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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 (*Pst*DV) 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 *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) 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 *Pst*DV in the Australian Indo-Pacific.

1. Introduction

Penaeus stylirostris densovirus (*Pst*DV) is a non-enveloped, single stranded DNA (ssDNA) virus in the genus *Brevidensovirus*, family Parvoviridae (Shike et al., 2000). Its genome (approximately 3.9 kbp) consists of 3 main overlapping open reading frames (ORFs): ORF1, ORF2 and ORF3 (Shike et al., 2000). This virus has been distributed in shrimp culture facilities worldwide (Rai et al., 2012) and is reported to be associated with high mortality in *Penaeus stylirostris* and stunting (called runt deformity syndrome or RDS) in *Penaeus vannamei* (Bell and Lightner, 1984; Castille et al., 1993) and *Penaeus monodon* broodstock (Primavera and Quinitio, 2000).

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). 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 after the first official report in 1991 despite the virus existing here, as demonstrated by positive results of PCR tests (Jaroenram and Owens, 2014a; Krabsetsve et al., 2004; Saksmerprome et al., 2010). 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, we analysed the genomic sequence of Australian *Pst*DV, and determined its genetic recombination and molecular evolution in relation to other geographical isolates of *Pst*DV. We discovered a genetic change that would be highly likely to be associated with the reduction of virulence in Australian *Pst*DV. Also, we were able to make inferences about levels of variation, recombination and evolutionary rate of this virus.

2. Material and methods

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 (Jaroenram and Owens, 2014a,b; Saksmerprome et al., 2011; Tang and Lightner, 2006). To ensure that our results were not derived from EVEs, we took *Pst*DV-infected

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P. monodon from our previous study (Jaroenram and Owens, 2014a). Briefly, this study sampled 130 prawns from 2 farms in northern Queensland in October 2012: 80 from 4 ponds of farm A (26, 25, 25 and 4 prawns/pond), and 50 from 2 ponds of farm B (25 prawns each). 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. All the 130 prawns were screened for the presence of infectious *Pst*DV using a set of 8 overlapping primer pairs designed to detect the whole *Pst*DV genome (minus the hairpin ends). Thirteen samples were positive for all the primer sets (i.e. the whole genome was amplified) indicating that they carried infectious *Pst*DV. Among these, 9 showing strong and specific PCR bands were chosen for sequencing here. The positive shrimp were divided arbitrarily into 3 groups. Gills or pleopods from each group were pooled for DNA extraction using Isolate II Genomic DNA Kit (Bioline). The concentration and quality of DNA were measured by spectrophotometer analysis at 260 and 280 nm, and then adjusted to 50 ng/μl by DNase-free water. Two microliters of the template were used in the following reactions.

2.2. PCR amplification, cloning and genome sequencing of Australian PstDV

Each DNA pool was subject to PCR using overlapping primers (Table 1). PCR reactions were performed in a 25-μl volume containing 1× MyFi PCR buffer (Bioline), 0.4 μM each forward and reverse primer, 2 U MyFi™ Taq DNA Polymerase (Bioline), and the specified amount of DNA template and DNase-free water. The PCR amplification profile consisted of initial denaturation at 94 °C for 3 min, 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. After PCR reactions were completed, the products were visualized by 1% agarose gel electrophoresis. Recombinant plasmids containing the DNA fragments of interest were constructed using pGEM®-T Easy Vector and amplified in JM109 *Escherichia coli* (Promega) following the manufacturer's protocol. The plasmids were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega). Sequencing of both strands of DNA plasmids (5-6 clones/PCR fragment/DNA pool) was performed by Macrogen Inc. (South Korea).

2.3. Bioinformatical analysis of PstDV genome

The overlapping sequences (above) were analysed using Sequencer 4.9, and the presence of potential open reading frames (ORFs) was determined using the AlignX tool in the Vector NTI program (Invitrogen). 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 the GenBank database (GenBank No. KM593913). Comparative analyses of the genome and the encoded ORFs of this *Pst*DV isolate were performed using a BLAST search against the National Center for Biotechnology Information (NCBI) database.

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, ORF 1 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 2) using Vector NTI.

2.5. Phylogenetic analysis

Our entire DNA sequence was aligned the sequences of 19 worldwide distributed *Pst*DV representatives (Table 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 edition of the phylogenetic tree were performed with TreeDyn 198.3 (Chevenet et al., 2006).

2.6. Recombination and selection analysis

Prior to analysis, the 20 *Pst*DV sequences (Table 2) were first aligned, 2 sequences containing gaps and/or incomplete ORFs (GQ411199 and DQ228358), were excluded from analysis as they were not recognized by the programs 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, and AY362547), and 2) inside Australia (KM593908, KM593909, KM593910, KM593911, KM593912, GQ475529, EU675312 and this study). 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

Table 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).