shown on the left side of the figures. The nt position in the alignment is shown on the *x*-axis. Insets: corresponding BPs and Akaike Information Criterion score (c-AIC) for the best model assigned to infer BPs. Δc-AIC: improvement relative to the model with one fewer breakpoint. Given the maximum number of BPs, the method searched for all possible locations for BPs, inferring phylogenies for each putative non-recombinant fragment and assessed goodness of fit by using c-AIC. On ORF1 Fr1 (Figure 6.4A), for example, 4 BPs (inset), corresponding to peaks from left to right, located respectively at the nt 139, 475, 846 and 1026 were found, and the sequences were divided into 5 non-recombination split partitions.

Table 6.4: Number of positive (+) and negative (−) selected codons on each ORF of the genome of *Pst*DVs circulating outside Australia (nAus Grp 1) and inside Australia (Aus Grp 2) as analyzed by SLAC, FEL and REL. For the purpose of analysis in relation to the genome organization of the AUS-*Pst*DV2012, ORF1 of Group-2 samples were divided into 2 fragments: ORF1 Fr1 and ORF1 Fr2. They were analysed and shown separately. Significance level: SLAC p-value: 0.1; FEL p-value: 0.1; REL Bayes Factor: 50.

Sample	Fragment analysed	SLAC		FEL		REL		Two methods consensus		Three methods consensus	
		+	−	+	−	+	−	+	−	+	−
nAus Grp 1	ORF1	-	4	-	33	2	3	-	1	-	3
	ORF2	-	1	-	10	-	-	-	1	-	-
	ORF3	-	1	-	31	21	-	-	1	-	-
	Total	**-**	**6**	**-**	**74**	**23**	**3**	-	**3**	-	**3**
Aus Grp 2	ORF1 Fr1	-	3	-	37	4	-	-	3	-	-
	ORF1 Fr2	-	6	-	58	-	82	-	52	-	6
	ORF2	-	3	-	20	3	-	-	1	-	-
	ORF3	-	3	-	72	-	-	-	3	-	-
	Total	**-**	**15**	**-**	**187**	**7**	**82**	-	**59**	-	**6**

6.3.6. Inference of the nucleotide substitution rate and the demographic history

To select the best-fit clock model, the marginal likelihood estimate of different models (strict, relaxed lognormal and relaxed exponential) were compared (Table 6.5). Using the harmonic smoothed mean estimator, the relaxed exponential model had the largest marginal likelihood (-9905.31). This model was favoured over other tested clocks according to the Bayes factors comparison (103.71; strict clock and 106.92; relaxed lognormal clock). Under this model (with a constant population size), the nt substitution rates were estimated to be 1.55×10^{-3} substitutions/site/year (95% highest posterior density, HPD: 6.93×10^{-4}– 2.46×10^{-3}, standard error of mean: 2.42×10^{-5}). Parallel estimation for all 20 dated sequences (Table 6.2) revealed the rate of 1.90×10^{-4} substitutions/site/year; approximately 12 times lower than that for the data set above.

Historical *Pst*DV population demography in the northern Queensland was inferred from Bayesian Skyline Plot (BPS). The analysis suggested that the *Pst*DV population decreased slowly after 1991, and reached the bottom in around 1996 prior to a gradual increase and leveled off since 2003 (Figure 6.5). A similar qualitative pattern of BSP was observed with individual ORF analyzed (data not shown). These results indicate that the population of *Pst*DV has reached an equilibrium state, supporting our hypothesis regarding viral accommodation.

Table 6.5: Evolutionary models compared by marginal likelihoods obtained from the harmonic smoothed mean estimator performed under different clock models: Strict (S), Relaxed lognormal (RL) and Relaxed exponential (RE).

			Bayes factor[a]		
Trace	ln P(data | model)[b]	S.E.	S	RL	RE
S	-10009.02	+/-0.09	-	3.21	-103.71
RL	-10012.23	+/-0.14	-3.21	-	-106.92
RE	-9905.31	+/-0.17	103.71	106.92	-

[a]The Bayes factor is the difference between the marginal likelihoods of the two models being compared**.** Positive values indicate better relative model fit of the row's model compared to the column's model, while negative values indicate better relative model fit of the row's model compared to the column's model. [b]Marginal likelihood of model data

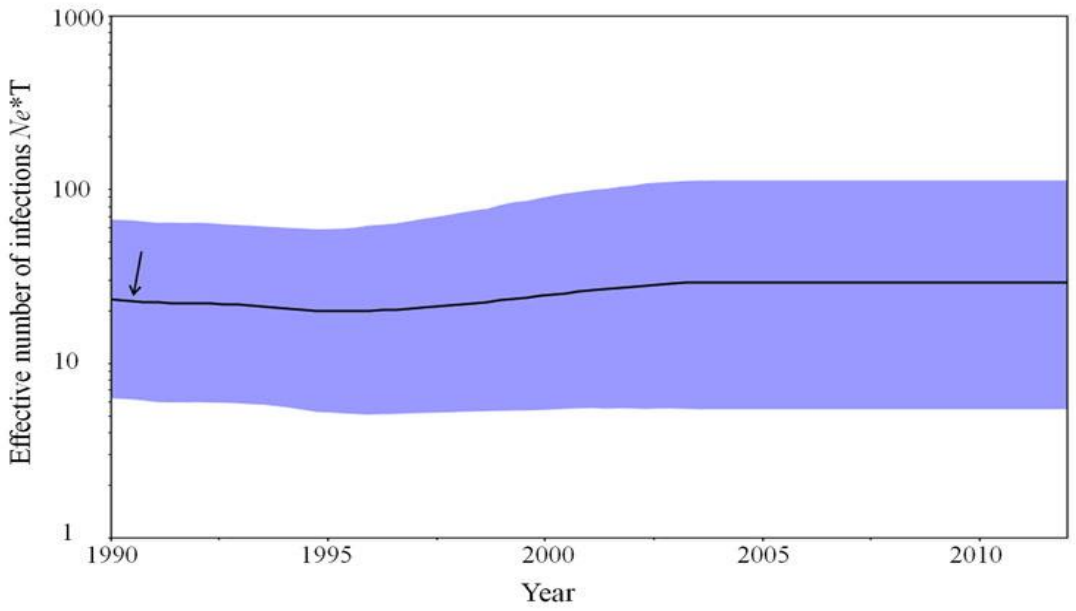


Figure 6.5: Bayesian skyline plot. The Y axis represents the effective number of infections, *Ne**T, where *Ne* is the effective number of infections and T is the generation time. The black line represents the mean estimate of the effective number of *Pst*DV infections through time, and the solid interval represents the 95% highest posterior density, HPD. The arrow indicates 1991, the year of *Pst*DV epizootic in Australia.

6.4. Discussion

The initial discovery of the 1305delA mutation within ORF1 of the Australian *Pst*DV genomes (Figure 6.2A, top) posed the question whether it is real or an artefact of technology. If it was real, was it consistently inherited over time? Sequencing of the genome of *Pst*DV from northern Queensland in 2012 found that all 9 samples examined were homogenous for the 1305delA mutation (Figure 6.2A, bottom). The sequence chromatograms showed clear DNA patterns, no overlapping peaks or noise at the A-missing nt for all the sequences examined (Figure 6.2C, highlighted). We discount polymerase errors since we used high fidelity enzymes and sequenced amplicons in both directions. Furthermore, this point mutation was also found in the other Australian *Pst*DV sequence (GenBank No. EU675312) collected from cultured shrimp in 1993; 19 years before this study. The sampling period, the sampling area, the research methodology and researchers for these viral isolates were independent. These facts combined with the number of individual *Pst*DV samples that possesses the mutation led to a logical conclusion that the A-deletion was real.

The 1305delA occurs at the NLS encoding sequence (Table 6.3, rows 1-2). As the NLS is involved with mediating proteins entry through the nuclear pore to be functional in the host cell nucleus (Jans *et al.*, 2000; Owens, 2013), incorrectly translated NLS caused by the 1305delA may compromise its NL signature, consequently affecting the DNA replication process of *Pst*DV. The mutation also creates a premature termination codon within ORF1 which leads to the predicted NS1 protein being shortened by 199 aa with 2 putative minor proteins (NS1 Fr1 and NS1 Fr2) produced instead, and separation of the replication initiator motifs from NTP-binding and helicase domains (see Section 6.3.2). This change possibly strongly modifies the characteristics of the NS1, and consequently affects the growth of *Pst*DV as NS1 was responsible for the majority of enzymatic activities involved in viral transcription and replication. However, if both NS1 Fr1 and Fr2 are associated post-translation, then some functionality would be returned with some considerable loss in speed and efficiency. Further research that elucidates the basis *Pst*DV NS1 malfunction would be particularly informative. The 1305delA does not

cause any frame shift or premature stop codon in other ORFs as its location does not overlap with those ORFs. The 1305delA has not been reported as responsible for ORF1 frame-shift and can therefore be regarded as novel. As it is unique for the Australian *Pst*DV strain, it serves as a genetic marker for identifying this viral strain. Both Australian *Pst*DV strains isolated in 2012 (this study) and 1993 (GenBank No. EU675312) contain this genomic marker. Therefore, they are assumed to originate from the same ancestor. The marker can also be useful in studying the relationship between the degree of virulence of *Pst*DV and the genetic causes as it is associated with defective NS1 production. This knowledge may be an important clue regarding viral accommodation and genes related to virulence. As this sequence information is from full length transcripts, it is clear that *Pst*DVs with the 1305delA are circulating in Australian prawn populations.

With *Pst*DV genome analysis, our sequence shared a comparable degree of identity with those in both lineage I (~93-94%) which originated from Australia and Madagascar, and lineage III (~91-92%) from some parts of Asia (Table 6.2) When a phylogenetic tree was constructed, AUS-*Pst*DV2012 clustered in lineage II, a different lineage to the isolate reported in 1993 (Figure 6.3). Both strains were discovered from prawn farms in the same geographic area separated by only a 19-year interval. In addition, they were assumed to originate from the same ancestor due to the presence of the genetic marker in their genomes (see above). Interestingly, several isolates from prawns cultured in northern Queensland were classified into lineage III. Hence, it can be concluded that different lineages of *Pst*DV have been co-circulating in Australia, one originated from Asia (GenBank No. KM593910, KM593912, GQ475529, KM593911, KM593909, and KM593908) and other endemic strains (this study and GenBank No. EU675312) (Figure 6.3).

With GARD analysis, 10 break points in the genome of Australian *Pst*DVs were revealed: 4 in ORF1 Fr1 (Figure 6.4A), 1 in ORF1 Fr2 (Figure 6.4B), 2 in ORF2 (Figure 6.4C) and 3 in ORF3 (Figure 6.4D). This indicated the exchange of genetic information between different isolates found in Australia, consequently contributing

to increased genetic diversity. This might be the reason for the two Australian isolates sampled from the same geographical area but in different years (1993 and 2012) are less similar in DNA sequence than expected and are in different lineages. The data herein are consistent with there being an older Australian endemic *Pst*DV (AUS-*Pst*DV2012) and an introduction of an Asian *Pst*DV around 1991 (GenBank No. KM593908). Signals of natural selection (both positive and negative) as analyzed using SLAC, FEL and REL were also found in many codons in *Pst*DV genomes both endemic (Table 6.4, Aus Grp2) and outside Australia (Table 6.4, nAus Grp1). SLAC and FEL detected only negative selection signals, while REL detected both positive and negative signals. It is known that different approaches to inferring the same effect can lead to slightly different conclusions, never with 100% accuracy (Poon *et al.*, 2009). SLAC is the most conservative; it may miss some sites, but it does not identify many neutral sites. Hence, the actual rate of false positives can be much lower than the significance level. FEL detects more sites, while REL selects most sites but yields the highest error rate (Pond and Frost, 2005; Poon *et al.*, 2009). With this in mind, we attempted not to translate our results into the actual signature of selection, but inferred the overall number of possible events instead. Thus, there are possible trends for both increasing the prevalence of adaptive traits in *Pst*DV populations, and decreasing prevalence of traits that diminish individual viruses' capacity to succeed reproductively. However, the latter case seems highly likely due to the negative consensus values across all three methods.

Using Bayesian coalescent approaches to evaluate the evolutionary rate of *Pst*DV circulating in Australia, the nt substitution rate was estimated to be 1.55×10^{-3} substitutions/site/year, which is unexpectedly high for a dsDNA virus but perhaps not for single-stranded (ss) DNA virus (see Robles-Sikisaka et al., 2010) and comparable to RNA viruses which evolve at a rate of 10^{-3} to 10^{-5} substitutions/site/year (Scholtissek, 1995; Jenkins *et al.*, 2002). It is generally assumed that dsDNA viruses evolve at a similar rate to that of their hosts due to viral dependence on the host's cellular machinery for replication (Shackelton and Holmes, 2006; Duffy and Holmes, 2008). This data set provides a good approximation of the rate of evolution due to the length of the sequences that cover

all ORFs, the range of sampling dates (from 1991 to 2012; the first year of an official record of *Pst*DV existence in Australia until the latest, a 22-year interval). We attribute this unexpectedly high estimate to the DNA recombination, a major evolutionary mechanisms driving diversity and adaptation in parvoviruses (Shackelton *et al.*, 2007). Our estimate is approximately 12 times and 9 times higher than that computed from 20 worldwide distributed isolates (this study) and the 18 isolates of a previous study (Robles-Sikisaka *et al.*, 2010). However, note that the study of Robles-Sikisaka et al. (2010) only looked at the capsid gene whilst we looked at all ORFs and what effect this had on this comparison is unknown. We also note that a number of other parvoviruses infecting mammals and begomoviruses infecting plants have been reported to evolve at roughly the same rate. These include human B19 erythrovirus ($1x10^{-4}$) (Shackelton and Holmes, 2006), human parvovirus B19 (Parsyan *et al.*, 2007), porcine circovirus 2 (Firth *et al.*, 2009), canine parvovirus (CPV) ($1.7x10^{-4}$), feline panleukopenia parvovirus (FPLV) ($9.4x10^{-5}$) (Shackelton *et al.*, 2005), and the ssDNA containing begomovirus, tomato yellow leaf curl virus (TYLCV) ($2.88x10^{-4}$) (Duffy and Holmes, 2008).

Regarding the history of *Pst*DV outbreak in Australia, high mortalities and obvious gross pathology caused by the virus in farmed prawn were not recorded since 1991 despite the virus existing here. When a BPS-derived *Pst*DV population growth was inferred, the effective number of infections of *Pst*DV decreased slowly after 1991, prior to recovery in 1996 and remained stable from 2003 onwards (Figure 6.5). Of considerable interest is that this time corresponded to the severe outbreaks of Midcrop Mortality Syndrome (MCMS) in Australia (Anderson and Owens, 2001) of which *Pst*DV was a component. Electron microscopy demonstrated a parvovirus was involved from March 1994 onwards and MCMS was nationwide by 1996 when the upturn in the BSP started to occur. MCMS continued unabated until 2003 when a combination of broodstock from the Northern Territory, restriction to one crop per year, longer dry-outs of ponds and partial harvests returned predictable production to the industry. We hypothesize that the high mortality rates in prawns infected with MCMS put selective pressure for "fit" biotypes to replicate before prawns succumbed (Figure 6.5). The apparent stability of the *Pst*DV population does not

correlate with the high substitution rate we found. The simple explanation to this contradiction is that mutation mostly produced negative traits (Table 6.4), resulting in a trend for decreasing prevalence of traits that diminished individual virions capacity to succeed reproductively and its genes to be present in the next generation. Therefore, the effective viral population (*Ne**T) did not increase much. Altogether, we interpret reduction in virulence across northern Queensland primarily in terms of pathogen evolution. Specifically, we hypothesize an inherent trade-off between transmission rate and virulence that has changed over time as *Pst*DV evolved. Future research that elucidates the basis of this trade-off in association with the effect of fragmented ORF1 caused by the 1305delA mutation would be particularly informative.

For clarification on awareness of *Pst*DV outbreaks in Australia, large amounts of commodity prawns from South East Asia (Krabsetsve *et al.*, 2004) have been imported into Australia which is believed to be the source of Asian-originated *Pst*DV currently spreading in northern Queensland. Recombination of this strain with an endemic strain could, in the worst case, lead to the emergence of virulent strains following changes in the host or environmental conditions. Furthermore, northern Australia shares the marine environment and fauna with the Indo-Pacific region. In conjunction with the fact that shrimp farms are generally located in coastal regions that also provide a natural habitat for wild shrimp, there are opportunities for admixture of wild and captive shrimp populations. Consequently, a lethal strain of *Pst*DV originating in the wild or derived from the Indo-Pacific region can also potentially be introduced into cultured populations, and vice versa. Routine genetic monitoring of *Pst*DV in both wild and cultured shrimp might aid management efforts in this regard.

In 1999–2000, a new strain of Taura syndrome virus (TSV Mx2000), one of the most serious shrimp pathogens, emerged in Mexican shrimp culture. It was reported to have escaped detection since it did not react with the monoclonal antibody (MAb) used for the routine screening of TSV by immunohistochemistry (ISH). Subsequent

cloning and sequencing showed that it contained distinctive capsid gene mutations that contributed to detection failure in the ISH method (Robles-Sikisaka *et al.*, 2002). For *Pst*DV, use of the BS4.5 DNA gene probe designed to specifically detect the virus also failed to react with Australian isolate (Owens *et al.*, 1992; Lightner, 1996c). A view was forming that the Australian isolate also has unique mutations that contributed to the dot-blotting detection failure. This suggestion is highly likely due to the presence of genetic recombination among Australian isolates (this study). Genetic screens may allow the identification of otherwise undetectable strains, as well as shed light on the unknown determinants of changes in virulence such as the discovered point mutation in ORF1.

In conclusion, Australian prawns have developed resistance to *Pst*DV and/or the virus has reached equilibrium with the host in terms of genes related to virulence. A 1-bp deletion (1305delA) within ORF1 has been identified as being responsible for 199-aa shortened NS1 synthesis in Australian *Pst*DV. Alteration of such a big aa portion may modify the characteristics of the NS1, consequently affecting the growth of *Pst*DV. Thus, the 1305delA mutation is believed to be associated with the reduction of virulence in the Australian strain. However, to experimentally prove this concept is exceptionally difficult at present due to the lack of *in vitro* cell cultures to support the growth of *Pst*DV and to facilitate intensive studies. In addition, the complete genome of the virus (including both hairpin ends) has not been reported. The 1305delA serves as a genetic marker to identify this Australian viral strain. The constant growth of Australian *Pst*DV population and signs of viral accommodation have also been revealed herein. However, we should not assume that *Pst*DV is a stable or static virus, and understate the potential for new virulence stains to arise. This is because of a high degree of evolutionary and genetic variation for *Pst*DV attributable to the viral recombination has been observed. Thus, awareness on the emergence of new virulent strains should be raised as preventative measures for future outbreaks of *Pst*DV in Australia and the Australian Indo-Pacific.

CHAPTER 7

GENERAL DISCUSSION

Penaeus stylirostris densovirus (*Pst*DV) is an important shrimp pathogen that contributes to the poor health and production losses of prawns across the globe (Rai *et al.*, 2012; Owens, 2013). Recently, *Pst*DV-related sequences were found in the genome of *P. monodon* and *P. vannamei* (Tang and Lightner, 2006; Saksmerprome *et al.*, 2011). This led to false positive results by PCR-based detection systems including those recommend by the World Organization for Animal Health (OIE) (the 309F/R PCR primer protocol and the IQ2000^{TM} PCR detection kit) (Saksmerprome *et al.*, 2011). Here, we examined and mapped the *Pst*DV insertion profile in the genome of Australian *P. monodon*. A DNA sequence which is likely to represent infectious *Pst*DV was also identified and used as a target sequence for RPA-LFD, developed for specifically detecting *Pst*DV. The protocol at 37 °C for 30 min followed by 5 min at room temperature showed no cross-reaction with other shrimp viruses, and was 10 times more sensitive than both the OIE protocols mentioned above. The major benefit of this protocol is that it could reduce false positive results arising from viral inserts to ~5%, compared to 76-78% by the OIE protocols above (Saksmerprome *et al.*, 2011). Its total assay time is approximately 35 min, about 6–8 times faster than the Saksmerphrome *et al.*'s interim 3-tube nested PCR-AGE detection of *Pst*DV.

The estimated cost for a single assay using the RPA–LFD protocol (excluding DNA extraction) is approximately US$ 8 or comparable to that of LAMP-LFD and the IQ2000^{TM} kit which we estimated for standard nested PCR using a DNA extraction kit, a nested PCR detection kit and gel electrophoresis followed by UV detection (Jaroenram *et al.*, 2009). This estimate does not include capital costs for equipment (i.e. heating block). The RPA–LFD cost could probably be further reduced by production-scale savings. The market force may also make it be more affordable in the future, similar to PCR reagents and relevant equipment (i.e. thermal cycler)

which were more expensive in the early days of the technology than they are now. Our RPA protocol reduced complications associated with reaction preparation found in PCR or LAMP as the RPA reaction system is provided in a stabilized dried format which permits transportation and limited storage without refrigeration (http://www.twistdx.co.uk/our_technology/). In addition, the reaction can be completely set up at room temperature. Together with the requirement of only a simple heating block for maintaining the reaction, our protocol provides qualitative DNA testing without the need of a laboratory, and that indicates the applicability for onsite detection. However, our protocol is a prototype and to use it in field, evaluation of its efficiency on a larger scale is first required. In addition, a simple but efficient-and-compatible DNA extraction method has to be established and combined in order to make the RPA-LFD described truly practical and robust. As our protocol is the first RPA-based method for the detection of a shrimp pathogen, it serves as a model platform that could be adapted easily to detect other pathogens or genetic markers of interest using basic laboratory equipment.

Implementation of monitoring and control measures for viral epizootic outbreaks requires an understanding of the factors that underlie molecular evolution and population dynamics (Robles-Sikisaka *et al.*, 2010). This is also true for *Pst*DVs that have been circulating in Australia. By screening *P. monodon* sampled from farms of northern Queensland in 2012 using our RPA-LFD, several shrimp were positive for infectious *Pst*DV. When whole genome of the virus from these shrimp was sequenced and analyzed, a single base deletion (1305delA) within the NLS-encoding sequence of ORF1; NS1 was detected and identified as being responsible for 199-aa shortened NS1 synthesis. This mutation is believed to reduce virulence as it strongly modifies the characteristics of NS1, a protein that plays major roles in *Pst*DV replication and transcription. This suggestion correlates with the constant growth of the Australian *Pst*DV population examined using Bayesian skyline plot (BPS), supporting our hypothesis regarding accommodation of *Pst*DV in Australian *P. monodon* in relation to viral genetics. Future research that elucidates the basis of *Pst*DV-NS1 malfunction in association with the viral accommodation would be particularly informative. Identification of the co-presence of a completely functional

genotype whose virions would be able to encapsidate the non-functional mutant genome, and understanding the consequences of these patterns of genetic variation for various evolutionary processes is interesting and should be further studied.

The 1305delA we found is unique for an Australian *Pst*DV strain. Therefore, it serves as a genetic marker for this viral strain, and provides usefulness in studying the relationship between the degree of virulence of *Pst*DV and the genetic causes. It is also beneficial for international seafood trade and quarantine management system. To illustrate this point, if any country having encountered an outbreak of *Pst*DV claims that the *Pst*DV is distributed from Australia via frozen seafood imported, this claim can be proven right or wrong using the marker described for this strain. With this approach, economic tension including trade restriction or sanction against Australia can be prevented. Having research on developing a protocol to specifically detect the 1305delA mutation would be of interest in this regard.

For clarification of awareness of *Pst*DV outbreaks in Australia, although the constant growth of Australian *Pst*DV population and signs of viral accommodation have been revealed herein, we should not assume that the *Pst*DV is a stable, and understate the potential for new virulence stains to arise. This is because of a high degree of evolution for the virus, which was attributable to the viral recombination, was also observed. With this finding, we primarily suggest that awareness of the emergence of new virulent strains should be raised as a preventative measure against future outbreaks of *Pst*DV in Australia and the Australian Indo-Pacific where fauna and marine environment are shared. However, analysis of a much larger number of native, wild samples of *P. monodon* would be particularly useful, and required to prove that the hypothesis regarding a faster rate of evolution and viral accommodation proposed herein are consistent and represent the evolution of *Pst*DV in the Australia Indo-Pacific region in general.

In conclusion, a more practical and efficient protocol to specifically detect *Pst*DV, and the factors that underlie the evolution of the Australian *Pst*DV have been established and identified here, respectively. Integration of such outputs and knowledge can help contribute toward better strategies to counter *Pst*DV.

REFERENCES

Alfaro Aguilera, R., Guevara Torres, M. and Gonzales Chávez, I. (2010) Prevalencia y distribución de los principales agentes etiológicos que afectan los langostinos silvestres en Tumbes, Perú. *Rev. peru biol* **17**: 359-364

Anderson, I.G. and Owens, L.G. (2001) The diagnosis and prevention of the mid-crop mortality syndrome of pond-reared black tiger prawns (Penaeus monodon), 56 pp. Oonoonba Veterinary Laboratory, Department of Primary Industries.

Arunrut, N., Prombun, P., Saksmerprome, V., Flegel, T.W. and Kiatpathomchai, W. (2011) Rapid and sensitive detection of infectious hypodermal and hematopoietic necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Journal of virological methods* **171**: 21-25

Astell, C. (1990) Terminal hairpins of parvovirus genomes and their role in DNA replication. *Handbook of parvoviruses* **1**: 59-80

Bell, T.A. and Lightner, D.V. (1984) IHHN virus: infectivity and pathogenicity studies in Penaeus stylirostris and Penaeus vannamei. *Aquaculture* **38**: 185-194

Bell, T. and Lightner, D. (1987) IHHN disease of Penaeus stylirostris: effects of shrimp size on disease expression. *Journal of fish diseases* **10**: 165-170

Boada, M. (2008) Detection of the Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) in Asymtomatic Cultured White Shrimp, Litopenaeus vannamei (Boone), in Venezuela. *REVISTA CIENTIFICA* **18**: 7-11

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A. and Drummond, A.J. (2014) BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS computational biology* **10**: e1003537

Boyle, D.S., Lehman, D.A., Lillis, L., Peterson, D., Singhal, M., Armes, N., Parker, M., Piepenburg, O. and Overbaugh, J. (2013) Rapid detection of HIV-1 proviral DNA for early infant diagnosis using recombinase polymerase amplification. *MBio* **4**: e00135-13

Bray, W.A., Lawrence, A.L. and Leung-Trujillo, J.R. (1994) The effect of salinity on growth and survival of Penaeus vannamei, with observations on the interaction of IHHN virus and salinity. *Aquaculture* **122**: 133-146

Braz, R.F.S., Rodrigues de Oliveira da Silva, C.P., Reis, L.G., Martins, P.C.C., Pereira de Sales, M. and Meissner, R.V. (2009) Prevalence of infectious

hypodermal and hematopoietic necrosis virus (IHHNV) in Penaeus vannamei cultured in northeastern Brazil. *Aquaculture* **288**: 143-146

Brock, J.A., Lightner, D.V. and Bell, T.A. (1983) A review of four virus (BP, MBV, BMN and IHHNV) diseases of penaeid shrimp with particular reference to clinical significance, diagnosis and control in shrimp aquaculture. *Proceedings of the 71st International. Council for the Exploration of the Sea, CM*: 1-18

Browdy, C.L., Holloway, J.D., King, C.O., Stokes, A.D., Hopkins, J.S. and Sandifer, P.A. (1993) IHHN virus and intensive culture of Penaeus vannamei: effects of stocking density and water exchange rates. *Journal of Crustacean Biology* **13**: 87-87

Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular biology and evolution* **17**: 540-552

Chayaburakul, K., Nash, G., Pratanpipat, P., Sriurairatana, S. and Withyachumnarnkul, B. (2004) Multiple pathogens found in growth-retarded black tiger shrimp Penaeus monodon cultivated in Thailand. *Diseases of aquatic organisms* **60**: 89-96

Chevenet, F., Brun, C., Bañuls, A.-L., Jacq, B. and Christen, R. (2006) TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC bioinformatics* **7**: 439

Claydon, K., Tahir, R.A.H., Said, H.M., Lakim, M.H. and Tamat, W. (2010) Prevalence of shrimp viruses in wild Penaeus monodon from Brunei Darussalam. *Aquaculture* **308**: 71-74

Coelho, M.G.L., Silva, A.C.G., Vila Nova, C., Neto, J.M.O., Lima, A.C.N., Feijó, R.G., Apolinário, D.F., Maggioni, R. and Gesteira, T.C.V. (2009) Susceptibility of the wild southern brown shrimp (*Farfantepenaeus subtilis*) to infectious hypodermal and hematopoietic necrosis (IHHN) and infectious myonecrosis (IMN). *Aquaculture* **294**: 1-4

Cordellier, M. and Pfenninger, M. (2008) Climate-driven range dynamics of the freshwater limpet, Ancylus fluviatilis (Pulmonata, Basommatophora). *Journal of Biogeography* **35**: 1580-1592

Crochu, S., Cook, S., Attoui, H., Charrel, R.N., De Chesse, R., Belhouchet, M., Lemasson, J.-J., de Micco, P. and de Lamballerie, X. (2004) Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of Aedes spp. mosquitoes. *Journal of general virology* **85**: 1971-1980

De la Vega, E. (2006) A molecular approach to study the interaction between environmental stress, immune response and disease in the black tiger prawn (Penaeus monodon). *Ph.D. Thesis. University of Queensland.*

Dhar, A.K., Lakshman, D.K., Natarajan, S., Allnutt, F. and van Beek, N.A.M. (2007) Functional characterization of putative promoter elements from infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimp and in insect and fish cell lines. *Virus Research* **127**: 1-8

Drummond, A.J., Rambaut, A., Shapiro, B. and Pybus, O.G. (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular biology and evolution* **22**: 1185-1192

Duffy, S. and Holmes, E.C. (2008) Phylogenetic evidence for rapid rates of molecular evolution in the single-stranded DNA begomovirus tomato yellow leaf curl virus. *Journal of virology* **82**: 957-965

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792-1797

Euler, M., Wang, Y., Heidenreich, D., Patel, P., Strohmeier, O., Hakenberg, S., Niedrig, M., Hufert, F.T. and Weidmann, M. (2013) Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. *Journal of clinical microbiology* **51**: 1110-1117

Euler, M., Wang, Y., Nentwich, O., Piepenburg, O., Hufert, F.T. and Weidmann, M. (2012) Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. *Journal of Clinical Virology* **54**: 308-312

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A.X. (2005) Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. Academic Press.

Feschotte, C. and Gilbert, C. (2012) Endogenous viruses: insights into viral evolution and impact on host biology. *Nature Reviews Genetics* **13**: 283-296

Firth, C., Charleston, M.A., Duffy, S., Shapiro, B. and Holmes, E.C. (2009) Insights into the evolutionary history of an emerging livestock pathogen: porcine circovirus 2. *Journal of virology* **83**: 12813-12821

Flegel, T.W. (2006) The special danger of viral pathogens in shrimp translocated for aquaculture. *Science Asia* **32**: 215-221

Flegel, T.W. (2009) Hypothesis for heritable, anti-viral immunity in crustaceans and insects. *Biol Direct* **4**: 32

Flegel, T.W. (2012) Historic emergence, impact and current status of shrimp pathogens in Asia. *Journal of Invertebrate Pathology* **110**: 166–173

Fontanella, F.M., Feldman, C.R., Siddall, M.E. and Burbrink, F.T. (2008) Phylogeography of *Diadophis punctatus*: Extensive lineage diversity and repeated patterns of historical demography in a trans-continental snake. *Molecular phylogenetics and evolution* **46**: 1049-1070

Formosa, T. and Alberts, B.M. (1986) Purification and characterization of the T4 bacteriophage uvsX protein. *Journal of Biological Chemistry* **261**: 6107-6118

Gillett R. (2008) Global study of shrimp fisheries. FAO Fisheries Technical Paper No. 475. Rome, FAO. 331p

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic biology* **59**: 307-321

Guzmán-Sáenz, F.M., Molina-Garza, Z.J., Pérez-Castañeda, R., Ibarra-Gámez, J.C. and Galavíz-Silva, L. (2009) Virus de la necrosis hipodérmica y hematopoyética infecciosa (IHHNV) y virus del síndrome de Taura (TSV) en camarón silvestre (Farfantepenaeus aztecus Ives, 1891 y Litopenaeus setiferus Linnaeus, 1767) de La Laguna Madre, Golfo de México. *Revista de biología marina y oceanografía* **44**: 663-672

Hazreen Nita, M., Kua, B., Bhassu, S. and Othman, R. (2012) Detection and genetic profiling of infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in wild berried freshwater prawn, Macrobrachium rosenbergii collected for hatchery production. *Molecular Biology Reports* **39** 3785-3790

Hsieh, C.Y., Chuang, P.C., Chen, L.C., Tu, C., Chien, M.S., Huang, K.C., Kao, H.F., Tung, M.C. and Tsai, S.S. (2006) Infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture* **258**: 73-79

Huang, S.W., Lin, Y.Y., Yu, E.M., Liu, T.T., Shu, H.Y., Wu, K.M., Tsai, S.F., Lo, C.F., Kou, G.H. and Ma, G.C. (2008) A first glimpse into he genome of he tiger shrimp, Penaeus monodon, Handbook and abstracts, 58.

Iskra-Caruana, M.L., Baurens, F.C., Gayral, P. and Chabannes, M. (2010) A Four-Partner Plant-Virus Interaction: Enemies Can Also Come from Within. *Molecular plant-microbe interactions* **23**: 1394-1402

Jans, D.A., Xiao, C.Y. and Lam, M.H.C. (2000) Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**: 532-544

Jaroenram, W., Arunrut, N. and Kiatpathomchai, W. (2012) Rapid and sensitive detection of shrimp yellow head virus using loop-mediated isothermal amplification and a colorogenic nanogold hybridization probe. *Journal of virological methods* **186**: 36-42

Jaroenram, W., Kiatpathomchai, W. and Flegel, T.W. (2009) Rapid and sensitive detection of white spot syndrome virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Molecular and cellular probes* **23**: 65-70

Jaroenram, W. and Owens, L. (2014) Separation of endogenous viral elements from infectious *Penaeus stylirostris* densovirus using recombinase polymerase amplification. *Molecular and Cellular Probes* **28**: 284-287

Jenkins, G.M., Rambaut, A., Pybus, O.G. and Holmes, E.C. (2002) Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. *Journal of molecular evolution* **54**: 156-165

Jiménez, R., Barniol, R., De Barniol, L. and Machuca, M. (1999) Infection of IHHN virus in two species of cultured penaeoid shrimp Litopenaeus vannamei (Boone) and Litopenaeus stylirostris (Stimpson) in Ecuador during El Niño 1997–98. *Aquaculture research* **30**: 695-705

Kalagayan, H., Godin, D., Kanna, R., Hagino, G., Sweeney, J., Wyban, J. and Brock, J. (1991) IHHN Virus as an Etiological Factor in Runt-Deformity Syndrome (RDS) of Juvenile Penaeus vannamei Cultured in Hawaii. *Journal of the World Aquaculture society* **22**: 235-243

Kersting, S., Rausch, V., Bier, F.F. and von Nickisch-Rosenegk, M. (2014) Rapid detection of Plasmodium falciparum with isothermal recombinase polymerase amplification and lateral flow analysis. *Malaria journal* **13**: 99

Khawsak, P., Deesukon, W., Chaivisuthangkura, P. and Sukhumsirichart, W. (2008) Multiplex RT-PCR assay for simultaneous detection of six viruses of penaeid shrimp. *Molecular and cellular probes* **22**: 177-183

Kiatpathomchai, W., Jaroenram, W., Arunrut, N., Jitrapakdee, S. and Flegel, T.W. (2008) Shrimp Taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *Journal of virological methods* **153**: 214-217

Kim, J.H., Choresca Jr, C.H., Shin, S.P., Han, J.E., Jun, J.W., Han, S.Y. and Park, S.C. (2011) Detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in Litopenaeus vannamei shrimp cultured in South Korea. *Aquaculture* **313**: 161–164

Kim, J.H., Kim, H.K., Nguyen, V.G., Park, B.K., Choresca, C.H., Shin, S.P., Han, J.E., Jun, J.W. and Park, S.C. (2012) Genomic sequence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) KLV-2010-01 originating from the first Korean outbreak in cultured Litopenaeus vannamei. *Archives of virology* **157**: 369-373

Koyama, T., Asakawa, S., Katagiri, T., Shimizu, A., Fagutao, F.F., Mavichak, R., Santos, M.D., Fuji, K., Sakamoto, T. and Kitakado, T. (2010) Hyper-expansion of large DNA segments in the genome of kuruma shrimp, Marsupenaeus japonicus. *BMC genomics* **11**: 141

Krabsetsve, K., Cullen, B.R. and Owens, L. (2004) Rediscovery of the Australian strain of infectious hypodermal and haematopoietic necrosis virus. *Diseases of aquatic organisms* **61**: 153-158

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A. and Lopez, R. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948

Lightner, D., Poulos, B., Bruce, L., Redman, R., Mari, J. and Bonami, J. (1992) New developments in penaeid virology: application of biotechnology in research and disease diagnosis for shrimp viruses of concern in the Americas. *Diseases of Cultured Penaeid Shrimp in Asia and the United States, Fulks W. & Main K., Eds. The Oceanic Institute, Makapuu Point, Honolulu, Hawaii, USA*: 233-253

Lightner, D., Redman, R. and Bell, T. (1983a) Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *Journal of invertebrate pathology* **42**: 62-70

Lightner, D., Redman, R., Bell, T. and Brock, J. (1983b) Detection of IHHN virus in Penaeus stylirostris and P. vannamei imported into Hawaii. *Journal of the World Mariculture Society* **14**: 212-225

Lightner, D.V. (1996a) Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. *Revue scientifique et technique (International Office of Epizootics)* **15**: 579-601

Lightner, D.V. (1996b) The penaeid shrimp viruses IHHNV and TSV: epizootiology, production impacts and role of international trade in their distribution in the Americas. *Rev. Scient. et Techni. Office Internat. des Epizoo* **15**: 579-601

Lightner, D.V. (1996c) The penaeid shrimp viruses IHHNV and TSV: epizootiology, production impacts and role of international trade in their distribution in the Americas. *Rev. sci. tech. Off. int. Epiz* **15**: 579-601

Lin, C.L., Lee, J.C., Chen, S.S., Wood, H.A., Li, M.L., Li, C.F. and Chao, Y.C. (1999) Persistent Hz-1 virus infection in insect cells: evidence for insertion of viral DNA into host chromosomes and viral infection in a latent status. *Journal of virology* **73**: 128-139

Liu, H., Fu, Y., Xie, J., Cheng, J., Ghabrial, S.A., Li, G., Peng, Y., Yi, X. and Jiang, D. (2011) Widespread endogenization of densoviruses and parvoviruses in animal and human genomes. *Journal of virology*: 9863-9876

Lotz, J.M. (1997) Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. *World Journal of Microbiology and Biotechnology* **13**: 405-413

Martínez-Cordova, L.R. (1992) Cultured blue shrimp (Penaeus stylirostris) infected with infectious hypodermal and hematopoietic necrosis virus in Northwestern Mexico. *The Progressive Fish-Culturist* **54**: 265-266

Martorelli, S.R., Overstreet, R.M. and Jovonovich, J.A. (2010) First report of viral pathogens WSSV and IHHNV in Argentine crustaceans. *Bulletin Of Marine Science* **86**: 117-131

Molthathong, S., Jitrakorn, S., Joyjinda, Y., Boonchird, C., Witchayachamnarnkul, B., Pongtippatee, P., Flegel, T. and Saksmerprome, V. (2013) Persistence of Penaeus stylirostris densovirus delays mortality caused by white spot syndrome virus infection in black tiger shrimp (Penaeus monodon). *BMC veterinary research* **9**: 33

Morales-Covarrubias, M., Nunan, L., Lightner, D., Mota-Urbina, J., Garza-Aguirre, M. and Chavez-Snachez, M. (1999) Prevalence of IHHNV in wild broodstock of Penaeus stylirostris from the upper Gulf of California, Mexico. *J. Aquat. Anim. Health* **11**: 296-301

Motte, E., Yugcha, E., Luzardo, J., Castro, F., Leclercq, G., Rodríguez, J., Miranda, P., Borja, O., Serrano, J. and Terreros, M. (2003) Prevention of IHHNV vertical transmission in the white shrimp *Litopenaeus vannamei*. *Aquaculture* **219**: 57-70

Nimitphak, T., Meemetta, W., Arunrut, N., Senapin, S. and Kiatpathomchai, W. (2010) Rapid and sensitive detection of Penaeus monodon nucleopolyhedrovirus (PemoNPV) by loop-mediated isothermal amplification combined with a lateral-flow dipstick. *Molecular and cellular probes* **24**: 1-5

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* **28**: e63-e63

Nunan, L., Poulos, B. and Lightner, D. (2000) Use of polymerase chain reaction for the detection of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp. *Marine Biotechnology* **2**: 319-328

Owens, L. (1987) Histological evidence for a IHHNV-like virus in Australia, First Australian Prawn Farming Research Workshop. NSW Department of Agriculture, Salamander Bay, New South Wales, Australia

Owens, L. (2013) Bioinformatical analysis of nuclear localisation sequences in penaeid densoviruses. *Marine genomics* **12**: 9-15

Owens, L., Anderson, I.G., Kenway, M., Trott, L. and Benzie, J.A.H. (1992) Infectious Hypodermal and Hematopoietic Necrosis Virus (Ihhnv) in a Hybrid Penaeid Prawn from Tropical Australia. *Diseases of aquatic organisms* **14**: 219-228

Owens, L. and Hall-Mendelin, S. (1989) Recent advances in Australian penaeid diseases and pathology. Advances in Tropical Aquaculture, Tahiti. Actes Colloq **9**:103–112

Pantoja, C.R., Lightner, D.V. and Holtschmit, K.H. (1999) Prevalence and geographic distribution of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in wild blue shrimp Penaeus stylirostris from the Gulf of California, Mexico. *Journal of Aquatic Animal Health* **11**: 23-34

Parsyan, A., Szmaragd, C., Allain, J.-P. and Candotti, D. (2007) Identification and genetic diversity of two human parvovirus B19 genotype 3 subtypes. *Journal of general virology* **88**: 428-431

Piepenburg, O., Williams, C.H., Stemple, D.L. and Armes, N.A. (2006) DNA detection using recombination proteins. *PLoS biology* **4**: e204

Pond, S.L.K. and Frost, S.D.W. (2005) Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Molecular biology and evolution* **22**: 1208-1222

Pond, S.L.K., Posada, D., Gravenor, M.B., Woelk, C.H. and Frost, S.D.W. (2006) Automated phylogenetic detection of recombination using a genetic algorithm. *Molecular biology and evolution* **23**: 1891-1901

Poon, A.F.Y., Frost, S.D.W. and Pond, S.L.K. (2009) Detecting signatures of selection from DNA sequences using Datamonkey, Bioinformatics for DNA Sequence Analysis, pp. 163-183. Springer.

Posada, D. (2008) jModelTest: phylogenetic model averaging. *Molecular biology and evolution* **25**: 1253-1256

Primavera, J.H. and Quinitio, E.T. (2000) Runt-deformity syndrome in cultured giant tiger prawn Penaeus monodon. *Journal of Crustacean Biology* **20**: 796-802

Puthawibool, T., Senapin, S., Kiatpathomchai, W. and Flegel, T.W. (2009) Detection of shrimp infectious myonecrosis virus by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *Journal of virological methods* **156**: 27-31

Rai, P., Pradeep, B. and Karunasagar, I. (2009) Detection of viruses in Penaeus monodon from India showing signs of slow growth syndrome. *Aquaculture* **289**: 231-235

Rai, P., Safeena, M.P., Karunasagar, I. and Karunasagar, I. (2011) Complete nucleic acid sequence of *Penaeus stylirostris* densovirus (*Pst*DNV) from India. *Virus Research* **158**: 37-45

Rai, P., Safeena, M.P., Krabsetsve, K., La Fauce, K., Owens, L. and Karunasagar, I. (2012) Genomics, Molecular Epidemiology and Diagnostics of Infectious hypodermal and hematopoietic necrosis virus. *Indian Journal of Virology* **23**: 203-214

Rambaut, A. and Drummond, A.J. (2007) Tracer v1. 4. Available from http://beast.bio. ed.ac.uk/Tracer.

Robles-Sikisaka, R., Bohonak, A.J., McClenaghan Jr, L.R. and Dhar, A.K. (2010) Genetic signature of rapid IHHNV (Infectious hypodermal and hematopoietic necrosis virus) expansion in wild Penaeus shrimp populations. *PloS one* **5**: e11799

Robles-Sikisaka, R., Hasson, K.W., Garcia, D.K., Brovont, K.E., Cleveland, K.D., Klimpel, K.R. and Dhar, A.K. (2002) Genetic variation and immunohistochemical differences among geographic isolates of Taura syndrome virus of penaeid shrimp. *Journal of general virology* **83**: 3123-3130

Rusaini, L.F., Kathy A., Elliman, J., Bowater, R.O. and Owens, L. (2013) Endogenous Brevidensovirus-like elements in *Cherax quadricarinatus*: Friend or foe? *Aquaculture* **396**: 136-145

Saksmerprome, V., Jitrakorn, S., Chayaburakul, K., Laiphrom, S., Boonsua, K. and Flegel, T.W. (2011) Additional random, single to multiple genome fragments of Penaeus stylirostris densovirus in the giant tiger shrimp genome have implications for viral disease diagnosis. *Virus Research* **160**: 180-190

Saksmerprome, V., Puiprom, O., Noonin, C. and Flegel, T.W. (2010) Detection of infectious hypodermal and haematopoietic necrosis virus (IHHNV) in farmed Australian *Penaeus monodon* by PCR analysis and DNA sequencing. *Aquaculture* **298**: 190-193

Scholtissek, C. (1995) Molecular evolution of influenza viruses. *Virus genes* **11**: 209-215

Shackelton, L.A., Hoelzer, K., Parrish, C.R. and Holmes, E.C. (2007) Comparative analysis reveals frequent recombination in the parvoviruses. *Journal of general virology* **88**: 3294-3301

Shackelton, L.A. and Holmes, E.C. (2006) Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. *Journal of virology* **80**: 3666-3669

Shackelton, L.A., Parrish, C.R., Truyen, U. and Holmes, E.C. (2005) High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 379-384

Shibata, T., Cunningham, R.P., DasGupta, C. and Radding, C.M. (1979) Homologous pairing in genetic recombination: complexes of recA protein and DNA. *Proceedings of the National Academy of Sciences* **76**: 5100-5104

Shike, H., Dhar, A.K., Burns, J.C., Shimizu, C., Jousset, F.X., Klimpel, K.R. and Bergoin, M. (2000) Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito brevidensoviruses. *Virology* **277**: 167-177

Staginnus, C. and Richert-Pöggeler, K.R. (2006) Endogenous pararetroviruses: two-faced travelers in the plant genome. *Trends in plant science* **11**: 485-491

Strimmer, K. and Pybus, O.G. (2001) Exploring the demographic history of DNA sequences using the generalized skyline plot. *Molecular biology and evolution* **18**: 2298-2305

Talavera, G. and Castresana, J. (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic biology* **56**: 564-577

Tang, K. and Lightner, D.V. (2001) Detection and quantification of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp by real-time PCR. *Diseases of aquatic organisms* **44**: 79-85

Tang, K. and Lightner, D.V. (2002a) Low sequence variation among isolates of infectious hypodermal and hematopoietic necrosis virus (IHHNV) originating from Hawaii and the Americas. *Diseases of aquatic organisms* **49**: 93-97

Tang, K.F. and Lightner, D.V. (2002b) Low sequence variation among isolates of infectious hypodermal and hematopoietic necrosis virus (IHHNV) originating from Hawaii and the Americas. *Diseases of aquatic organisms* **49**: 93-97

Tang, K.F., Poulos, B.T., Wang, J., Redman, R.M., Shih, H.-H. and Lightner, D.V. (2003) Geographic variations among infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection. *Diseases of aquatic organisms* **53**: 91-99

Tang, K.F.J., Durand, S.V., White, B.L., Redman, R.M., Pantoja, C.R. and Lightner, D.V. (2000) Postlarvae and juveniles of a selected line of Penaeus stylirostris are resistant to infectious hypodermal and hematopoietic necrosis virus infection. *Aquaculture* **190**: 203-210

Tang, K.F.J. and Lightner, D.V. (2006) Infectious hypodermal and hematopoietic necrosis virus (IHHNV)-related sequences in the genome of the black tiger prawn *Penaeus monodon* from Africa and Australia. *Virus Research* **118**: 185-191

Tang, K.F.J., Navarro, S.A. and Lightner, D.V. (2007) PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and virus-related sequences in the genome of Penaeus monodon. *Diseases of aquatic organisms* **74**: 165

Unzueta-Bustamante, M.L., Holtschmit, K., Olivas-Valdez, J., Martínez-Córdova, L., Porchas-Cornejo, M. and Lizárraga-Partida, M. (1998) Infectious Hypodermal And Hematopoietic Necrosis Virus (Ihhnv) In Wild Parent Stocks Of Blue Shrimp, Penaeus Styfirostris (Stimpson), In Guaymas Bay, Sonora, Mexico. *Ciencias Marinas* **24**: 491-498

Vega-Heredia, S., Mendoza-Cano, F. and Sánchez-Paz, A. (2012) The Infectious Hypodermal and Haematopoietic Necrosis Virus: A Brief Review of What We Do and Do Not Know. *Transboundary and Emerging Diseases* **59**: 95-105

Xiang, J. (2001) Recent advance of research and development on marine biotechnology in China, 6-8. In *Postgraduate conference on marine biology and biotechnology, June*, pp. 6-8

Yang, B., Song, X.L., Huang, J., Shi, C.Y. and Liu, L. (2007) Evidence of existence of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp cultured in China. *Veterinary microbiology* **120**: 63-70

Yonesaki, T., Ryo, Y., Minagawa, T. and Takahashi, H. (1985) Purification and some of the functions of the products of bacteriophage T4 recombination genes, uvs X and uvs Y. *European Journal of Biochemistry* **148**: 127-134

Zabbey, N., Erondu, E. and Hart, A. (2010) Nigeria and the prospect of shrimp farming: critical issues. *Livestock Res Rural Dev.* **22**

Zhang, C., Yuan, J. and Shi, Z. (2007) Molecular epidemiological investigation of infectious hypodermal and hematopoietic necrosis virus and Taura syndrome virus in Penaeus Vannamei cultured in China. *Virologica Sinica* **22**: 380-388

Zhang, J.X. and Sun, X.Q. (1997) A preliminary study on the virus in the eggs of Penaeus chinensis. *J Oceanogr Huanghai and Bohai Seas* **15**: 48-51

APPENDIX A

Reagents and bacteriological media

1. Reagents for bacterial cell transformation

1.1. 20 mM Isopropyl thio-β-D-galactoside (IPTG)

Isopropyl thio-β-D-galactoside	0.0477	g
Deionized water	10	ml

Mix homogenously and filter through 0.2 µm filter paper. Then, keep at -20 ^{o}C in a foil-wrapped tube until use.

1.2. 50 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal)

X-Gal	100	mg
Dimethylformamide	2	ml

Mix homogenously and filter through 0.2 µm filter paper. Then keep at -20 ^{o}C in a foil-wrapped tube until use.

1.3. Ampicillin stock solution (100 mg/mL)

Dissolve 1 g of sodium ampicillin (MW: 371.40 g/mol) in sufficient distilled water to make a final volume of 10 ml. Mix homogenously and filter through 0.2 or 0.45 µm filter paper. Then, store the ampicillin in aliquots at -20°C for 1 year or at 4 °C for 3 months.

2. Reagents for agarose gel electrophoresis

2.1. 0.5M EDTA

EDTA.Na_2.$2H_2O$	186.12	g
Deionized water	800	ml

Stir well and adjust pH to 8.0 using NaOH pellet or 10N NaOH solution. Then, add distilled water up to 1 L followed by autoclaving at 121^{o}C, 15 lb pressure for 15 min. Keep at room temperature

2.2. 50X TAE Buffer, pH 8.3

Trizma® Base	242	g
Glacial acetic acid	57.1	ml
0.5M EDTA	100	ml
Deionized water	750	ml

Stir well and adjust pH to 8.3 if required. Then, add distilled water up to 1 L followed by autoclaving at 121^{o}C, 15 lb pressure for 15 min. Dilute Stock buffer to 1X final concentration before use.

3. LFD Buffer (Phosphate Buffered Saline containing 0.1% Tween20)

NaC	18	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
Deionized water	800	ml

Stir well and adjust pH to 7.4. Then, add deionized water up to 1 l followed by autoclaving at 121^{o}C, 15 lb pressure for 15 min. Then, add Tween 20 to a final concentration of 0.1% (v/v) then keep at 4 ^{o}C until use.

4. Bacterial cell culture media

4.1. LB Broth solution

Difco™ LB Broth Miller powder	25	g
Deionized water	1	l

Mix thoroughly and then autoclave at 121^{o}C, 15 lb pressure for 15 min. Keep at 4^{o}C until used.

4.2 LB agar plate

Difco™ LB Broth Miller powder	25	g
Difco™ Solidifying agent	15	g
Deionized water	1	l

Mix thoroughly and then autoclave at 121^{o}C, 15 lb pressure for 15 min. Pour plates then keep at 4^{o}C until use.

APPENDIX B

A preliminary study on *Pst*DV status in Australian and imported prawns by Krabsetsve and Owens (unpublished)

Table B.1: Details of shrimp used for *Pst*DV screening by PCR using multiple primers

Year	Species	Stage of shrimp/ type of shrimp (farmed/caught)	Origin	Number of Samples
Australian sample				
1991	*P.monodon/P. esculentus*	Hybrids	AIMS	3
1993	*P. monodon*	Wild caught (B)	AIMS	10
1994	*P. monodon*	Broodstock	AIMS	12
1995A	*P. monodon*	Broodstock	AIMS	7
1995B	*P. monodon*	Farmed/Juveniles	Mossman/AIMS	3*
1996	*P. monodon*	Farmed/Wild caught	AIMS	7
1997	*P.monodon*	Farmed	Seafarm	5
1997	*P. monodon*	Juveniles	Burdekin	10
1999	*P. monodon*	Wild caught (B)	Jan Glade	10
2001	*P. monodon*	Farmed	QLD Farm, Cardwell	10
2003	*P. monodon*	Farmed	QLD Farm, Cardwell	10
2003/2004	*P. monodon*	N/A	AIMS	10
2004	*P. monodon*	Adults/Wild caught	Wild caught, QLD	9
2004	*P. semisulcatus*	Adult/Wild caught	Wild caught, QLD	1
2005 March	*P. monodon*	Grow-out	QLD Farm, Cardwell	6

2005 July	*P. monodon*	Grow-out	QLD Farm, Cardwell	20
2009	*P. monodon*	Post larvae	QLD Farm, Ayr	6
2009	*P. monodon*	Grow-out	QLD Farm, Ayr	12
2009	*P. monodon*	Broodstock	QLD Farm, Ayr	7
Imported samples				
2002	*P. stylirostris*	Adults	New Caledonia	5
2005	*P. vannamei*	Adults	China I	5
2005	*P. vannamei*	Adults	China II	5
2009	*P. vannamei*	Adults	Malaysia	10
2009	*P. vannamei*	Adults	Indonesia	10
2009	*P. monodon*	Broodstock	Brunei	3

Table B.2: PCR results corresponding to the samples listed in Table B.1. Plus (+) and minus (-) signs denote positive and negative result, respectively.

Year	**Sample No.**	**Primers**				
		392F/R	**389F/R**	**309F/R**		**MG831F/R**
1991						
	1A	+ve	+ve	+ve	+ve	-ve
	1B	+ve	-ve	-ve	-ve	-ve
	2	+ve	+ve	+ve	+ve	-ve
1993						
	1 to 3	+ve	-ve	-ve	-ve	-ve

	4 to 10	+ve	+ve	-ve	-ve	-ve
1994						
	1	-ve	-ve	-ve	-ve	-ve
	2	+ve	-ve	-ve	-ve	-ve
	3	+ve	+ve	-ve	-ve	-ve
	4	+ve	+ve	-ve	-ve	-ve
	5	+ve	-ve	-ve	-ve	-ve
	6	+ve	-ve	+ve	-ve	-ve
	7	+ve	+ve	-ve	-ve	+ very weak/double band
	8	+ve	-ve	-ve	-ve	-ve
	9	+ve	+ve	-ve	-ve	+ very weak/double band
	10	+ve	+ve	-ve	-ve	-ve
	11	+ve	-ve	-ve	-ve	+ very weak/double band
	12	+ve	+ve	-ve	-ve	-ve
1995 A						
	1	+ve	+ve	-ve	-ve	-ve
	2	+ve	+ve	+ve	+ve	-ve
	3	+ve	-ve	-ve	-ve	-ve
	4	+ve	+ve	-ve	-ve	-ve
	5	+ve	+ve	-ve	-ve	-ve

1995 B						
	6*	+ve	+ve	-ve	-ve	+ weak/double bands
(Pooled *)	7*	+ve	+ve	-ve	-ve	+ weak/double bands
	8*	+ve	+ve	+ve	+ve	+ double bands
1996						
	1	+ve	+ve	-ve	-ve	+ very weak/double bands
	2	+ve	+ve	-ve	-ve	+ very weak/double bands
	3	+ve	+ve	+ve	+ve	+ very weak/double bands
	4	+ve	-ve	-ve	-ve	+ very weak/double bands
	5	+ve	+ve	-ve	-ve	+ very weak/double bands
	6	+ve	+ve	-ve	-ve	+ very weak/double bands
	7	+ve	+ve	-ve	-ve	+ very weak/double bands
1997 A						
	1	+ve	+ve	-ve	-ve	+ weak/double bands
	2	+ve	-ve	-ve	-ve	+ weak/double bands
	3	+ve	-ve	-ve	-ve	+ weak/double bands
	4	+ve	+ve	-ve	-ve	+ weak/double bands
	5	+ve	+ve	-ve	-ve	+ weak/double bands
1997 B						

	6	*+ve*	*+ve*	*+ve*	*-ve*	-ve, only 600 bp band
	7	+ve	+ve	-ve	-ve	+ve double bands
	8	+ve	+ve	-ve	-ve	+ve double bands
	9	+ve	+ve	+ve	-ve	+ve double bands
	10	+ve	+ve	+ve	-ve	+ve double bands
	11	+ve	+ve	+ve	-ve	+ve double bands
	12	+ve	+ve	-ve	-ve	+ve double bands
	13	+ve	+ve	+ve	-ve	+ve double bands
	14	+ve	-ve	-ve	-ve	+ve double bands
	15	+ve	+ve	-ve	-ve	+ve
1999						
	1 to 4	+ve	+ve	-ve	-ve	+ve double bands, very weak
	5	+ve	+ve	+ve	-ve	+ve double bands
	6	+ve	-ve	-ve	-ve	+ve double bands
	7	+ve	-ve	-ve	-ve	-ve
	8	+ve	-ve	-ve	-ve	+ve double bands
	9	+ve	+ve	+ve	-ve	+ve
	10	+ve	-ve	-ve	-ve	-ve
2001						
	1 to 10	+ve	+ve	-ve	-ve	+ve double bands

2003						
	1	+ve	+ve	-ve	-ve	+ve double bands
	2	+ve	-ve	-ve	-ve	+ve double bands
	3	+ve	+ve	-ve	-ve	+ve double bands
	4	+ve	+ve	-ve	-ve	+ve double bands
	5	+ve	-ve	-ve	-ve	+ve double bands
	6	+ve	-ve	-ve	-ve	+ve double bands
	7	+ve	-ve	-ve	-ve	+ve double bands
	8	+ve	+ve	-ve	-ve	+ve double bands
	9	+ve	+ve	-ve	-ve	+ve double bands
	10	+ve	+ve	-ve	-ve	+ve double bands
2003/ 2004 Aims						
	1 to 9	+ve	+ve	-ve	-ve	+ very weak/double bands
	10	+ve	-ve	-ve	-ve	+ very weak/double bands
2004 wild						
	1	+ve	+ve	-ve	-ve	+ve double bands
	2	+ve	+ve	-ve	-ve	-ve
	3	+ve	-ve	-ve	-ve	+ve double bands
	4	+ve	-ve	-ve	-ve	+ve double bands

	5	+ve	+ve	-ve	-ve	+ve double bands
	6	+ve	+ve	+ve	+ve	+ve double bands
	7	+ve	-ve	-ve	-ve	+ve double bands
	8	+ve	+ve	-ve	-ve	+ve double bands
	9	+ve	+ve	+ve	-ve	+ve double bands
Semisulcatus	S10	+ve	-ve	-ve	-ve	-ve
2005 A						
	M1 to M5	+ve	+ve	-ve	-ve	+ve double bands
	M6	+ve	+ve	+ve	-ve	+ve
	M7 to M9	+ve	+ve	-ve	-ve	+ve double bands
	M10	+ve	+ve	+ve	+ve	+ve double bands
	M11	+ve	+ve	-ve	-ve	+ve double bands
	M12	+ve	+ve	-ve	-ve	+ve double bands
	M13	+ve	+ve	-ve	-ve	+ve double bands
	M14	+ve	+ve	+ve	+ve	+ve double bands
	M15	+ve	+ve	-ve	-ve	+ve double bands
	M16	+ve	+ve	-ve	-ve	+ve double bands
	M17	+ve	+ve	+ve	-ve	+ve
	M18	+ve	+ve	-ve	-ve	+ve double bands

	M19	+ve	+ve	+ve	-ve	+ve
	M20	+ve	+ve	-ve	-ve	+ve double bands
2005 B						
	5	+ve	+ve	-ve	-ve	+ve double bands
	275	+ve	+ve	-ve	-ve	+ very weak
	276	+ve	+ve	+ve	-ve	+ve
	277	+ve	+ve	+ve	-ve	+ve
	278	+ve	+ve	-ve	-ve	+ very weak
	271	+ve	+ve	-ve	-ve	+ve double bands
2009						
Postlarvae	Pl1 to Pl5	+ve	+ve	+ve	-ve	+ve
	Pl6	+ve	+ve	-ve	-ve	+ve
Runts/Stunts	1	+ve	+ve	-ve	-ve	+ve double bands
-Ayr	2	+ve	-ve	-ve	-ve	+ve double bands
	3 to 4	+ve	-ve	-ve	-ve	+ve double bands
	5 to 12	+ve	+ve	-ve	-ve	+ve double bands
Spawners	1 to 4	+ve	+ve	-ve	-ve	-ve
	5	+ve	-ve	-ve	-ve	-ve

	6	+ve	-ve	-ve	-ve	+ve
	7	+ve	-ve	-ve	-ve	-ve
Imported shrirmp						
P. stylirostris						
New Caledonia	STY1	+ve	+ve	+ve	+ve	-ve
	STY2	+ve	+ve	+ve	+ve	-ve
	STY3	+ve	+ve	+ve	+ve	-ve
	STY4	+ve	+ve	+ve	+ve	-ve
	STY5	+ve	+ve	+ve	+ve	-ve
P. vannamei						
China	CV1 to CV5	+ve	+ve	+ve	+ve	-ve
	TV1 to TV5	+ve	+ve	+ve	+ve	-ve
Malaysia	1-10	-ve	-ve	-ve	-ve	-ve
Indonesia	1-10	+ve	-ve	-ve	-ve	-ve
P. monodon						
Brunei	B1	+ve	+ve	-ve	-ve	+ve
	B2	+ve	-ve	-ve	-ve	+ve
	B3	+ve	+ve	+ve	+ve	-ve

APPENDIX C

Detail of *Pst*DV insertion patterns in the genome of shrimp from Farm A (Table C.1) and B (Table C.2) and the background of prawn (Table C.3) examined in Chapter 4.

Table C.1: Detail of *Pst*DV insertion patterns examined from shrimp from Farm A. In column 1, the number in brackets indicate the number of shrimp samples with the same pattern of positive (+) and negative (-) results as that for the sample number given. For example, in "3 inserts or 5 gaps (5 samples)", a sample in Pond 53 has an identical pattern to that of Pond 61.

Isolate (#)	Primer pairs used, position and expected size relative to Mexican *Pst*DV isolate (AF273215)							
80 samples in total:	Pair 1	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6	Pair 7	Pair 8
-Pond 66: 25 shrimp -Pond 53: 25 shrimp -Pond 41: 26 shrimp -Pond 48: 4 shrimp	158-723	702-1260	1059-1507	1451-2027	2002-2569	2544-3100	3031-3625	3168-3759
	566 bp	559 bp	449 bp	577 bp	568 bp	557 bp	595 bp	592 bp
Infective *Pst*DV (13 samples) -Pond 41: 11 samples -Pond 53: 2 samples	+	+	+	+	+	+	+	+
7 inserts or 1 gap (1 sample) -Pond 53	+	+	-	+	+	+	+	+
6 inserts or 2 gaps (4 samples) -Pond 41	+	+	+	+	+	+	-	-
4 inserts or 4 gaps (2 samples) -Pond 41: 1 sample	+	+	-	-	-	+	-	+

-Pond 41: 1 sample	+	+	-	+	-	+	-	-
3 inserts or 5 gaps (5 samples)								
-Pond 41: 1 sample	+	-	-	-	-	+	-	+
-Pond 53: 1 sample	+	+	+	-	-	-	-	-
-Pond 53 & 66: 1 each	+	+	-	-	-	-	-	+
-Pond 66: 1 sample	+	+	-	+	-	-	-	-
2 inserts or 6 gaps (48 samples)	+	+	-	-	-	-	-	-
-Pond 41: 6 samples								
-Pond 48: 4 samples								
-Pond 53: 17 samples								
-Pond 66: 21 samples								
1 insert or 7 gaps (6 samples)	-	+	-	-	-	-	-	-
-Pond 41: 2 samples								
-Pond 53: 2 samples								
-Pond 66: 2 samples								
No insert (1 sample): Pond 53	-	-	-	-	-	-	-	-

Table C.2: Detail of *Pst*DV insertion patterns examined from shrimp from Farm B. In column 1, the number in brackets indicate the number of shrimp samples with the same pattern of positive (+) and negative (-) results as that for the sample number given. For example, in "1 insert (7 gaps) (3 samples)", 2 samples share an identical pattern (pattern 1) and that differs from pattern 2 which also has 1 insert.

Isolate (#) 50 samples in total: -Pond 22A: 25 shrimp -Pond 33A: 25 shrimp	Primers used, position and expected size relative to Mexican *Pst*DV isolate (AF273215)							
	Pair 1	Pair 2	Pair 3	Pair 4	Pair 5	Pair 6	Pair 7	Pair 8
	158-723	702-1260	1059-1507	1451-2027	2002-2569	2544-3100	3031-3625	3168-3759
	566 bp	559 bp	449 bp	577 bp	568 bp	557 bp	595 bp	592 bp
Infective *Pst*DV (0 sample)								
4-7 inserts or 1-4 gaps (0 sample)								
3 inserts or 5 gaps (1 sample) -Pond 22A	+	+	-	-	-	-	-	+
2 inserts or 6 gaps (43 samples) -Pond 22A: 22 samples -Pond 33A: 21 samples	+	+	-	-	-	-	-	-
1 insert or 7 gaps (3 samples)								
-Pond 33A (pattern1): 2 samples	-	+	-	-	-	-	-	-
-Pond 33A (pattern2): 1 sample	+	-	-	-	-	-	-	-
No insert (3 samples) -Pond 22A: 2 samples -Pond 33A: 1 sample	-	-	-	-	-	-	-	-

Table C.3: Details of shrimp used for *Pst*DV random insert examination as determined by the 8-overlapinng PCR primer method (see Table C.1 and C.2). The shrimp were collected in October 2012.

Farm	Pond name	Origin of seed	size	Number of shrimp tested	Number of *Pst*DV infected shrimp	% Prevalence
A	66 East Coast	Wild caught broodstock	5.7 g	25	0	0.00
	53 East Coast	Wild caught broodstock	5.9 g	25	2	8.00
	48 North Territory	Wild caught broodstock	1.0 g	4 (all we have)	0	0.00
	41 North Territory	Wild caught broodstock	0.5 g	26 (all we have)	11	42.31
	Total:			80	13	16.25
B	22A	Wild caught broodstock	10.0 g	25	0	0.00
	33A	Wild caught broodstock	8.0 g	25	0	0.00
	Total:			50	0	0.00

APPENDIX D

The genome sequence of Australian *Pst*DV (AUS-*Pst*DV2012) reported in this thesis (GenBank No. KM593913)

ATGGAAGATACGAACAACCACCCATGGCAATCAATACCTAGTCCGTCAT
TATTTGGATCATCAAGTAACAGTGAACCAACAGAAGTCTTTGAAAACGT
CTTCGGGGAAAGACAAACCCAAGGATACAAATGTAAGTACAAGTGACTG
ACTAAGTGACGATCCGTTAAATTCCTGATTGACGCAAATGACGACGTCA
TATGCGTCACTTACAAAAGACGTAACCGCTTTCGTCCATCACTCACTTAA
ATCTTTCTCTACCTTTCAGACGACATACCCCAACAAATATCGCTGCGCTA
CTGCCCAGATCACATTCTACCGTGGTGCTTCATAGGGAACAGACCCGTTC
TCTACTGCCTCTGCAACGAGTGTTTTATAGACAATCTCAATGTCAACGGA
CAGTGTCAACACTGTCATCCCGACGACGAAGAATGGACAGAAAATATGG
CCAAGAACGTACTGCATACATGTGAGGGCAAACCAGACTCACTTAGTGA
AATGCTTCGACGAAGCACGGAATCGACTGACTCCAGCCAGGGAATTTCT
CCAAGCCTTCTCACCCCAGGTCCAAATCAAGAGCCTGGACCCACTACCG
AACAACTTCTTAATATGTCTGAAGAATTGTTCCAGTTTTCAGACGAGGAA
GACAATTCTCAAACTCCTCCAAGAACTTCAACACCAGAACAAACTGATC
CTAAGGTCTGCGTGGATAACCTGGGAATACGAGAGGGAACAGGAAACG
GAACAATTCAACTTGGAAGTGAATCAGAAACCTCCCTTGGAAGTGTTGG
AAACAGTAATAACAGGCGTCAAAAGAGACAGAGAGGAATTACTTACAT
CAGTGACACATCAGATTCCTCTGGATCAGATGACGAAAATCTGGGTGCA
TCACATAGAAATAAAAGAAGCAAACACTCCGACACCACAAAAGAGACA
GGCGGAGGAGACGACCGACGACATCAGGAAAGCGATCAAGGAAGCAAT
GGAAATCGACTGGAACCTACCGATGGAGGAGAGAGCTATAGTAGCGGA
ACACAACCCGACTTTATTGAAGGGACTCCCGACGGACCGGACGAAATGG
ACGGAAGGCGACTGGAAGAGAGTGAGATTGAAAAACAAGTGGAAAGTA
CAACATGGTACACCTTCGTCATCAGAGAAAAACCACAACCAAGAAGACT
CTCCGGACGAACGCCAAACTTCACCATTACAGATCATGGTGACCACTGG
CACATCACATACTCCGGACACCCAACCAATAAGACCAGACATAGAGCTA
CAATCCTCGCCTATTTGGGAGTTACCTTTGCTGCCAGAGCCGAAGCTGAA
GCGACTACGGTACTTATTAAAGATATCAAGAGATGGATACTCTATCTTAT
CAGATACGGTATTGAACGGCTTTCGTATTTTGGTCTTGGCCACACCATTT
TTAGAAGAATCATCAAGTACTTCCAACAATACCGAAGAGAAGAGGACAC
CATAGACGGACCATGTCCATACATGTCCAGCACAAGAGATGAAAGAGGA
GAAGAAAAATCCAAAGAAAACAGTGCTGAATACGACTACCTGAGGCAC
CTCGTCAAAAGTAAAGCAGCAAGAACAGTACAAGAACTGGTTAATAAAC
TAGACGATGAAGAATACAAACAACTCTGGACACGTACAAGGGGACAAT
ATAAAGACAAATTAAGAGGAATCCTCACTTACTACAACAACAAGAAGAA
ATTCAGTCAAAGTCAACTATCACTCATTACAATCTACACAATATATCAAA
AAGGAAACCAGACTTCGAAAACATGCAATGGCTCAAGTACATGTTCGCC
AACAATGACATCCGTGTACCAGAAATCTTAGCATGGATAATTATAGTTG
CTGACAAGAAACTTGATAAAATTAACACTCTAGTTCTACAAGGACCAAC
AGGAACAGGCAAATCTCTGACCATTGGAGCATTACTCGGCAAGTTAAAC
ACTGGCTTAGTAACAAGAACAGGAGATTCAAACACCTTCCATCTTCAAA
ACCTCATCGGAAAATCCTATGCACTATTCGAAGAACCAAGAATTAGTCA
AATAACAGTGGACGACTTCAAACTCCTTTTCGAAGGATCAGACTTAGAA

GTAAACATAAAACACCAAGAGTCAGAAATTATGGGACGAATACCAATCT
TCATATCAACAAACAAGGACATAGACTACTGGGTACCTCCAGCTGATGG
TAAAGCTCTACAAACAAGAACAATAACCTTCCACCTGACAAGACAAATA
AAAGGCCTCTCAGACAGGATGAACAGCCAGTACGACATCAACCCTCCAC
CAGACAAGATCACCAGCGACGACTTCCTAGGACTCTTCCAAGAATACGA
AAAGGAAATCGACGACATCATAGACAACCATGTGCGCCGATTCAACAAG
AGCAAGCCCAAGGAAAAGATCCAGGAGGGATGCACATAATGAAGACGA
AGAACACGCCGAAGGATCAAGTGGACCAGACCCACACAGATGTCTACA
ATTCAATACTGGAGACTCAATACATATTACTTTCCAAACAAGAAGATACT
TCGAATTCGACGCTGCCAATGATGGAAACTTCGACGGAAAAAATTTATA
CTGCCTCCCACTACATTGGATGAACTTATATCTCTATGGTCTAAAGAGCA
GCGACAGTTCAGCATCAGAAACACAACGATATAAAATGGTAAAATCAAT
GATGAAGACCTACGGATGGAAAGTACACAAAGCAGGCGTAGTGATGCA
CTCGATGGTACCCCTTATGAAAGACTTAAAAGTATCAGGGGGAACATCA
TTTGAGACTCTCACATTTACAGACACCCCATATTTAGAAATATTTAAGGA
TACTACTGGACTACATAATCAACTATCAACTAAGGAAGCCGACGTAACA
TTGGCTAAATGGATTCAAAATCCCCAACTTGTAACGGTACAATCCACAG
CTGCAAATTATGAAGACCCTCTCCAACAATTTGGCTTCATGGAACAAATG
CGAACAGGAGATAGAAAGGCTTATACAATCCATGGTGATACAAGAAACT
GGTATGGAGGAGAAATAGCTACAACTGGACCGATCTTTATTCCAAAGTG
GGGAGGCCAAATCAAATGGGACAAACCATCCCTTGGAAACCTTGTCTAC
CCAGCGGAACATCATACCAACGATTGGCAACAAATCTTCATGAGAATGT
CACCAATCAAAGGACCATCCGGAGAAGAACTAAAGCTAGGCTGCAGAG
TACAAGCAGACTTCTTCCTACACCTAGAAGTTCGACTCCCACCGCAAGG
ATGTGTAGCAAGTCTGGGGATGCAGCAATATCTCCATAAACCATGTTCA
GGACAACTTAACAAATGTTATGTTATGCATACTAACTAATATACTTAATC
TGTGCAATATGTAACCAACTATAACCTGCTTTATACCAATAAAAACCCAC
AAAAAGCAAATATATCTCACTATCCTTTACACAAACCGGCTACCCAGGC
ATGGTGGGAAACCTGCTACCCAGGCAAGGTGGGACAATAATCTCATAAT
GACAAAATAAGTCAGTCATATAAGTCATTCACAAAAGACTCGTAACTCC
ACCGTCAGTCAGTCATTTAGAGTC

APPENDIX E

The host origins of Australian endemic *Pst*DVs

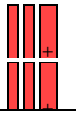
Table E.1: Detail of the host origins of Australian endemic *Pst*DVs

GenBank No.	Year of isolation	Host origin
KM593908	1991	*P. esculentus/ P. monodon* hybrid obtained from research facility in northern Queensland
EU675312	1993	*P. monodon* from East Coast prawn farm
KM593909	1995	Farmed *P. monodon* broodstock
KM593910	1996	Farmed *P. monodon* originated from wild captured broodstock (personal communication with prawn farm)
KM593911	2004	Wild captured *P. monodon* broodstock
KM593912	2005	Wild captured *P. monodon* broodstock
GQ475529	2008	Farmed *P. monodon*
KM593913 (this study)	2012	Farmed *P. monodon* originated from wild captured *P. monodon* broodstock

APPENDIX F

Detail of natural selection analysis using SLAC, FEL, and REL approaches

Table F.1: Detail of positively and negatively selected sites for ORF1, 2 and 3 of the genome of non-Australian *Pst*DV isolates (nAus Grp 1) as analyzed by method consensus (SLAC, FEL and REL). Significance level: SLAC p-value: 0.1; FEL p-value: 0.1; REL Bayes Factor: 50. Color legend in the tables: Codon is non-neutral according to a given method at the specified significance level. In the consensus column, Codon has dN>dS '+' inside the box means that the difference is significant. In the consensus column, Codon has dN<dS '+' inside the box means that the difference is significant.

Codon	SLAC dN-dS	SLAC p-value	FEL dN-dS	FEL p-value	REL dN-dS	REL Bayes Factor	Consensus
ORF1: 2 positively selected sites (at least 1 method)							
28	9.348	0.764	164.054	0.349	0.025		
193	11.072	0.657	129.346	0.301	0.082		
ORF1: 33 negatively selected sites (at least one method)							
249	-24.507	0.111	-204.917		-8.061	2.786	
320	-23.780	0.205	-288.331		-2.355	1.434	
359	-34.492		-514.138		-15.054		
381	-15.890	0.267	-160.344		-2.183	1.437	
386	-12.254	0.333	-191.963		-2.468	1.501	
396	-19.273	0.216	-531.647		-4.478	1.844	
398	-25.177	0.196	-284.854		-2.450	1.446	
400	-19.273	0.243	-531.647		-4.471	1.725	
403	-12.254	0.333	-263.267		-2.703	1.497	
404	-12.254	0.333	-163.252		-1.410	1.360	
414	-13.699	0.309	-272.539		-3.473	1.685	
418	-12.254	0.333	-353.725		-2.936	1.530	
443	-13.233	0.318	-138.520		-1.907	1.462	
449	-25.177	0.162	-284.854		-2.452	1.494	
454	-38.265		-1684.100		-13.734	13.804	

482	-30.588	0.134	-429.351		-3.850	1.713
483	-32.193	0.127	-758.425		-3.200	1.607
503	-34.108		-601.924		-15.101	
508	-12.254	0.333	-226.093		-2.022	1.408
509	-12.254	0.335	-189.235		-2.367	1.534
527	-12.254	0.339	-191.963		-2.471	1.546
534	-19.532	0.213	-3145.630		-4.152	1.786
558	-31.984	0.128	-649.646		-4.775	1.883
570	-30.588	0.134	-429.351		-3.850	1.713
579	-25.177	0.162	-284.854		-2.455	1.534
585	-12.254	0.333	-226.093		-2.020	1.385
591	-12.254	0.333	-200.876		-1.835	1.422
601	-26.819		-371.419		-14.905	
604	-19.273	0.243	-531.647		-4.471	1.725
607	-12.254	0.333	-148.660		-1.440	1.423
632	-12.254	0.339	-191.963		-2.471	1.546
645	-26.068	0.157	-267.760		-3.062	1.566
647	-29.879	0.137	-307.014		-2.568	1.510

ORF2: 10 negatively selected sites (at least one method) (No positively selected site)

26	-26.123	0.162	-1390.360		-4.116	1.641
46	-23.449	0.131	-1003.320		-14.053	13.017
141	-12.720	0.333	-359.967		-2.680	1.449
169	-24.262	0.175	-980.074		-4.042	1.547
183	-30.938		-631.957		-11.177	4.556
194	-12.720	0.333	-308.374		-2.423	1.440
211	-12.720	0.333	-544.141		-2.920	1.507
301	-13.633	0.311	-698.707		-3.716	1.547
325	-12.720	0.333	-269.023		-1.885	1.502
326	-47.222	0.119	-4394.120		-4.860	1.666

ORF3: 21 positively selected sites (at least one method)

8	12.924	0.430	80.958	0.212	0.352	
35	5.062	0.810	26.426	0.526	0.300	

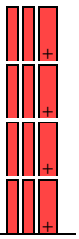
44	5.026	0.816	21.713	0.569	0.291	
51	-11.384	0.925	-930.813		0.307	
53	6.158	0.699	22.119	0.525	0.292	
105	8.444	0.952	35.154	0.616	0.352	
113	-6.148	0.889	-75.432	0.391	0.301	
163	7.454	0.623	42.507	0.354	0.312	
167	6.148	0.667	30.558	0.374	0.304	
185	7.871	0.610	40.868	0.361	0.311	
188	6.148	0.667	26.915	0.440	0.300	
240	13.690	0.440	94.149	0.319	0.352	
246	11.755	0.488	85.510	0.195	0.352	
247	6.064	0.676	33.568	0.344	0.307	
292	5.024	0.816	26.030	0.536	0.299	
303	12.290	0.445	70.562	0.195	0.352	
304	12.924	0.430	82.087	0.210	0.352	
314	6.148	0.667	27.249	0.438	0.301	
319	-12.048	0.930	-495.996	0.145	0.307	
322	5.690	0.758	22.857	0.563	0.294	
325	5.793	0.707	33.882	0.348	0.307	

ORF3: 31 negatively selected sites (at least one method)

27	-18.741	0.225	-269.663		-0.923	2.325
42	-12.296	0.333	-78.320		-0.916	2.098
51	-11.384	0.474	-930.813		0.307	0.005
63	-21.788	0.188	-138.386		-0.927	2.453
70	-21.877	0.187	-119.249		-0.926	2.420
85	-12.296	0.335	-97.296		-0.926	2.408
89	-22.152	0.185	-125.874		-0.935	2.771
93	-12.296	0.333	-130.322		-0.916	2.113
100	-29.028		-172.774		-0.919	2.189
103	-17.692	0.244	-75.099		-0.921	2.242
114	-21.877	0.187	-119.242		-0.916	2.109
118	-12.296	0.333	-97.059		-0.915	2.081

122	-24.592	0.148	-207.472	-0.897	1.678	
131	-18.741	0.230	-269.663	-0.922	2.290	
135	-12.296	0.335	-93.480	-0.926	2.408	
145	-22.239	0.184	-274.273	-0.928	2.486	
152	-21.781	0.188	-192.662	-0.916	2.110	
172	-12.296	0.333	-117.591	-0.916	2.113	
173	-18.749	0.230	-494.847	-0.923	2.299	
178	-24.592	0.111	-187.052	-0.905	1.830	
182	-12.296	0.333	-93.480	-0.916	2.099	
189	-22.152	0.185	-125.881	-0.926	2.427	
199	-22.152	0.185	-125.881	-0.916	2.101	
206	-12.296	0.335	-97.296	-0.926	2.408	
208	-12.732	0.349	-106.807	-0.912	2.003	
211	-21.795	0.220	-203.735	-0.909	1.910	
219	-21.877	0.187	-119.249	-0.926	2.420	
231	-12.296	0.333	-201.700	-0.916	2.099	
238	-12.296	0.333	-64.922	-0.927	2.454	
241	-17.692	0.244	-75.099	-0.921	2.242	
255	-22.152	0.185	-125.874	-0.927	2.443	

Table F.2: Detail of positively and negatively selected sites for ORF1 Fr1 and Fr2, ORF2 and ORF3 of the genome of Australian *Pst*DV isolates (Aus Grp 2) as analyzed by method consensus (SLAC, FEL and REL). Significance level: SLAC p-value: 0.1; FEL p-value: 0.1; REL Bayes Factor: 50. Color legend in the tables: Codon is non-neutral according to a given method at the specified significance level. In the consensus column, Codon has dN>dS '+' inside the box means that the difference is significant. In the consensus column, Codon has dN<dS '+' inside the box means that the difference is significant.

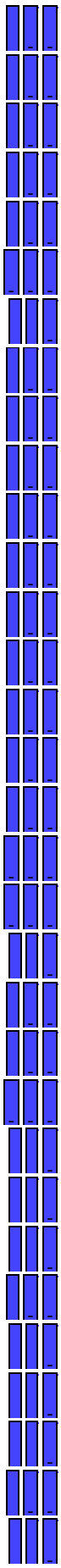
Codon	SLAC dN-dS	SLAC p-value	FEL dN-dS	FEL p-value	REL dN-dS	REL Bayes Factor	Consensus
ORF1 Fr1: 4 positively selected sites (at least one method)							
110	10.045	0.414	55.742	0.124	1.004		
159	7.981	0.587	73.579	0.204	0.954		
177	7.799	0.548	49.489	0.555	0.833		
193	7.410	0.569	52.875	0.197	0.946		
ORF1 Fr1: 37 negatively selected sites (at least one method)							
69	-36.208		-176.943		-7.386	6.204	
93	-11.848	0.211	-87.088		-1.699	2.871	
109	-12.461	0.204	-103.983		-1.945	3.341	
139	-14.968	0.111	-94.834		-5.257	4.012	
170	-10.348	0.186	-85.697		-4.084	2.684	
185	-9.877	0.209	-74.126		-1.524	3.958	
221	-8.883	0.232	-39.137		-0.766	3.605	
257	-32.072		-726.351		-11.990	27.673	
269	-6.076	0.333	-42.465		-0.875	3.482	
271	-12.178	0.166	-131.163		-1.788	3.999	
282	-13.311	0.186	-53.991		-1.124	2.962	
330	-198.191	0.295	-318.190		-2.712	1.478	
333	-214.249	0.273	-203.936		-2.988	1.730	
342	-21.986	0.193	-2335.770		-2.752	6.512	
343	-24.905	0.167	-42.937		-0.964	5.124	
349	-25.383	0.190	-174.357		-2.064	5.610	

351	-24.905	0.201	-42.937		-0.953	4.005
357	-14.738	0.377	-365.508		-5.835	1.140
359	-27.144	0.137	-193.245		-12.475	21.545
369	-12.504	0.333	-42.556		-0.920	5.001
371	-24.905	0.201	-42.937		-0.953	4.005
377	-3.881	0.646	-73.758		-2.536	0.635
381	-23.328	0.189	-1032.240		-3.714	6.676
386	-25.008	0.111	-370.962		-10.142	15.431
389	-12.309	0.339	-43.298		-0.988	4.070
393	-12.309	0.339	-34.235		-0.730	4.066
403	-12.504	0.333	-29.240		-0.640	4.420
405	-12.504	0.333	-29.240		-0.640	4.420
406	-14.756	0.298	-29.322		-0.717	4.675
407	-12.504	0.339	-134.018		-1.577	5.508
413	-6.253	0.556	-60.002		-1.665	0.631
416	-25.599	0.163	-45.011		-0.984	4.028
417	-27.969		-199.838		-12.311	30.432
418	-12.504	0.333	-42.556		-0.914	4.511
424	-23.328	0.189	-54.111		-1.188	4.515
425	-16.386	0.269	-32.744		-0.707	4.230
429	-33.692	0.124	-44.632		-1.012	6.055

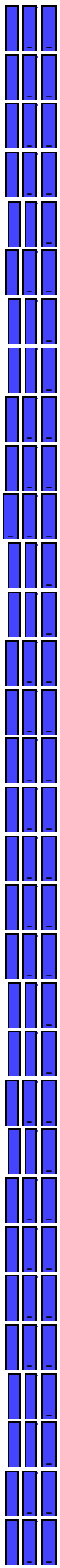
ORF1 Fr2: 82 negatively selected sites (at least one method). No positively selected found

2	-104.865	0.342	-83.465		-1.208	
4	-93.227	0.408	-10.424	0.529	-1.119	
5	-298.453	0.112	-35.551		-1.207	
6	-201.121	0.186	-7118.060		-1.207	
11	-213.300	0.144	-23.045		-1.208	
20	-92.051	0.333	-11.367		-1.208	
23	-95.604	0.321	-9.487	0.137	-1.207	
24	-95.604	0.321	-13.729		-1.207	
25	-92.051	0.333	-14.950	0.104	-1.207	

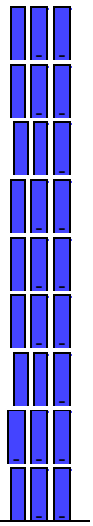
26	-92.051	0.333	-14.950		-1.208
28	-298.453	0.112	-35.551		-1.207
30	-128.602	0.228	-40.710		-1.207
31	-213.300	0.144	-23.045		-1.208
32	-25.860	0.157	-78.749		-1.208
33	-39.883		-52.324		-1.208
35	-16.372	0.333	-5.482	0.149	-1.209
36	-27.141	0.162	-24.110		-1.207
37	-32.743	0.111	-29.792		-1.207
38	-27.141	0.162	-28.712		-1.207
45	-32.743	0.113	-37.393		-1.206
48	-27.141	0.162	-34.037		-1.207
50	-34.019	0.103	-14.754		-1.208
51	-32.743	0.113	-31.763		-1.206
53	-29.085	0.167	-21.308		-1.207
54	-22.946	0.291	-47.839		-1.207
55	-32.743	0.113	-37.393		-1.206
56	-56.380		-117.240		-1.208
57	-55.239		-51.701		-1.207
59	-16.372	0.333	-4.826	0.164	-1.209
60	-32.743	0.113	-37.393		-1.206
61	-29.085	0.167	-21.308		-1.207
67	-71.718		-75.467		-1.208
68	-16.372	0.392	-11.341	0.104	-1.208
73	-15.029	0.363	-5.418	0.175	-1.208
78	-16.372	0.339	-11.341	0.147	-1.206
79	-38.404	0.155	-92.140		-1.208
81	-34.612	0.191	-9.836	0.107	-1.208
82	-16.372	0.333	-9.787	0.105	-1.208
83	-12.197	0.447	-7.235	0.159	-1.207
87	-16.372	0.333	-10.478		-1.209
89	-17.013	0.321	-5.364	0.178	-1.208



94	-32.743	0.111	-25.829		-1.207
95	-34.626	0.158	-14.109		-1.208
96	-34.626	0.158	-14.109		-1.208
99	-22.897	0.227	-18.947		-1.207
100	-12.211	0.447	-6.494	0.157	-1.208
101	-34.626	0.158	-14.109		-1.209
106	-19.280	0.336	-10.780	0.114	-1.207
108	-16.372	0.333	-9.676	0.126	-1.207
110	-18.076	0.302	-11.048		-1.208
114	-38.221	0.146	-17.534		-1.208
117	-65.605		-164.204		-1.208
119	-16.372	0.340	-17.883	0.123	-1.206
120	-24.120	0.218	-13.555	0.102	-1.206
121	-18.076	0.302	-11.048		-1.208
122	-16.372	0.333	-9.676		-1.209
125	-18.076	0.302	-11.048		-1.208
128	-34.626	0.158	-14.109		-1.209
129	-38.221	0.156	-17.534		-1.208
130	-34.626	0.158	-14.109		-1.208
136	-32.743	0.111	-18.513		-1.209
138	-16.372	0.333	-7.574	0.123	-1.208
140	-16.372	0.333	-6.032	0.214	-1.206
141	-21.601	0.276	-20.947		-1.208
143	-15.008	0.364	-7.270	0.148	-1.207
147	-16.372	0.333	-9.403		-1.209
149	-16.372	0.333	-9.178		-1.209
152	-15.008	0.364	-12.463		-1.207
153	-32.743	0.111	-30.035		-1.209
158	-16.372	0.339	-9.178	0.160	-1.206
159	-12.211	0.447	-7.536	0.142	-1.208
162	-22.050	0.312	-14.559		-1.206
172	-16.372	0.333	-8.249		-1.209



173	-16.372	0.333	-8.249		-1.209	
178	-34.626	0.158	-14.109		-1.208	
183	-16.372	0.339	-10.930	0.150	-1.206	
184	-10.263	0.541	-20.554		-1.205	
189	-21.601	0.258	-20.947		-1.208	
190	-38.221	0.156	-17.534		-1.208	
192	-17.013	0.321	-6.545	0.153	-1.208	
196	-35.213		-88.658		-1.208	
197	-34.626	0.158	-14.109		-1.208	

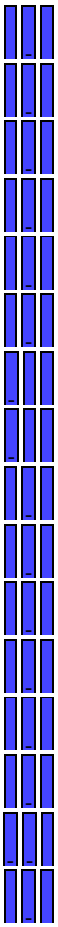


ORF2: 3 positively selected sites (at least one method)

158	11.908	0.360	52.541	0.102	0.975
175	6.981	0.480	58.808	0.321	1.148
196	9.133	0.639	88.931	0.623	1.136

ORF2: 22 negatively selected sites (at least one method)

27	-22.708	0.157	-155.344		-0.795	2.654
78	-22.708	0.157	-155.344		-0.793	2.597
86	-13.621	0.272	-329.826		-0.778	2.388
115	-16.551	0.218	-62.429		-0.792	2.702
140	-10.715	0.333	-46.519		-0.802	2.999
141	-21.430	0.111	-206.937		-0.822	2.359
147	-0.657		0.000	1.000	-0.678	2.658
152	-0.383		0.000	1.000	-0.640	2.201
164	-16.460	0.217	-72.844		-0.770	2.194
167	-22.708	0.157	-155.344		-0.795	2.654
169	-23.530	0.152	-720.400		-0.802	2.840
177	-12.170	0.111	-135.328		-0.865	3.631
179	-6.085	0.333	-45.494		-0.816	3.514
183	-10.089	0.224	-129.294		-0.805	3.148
186	-17.807		-111.257		-0.867	3.841
187	-6.085	0.333	-55.668		-0.823	3.350



201	-9.567	0.212	-50.931	-0.794	2.780	
211	-6.085	0.333	-58.237	-0.817	3.871	
228	-8.443	0.231	-86.546	-0.844	3.065	
234	-6.085	0.335	-34.147	-0.829	4.341	
235	-15.392	0.132	-120.116	-0.839	4.488	
357	-178.812	0.333	-101.808	-0.745	1.800	

ORF3: 72 negatively selected sites (at least one method). No positively selected site

13	-11.914	0.133	-43.226	-0.944	0.000	
14	-6.071	0.350	-25.887	-0.949	0.000	
22	-11.329	0.179	-25.997	-0.949	0.000	
23	-10.566	0.194	-41.178	-0.948	0.000	
35	-11.329	0.179	-25.996	-0.951	0.000	
36	-8.146	0.275	-91.448	-0.946	0.000	
39	-10.566	0.232	-41.178	-0.947	0.000	
40	-11.329	0.179	-25.997	-0.952	0.000	
41	-10.906	0.186	-84.485	-0.951	0.000	
42	-6.071	0.333	-59.215	-0.950	0.000	
45	-6.071	0.370	-23.470	-0.947	0.000	
59	-11.329	0.179	-25.997	-0.949	0.000	
62	-11.144	0.182	-24.032	-0.951	0.000	
65	-11.329	0.179	-25.997	-0.951	0.000	
66	-11.329	0.179	-25.997	-0.952	0.000	
67	-11.329	0.179	-25.997	-0.949	0.000	
70	-11.499	0.176	-84.555	-0.951	0.000	
72	-11.329	0.211	-25.996	-0.948	0.000	
73	-11.587	0.200	-92.656	-0.948	0.000	
82	-11.144	0.214	-24.032	-0.948	0.000	
85	-12.142	0.112	-48.235	-0.945	0.000	
87	-12.162	0.163	-27.079	-0.956	0.000	
89	-19.967		-24690.900	-0.958	0.000	
93	-5.384	0.333	-12.346	-0.955	0.000	

96	-5.384	0.333	-12.346	-0.957	0.000	
97	-12.839	0.169	-93.412	-0.955	0.000	
99	-9.946	0.213	-13.038	-0.955	0.000	
100	-6.654	0.298	-16.986	-0.955	0.000	
103	-8.765	0.226	-24.891	-0.956	0.000	
107	-8.445	0.235	-11.498	-0.955	0.000	
108	-5.384	0.333	-15.586	-0.957	0.000	
114	-9.984	0.180	-14.380	-0.958	0.000	
116	-5.384	0.333	-12.346	-0.955	0.000	
119	-10.768	0.111	-35.102	-0.954	0.000	
122	-27.686	0.148	-37.076	-0.945	0.000	
126	-24.270	0.145	-37.042	-0.948	0.000	
131	-26.087	0.195	-113.285	-0.948	0.000	
134	-27.686	0.113	-43.593	-0.943	0.000	
136	-13.843	0.333	-27.143	-0.949	0.000	
144	-13.843	0.333	-27.916	-0.949	0.000	
148	-24.834	0.218	-35.871	-0.947	0.000	
175	-17.293	0.267	-21.607	-0.949	0.000	
176	-28.213	0.198	-171.282	-0.946	0.000	
181	-13.843	0.333	-27.916	-0.946	0.000	
182	-27.686	0.111	-40.932	-0.949	0.000	
189	-25.526	0.181	-26.543	-0.950	0.000	
193	-13.843	0.333	-18.146	-0.949	0.000	
202	-28.213	0.198	-171.282	-0.946	0.000	
207	-24.959	0.185	-79.559	-0.949	0.000	
209	-21.331	0.239	-28.790	-0.947	0.000	
216	-25.526	0.181	-26.543	-0.949	0.000	
219	-26.421	0.175	-374.369	-0.951	0.000	
228	-27.686	0.111	-36.074	-0.949	0.000	
230	-27.686	0.111	-48.727	-0.949	0.000	
232	-21.613	0.175	-20.350	-0.955	0.000	
234	-11.336	0.333	-46.645	-0.953	0.000	

235	-25.587	0.163	-35.163	-0.952	0.000	
241	-33.592		-1042.660	-0.952	0.000	
245	-11.336	0.333	-32.565	-0.953	0.000	
253	-11.336	0.333	-32.565	-0.953	0.000	
254	-22.672	0.111	-79.957	-0.951	0.000	
256	-21.613	0.175	-20.351	-0.954	0.000	
258	-11.336	0.333	-17.678	-0.953	0.000	
260	-21.613	0.175	-20.350	-0.953	0.000	
265	-21.412	0.176	-63.368	-0.956	0.000	
280	-25.587	0.163	-35.163	-0.952	0.000	
283	-41.427		-53727.500	-0.951	0.000	
287	-22.672	0.111	-41.256	-0.951	0.000	
291	-15.958	0.224	-27.390	-0.948	0.000	
295	-11.336	0.333	-17.482	-0.950	0.000	
299	-11.336	0.333	-14.121	-0.953	0.000	
313	-21.613	0.175	-20.351	-0.954	0.000	

APPENDIX G

List of Oral presentations and workshop

2015 "One base pair deletion is associated with reduction of virulence in australian *Penaeus stylirostris* densovirus", World Aquaculture Conference 2015, 26-30 June, Jeju, South Korea

2014 "Rapid and sensitive detection of *Penaeus stylirostris* densovirus by recombinase polymerase amplification (RPA) combined with a lateral flow dipstick (LFD)", Aquaculture America 2014, 9-12 Feb, Seattle, USA

Workshop

2008 Biosafety Course, College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland, Australia

APPENDIX H

Publication 1

Molecular and Cellular Probes 28 (2014) 284–287

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Short communication

Separation of endogenous viral elements from infectious *Penaeus stylirostris* densovirus using recombinase polymerase amplification

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ABSTRACT

Non-infectious *Penaeus stylirostris* densovirus (*PstDV*)-related sequences in the shrimp genome cause false positive results with current PCR protocols. Here, we examined and mapped *PstDV* insertion profile in the genome of Australian *Penaeus monodon*. A DNA sequence which is likely to represent infectious *PstDV* was also identified and used as a target sequence for recombinase polymerase amplification (RPA)-based approach, developed for specifically detecting *PstDV*. The RPA protocol at 37 °C for 30 min showed no cross-reaction with other shrimp viruses, and was 10 times more sensitive than the 309F/R PCR protocol currently recommended by the World Organization for Animal Health (OIE) for *PstDV* diagnosis. These features, together with the simplicity of the protocol, requiring only a heating block for the reaction, offer opportunities for rapid and efficient detection of *PstDV*.

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APPENDIX H

Publication 2

Journal of Virological Methods 208 (2014) 144–151

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Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocol

Recombinase polymerase amplification combined with a lateral flow dipstick for discriminating between infectious *Penaeus stylirostris* densovirus and virus-related sequences in shrimp genome

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RPA
LFD

ABSTRACT

Penaeus stylirostris densovirus (*Pst*DV) is an important shrimp pathogen that causes mortality in *P. stylirostris* and runt deformity syndrome (RDS) in *Penaeus vannamei* and *Penaeus monodon*. Recently, *Pst*DV-related sequences were found in the genome of *P. monodon* and *P. vannamei*. This led to false positive results by PCR-based detection system. Here, a more efficient detection platform based on recombinase polymerase amplification (RPA) and a lateral flow dipstick (LFD) was developed for detecting *Pst*DV. Under the optimal conditions, 30 min at 37 °C for RPA followed by 5 min at room temperature for LFD, the protocol was 10 times more sensitive than the Saksmerphrome et al's interim 3-tube nested PCR and showed no cross-reaction with other shrimp viruses. It also reduced false positive results arising from viral inserts to ~5% compared to 76–78% by the IQ2000™ nested PCR kit and the 309F/R PCR protocol currently recommended by World Organization for Animal Health (OIE) for *Pst*DV detection. Together with simplicity and portability, the protocol serves as an alternative tool to PCR for primarily screening *Pst*DV, which is suitable for both laboratory and field application.

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APPENDIX H

Publication 3

Aquaculture 448 (2015) 40–48

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Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

One base pair deletion and high rate of evolution: Keys to viral accommodation of Australian *Penaeus stylirostris* densovirus

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ABSTRACT

Penaeus stylirostris densovirus (PstDV) has caused large economic losses to shrimp farming worldwide. Here, a 1-bp deletion within the nuclear localization signal-encoding sequence of open reading frame 1 (ORF1; NS1) of Australian PstDV was identified as a cause of 199-amino acid shortened NS1 protein and production of a second protein, C-terminal NS1. This mutation is believed to reduce virulence as it strongly modifies the characteristics of NS1, which is responsible for the majority of enzymatic activities in PstDV. This finding supports a hypothesis regarding accommodation of PstDV in Australian prawns in relation to viral genetics. However, a high degree of evolution (1.55×10^{-3} substitutions/site/year) and genetic variation for the virus was attributable to the viral recombination observed with 10 potential genomic breakpoints identified. With this finding, we suggest that awareness of the emergence of new virulent strains should be increased as a preventative measure against future outbreaks of PstDV in the Australian Indo-Pacific.

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