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Short Communication

Mixed-genotype WSSV infections of shrimp are inversely correlated with disease outbreaks in ponds

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Abstract

Outbreaks of white spot syndrome virus (WSSV) in shrimp culture and the relation between the virus and virulence are not well understood. Here we provide evidence that WSSV mixed-genotype infections correlate with lower outbreak incidence and that disease outbreaks correlate with single-genotype infections. We tested 573 shrimp samples from 81 shrimp ponds in the Mekong delta with outbreak or non-outbreak status. The variable number tandem repeat (VNTR) loci of WSSV were used as molecular markers for the characterization of single- and mixed-genotype infections. The overall prevalence of mixed-genotype WSSV infections was 25.7%. Non-outbreak ponds had a significantly higher frequency of mixed-genotype infections than outbreak ponds for all VNTR loci, both at the individual-shrimp as well as at the pond level. The genetic composition of WSSV populations appears to correlate with the health status of shrimp ponds. The causal relationship between genotypic diversity and disease outbreaks can now be experimentally approached.

White spot disease (WSD) is a highly contagious viral disease that can rapidly spread throughout an area and cause high levels of mortality in many species of cultured penaeid shrimp, especially *Penaeus monodon* (Flegel & Alday-Sanz, 1998; Walker & Mohan, 2009). White spot syndrome virus (WSSV) is the causative agent of this disease (Inouye *et al.*, 1994). WSSV has a large dsDNA genome of about 300 kbp which shows relatively little genetic variation, except for significant nucleotide sequence variation at five loci (Marks *et al.*, 2004; Dieu *et al.*, 2004). Comparison of the complete genome sequence of shrimp-derived WSSV isolates from Thailand, Taiwan and China (GenBank Accession numbers: AF369029.2, AF440570.1 and AF332093.1) revealed three variable number tandem repeat (VNTR) loci on the WSSV genome, overlapping with ORF75, ORF94 and ORF125, and two variable regions in which major deletions occur, the ORF14/15 and ORF23/24 regions (Marks *et al.*, 2004). These variable regions have now been used as genetic markers in a considerable body of epidemiological research. Studies employing one or more VNTR loci have found high levels of variation, even at very small spatial scales, suggesting that these loci are useful markers at such small scales to study genomic variation (Wongteerasupaya *et al.*, 2003; Hoa *et al*., 2005; Pradeep *et al.*, 2008a). On the other hand, the ORF14/15 and ORF23/24 variable regions have proven useful for inferring patterns of spread on larger spatial scales, such as the spread of WSSV to and in Vietnam (Dieu *et al.*, 2004, 2010a) and in India (Pradeep *et al*., 2008b). These results show that PCR genotyping is a useful tool for tracing the movement of WSSV and to better understand the epidemiology of this virus.

Hoa *et al*. (2005) and Pradeep *et al.* (2008a) reported different PCR amplicons in a single reaction when amplifying VNTR loci from viral DNA from individual shrimp. These different amplicons could indicate mixed-genotype WSSV infections of individual shrimp, although this was never confirmed by further molecular analysis. Mixed-genotype infections are common for many viruses (e.g., Smith & Crook, 1988; Davis *et al.*, 1999; Cory *et al.*, 2005). Moreover, complementation between genotypes within the host can increase virulence (Símon *et al.*, 2006; Vignuzzi *et al*., 2006). However, for WSSV the following information is needed for further studies: (i) confirmation of whether mixed-genotype infections occur, and if so, whether they are rare or abundant, and (ii) what the role of mixed-genotype infections is in WSSV epidemiology and evolution. In this report, we first investigated whether VNTR loci

are suitable for detecting mixed-genotype WSSV infections by a PCR-based method. We then tested whether mixed-genotype WSSV infections occur in shrimp ponds using WSSV variable loci as molecular makers, and estimated the prevalence of mixed-genotype infections in individual shrimp. The disease status of these shrimp ponds was also recorded, allowing us to test to what extent single- or mixed-genotype infections were correlated with the occurrence of disease outbreaks.

P. monodon juveniles and wild crabs were collected from grow-out ponds in Bac Lieu and Ca Mau provinces, Vietnam, during 2006–2009 and were transported immediately to Can Tho University, Vietnam. During that period, a total of 573 shrimp samples were taken from 81 sites (ponds). The samples were stored either in 100% ethanol at room temperature, or in a liquid nitrogen container before transfer to -80 °C. DNA was extracted from gill tissues and WSSV DNA was detected with a two-step PCR using the IQ2000TM WSSV kit, according to the manufacturer's instructions (Farming IntelliGene Technology Corporation, Taipei, Taiwan). The primer sets were designed to yield WSSV-specific amplicons of 296 bp and/or 550 bp, respectively. Amplification of a 848-bp amplicon from a crustacean housekeeping gene allowed for confirmation of correct DNA isolation, when no WSSV-specific sequences were detected.

Genotyping of WSSV-positive samples was done using a PCR-based method, based on different molecular markers, tandem repeat sequences (ORF94 and ORF125) and compound repeat units (ORF75) located along the WSSV genome. In detail, PCR with specific primers and conditions (Supplementary Table S1, available at JGV online; Dieu *et al*., 2010a) was used to amplify the VNTR loci ORF94 (54bp repeat unit = RU), ORF125 (69bp RU), and ORF75 (compound RUs of 45bp and 102bp). All PCR reactions were performed in an Applied Biosystems (Foster City, USA) thermal cycler using GoTaq Flexi DNA Polymerase (Promega; Madison, USA), and the PCR products were analyzed by electrophoresis in 1% agarose gels.

To determine whether different PCR products are really indicative of mixed-genotype infection, the ORF75 PCR products of different sizes derived from one individual shrimp were cloned. This analysis was performed for two WSSV-positive samples, selected because they rendered multiple PCR amplicons. PCR products were ligated

into the pGEM-T easy vector (Promega) and transformed into competent *Escherichia coli* DH5α by electroporation. White colonies were selected for plasmid isolation and colony PCR with the ORF75-flank primer pair (Table S1). Plasmid DNA was purified from clones representative of all insert sizes observed by PCR with the GeneJET^{TM} plasmid miniprep kit (Fermentas, Burlington city, Ontario, Canada). Plasmid clones were sequenced using universal T7 and/or Sp6 primers, and sequence chromatograms were analyzed using Chromas software (version 1.45). The sequences were then aligned using NCBI BLAST and Genedoc software. Referenced WSSV sequences were obtained from the NCBI Genbank database (AF369029.2, AF440570.1 and NC_003225.1). Molecular cloning can induce variation in WSSV RU number (Dieu *et al.*, 2010b), but the frequency of variants is low (< 10% of clones) and these variants only have a decreased number of RUs.

Both WSSV samples analyzed by cloning and sequencing of ORF75 PCR products gave evidence of patent mixed-genotype infections. Individual *E. coli* clones contained WSSV variants with a different number of repeat units (Fig. 1), which corresponded to the amplicon lengths observed in the initial PCR reaction on the shrimp samples. Two distinct genotypes were present in sample A (Fig. 1A), and three genotypes were present in sample B (Fig. 1B). Sequencing of selected clones confirmed (Fig. 2) that the variants originating from a single shrimp sample were so different to each other that they are not likely to be artifacts from the experimental procedure. i.e., these variants could not be generated by the addition or deletion of a single repeat unit, or a single recombination event. Identical cloning and sequencing analyses were performed for two WSSV isolates which had rendered multiple PCR bands for ORF94 (4 and 9 RU variants) and ORF125 (4 and 7 RU variants), resulting in sequences of both variants being found for each isolate (data not shown). These data demonstrate that this approach works for all three WSSV VNTRs and, moreover, confirms that mixed-genotype WSSV infections occur.

To determine the sensitivity of the VNTR PCR assay, PCR products from different RU variants were purified (Gel Band Purification kit, GE Healthcare kit, Buckinghamshire, UK) and mixed in the following ratios of variants: 1:1000, 1:100; 1:10, 1:1, 10:1, 100:1, 1000:1. A total of 50 ng of DNA was then used as the template

for standard individual PCR-genotyping (ORF75, ORF94, ORF125). The detection threshold ranged from 1:10 (ORF75, ORF94) to < 1:1000 (ORF125). The smaller RU variant was more readily detected for all three VNTRs, indicating that the sensitivity of the assay depends on the exact combination of RU variants. When a large number of field samples were analyzed, more mixed-genotype infections were detected for ORF125 than for ORF75 or ORF94 (see Table 1). The rate of failure for PCR amplification of the VNTR is also lower for ORF125 (15/573) than for ORF75 (17/573) and ORF94 (33/573). Although PCR for all three VNTRs can be used to detect mixed genotype infections, the ORF125 PCR has the highest sensitivity, also for mixed-genotype infections. ORF125 is therefore probably best suited for investigating mixed-genotype WSSV infections and for epidemiological studies tracking virus populations over space and time at small scales.

The three WSSV VNTR loci were then analyzed for a large number of virus isolates (573) collected in the MeKong Delta from 2006 to 2009 (Table 1). The results show that a number of single shrimp samples contained multiple WSSV genotypes. The number of samples in which mixed-genotype infections were detected was different for each molecular marker, and the highest for ORF125 (18.8%). For ORF125, we found 3 cases of infections with 4 WSSV genotypes detected in a single shrimp sample. WSSV mixed-genotype infections therefore not only occur in the field, they are even common at an overall prevalence of 25.7% (This is the prevalence of mixed-genotype infections for ORF75, ORF94 or ORF125, a mixed-genotype prevalence higher than that of any one VNTR locus). As the sensitivity of our assay varies depending on the VNTR locus and RU variants present, the actual frequency of mixed-genotype infections may be higher than our estimates. Others have reported evidence for the occurrence of mixed-genotype infections in the field (Hoa *et al.*, 2005; Pradeep *et al.*, 2008a), and mixed-genotype infections have been studied in laboratory settings (Marks *et al.*, 2005; Pradeep *et al.*, 2009). The work presented here provides the first confirmation – by cloning and sequence analysis – that mixed-genotype WSSV infections occur in the field.

The disease status of ponds - either ‘outbreak’ or ‘non-outbreak’ - was determined during sample collection. Determination of a pond's disease status was done by means of examining shrimp from the (emergency) harvest, and by information from farmers.

An outbreak pond is a pond in which virtually all shrimp are moribund or dead. In a non-outbreak pond, healthy shrimp are present and a high percentage of moribund or dead shrimp (> 50%) was not observed. Disease outbreaks are the result of interactions between the pathogen, host and environment. We found that individual shrimp in non-outbreak ponds were more likely to contain mixed-genotype WSSV infections. The frequency of mixed-genotype infections ranged from 0.8% (ORF75) to 8.7% (ORF125) in outbreak ponds, and from 4.4% (ORF75) to 26.8% (ORF125) in non-outbreak ponds (Table 1). There were significantly more mixed-genotype infections in non-outbreak ponds than in outbreak ponds ($\chi^2 = 51.254$, $P < 0.001$), as determined with a test of equal proportions (R2.7.0; The R Foundation, Vienna, Austria). The WSSV variants typically detected in outbreak ponds (i.e., ORF94: 5, 6, 7, 8 and 9 RUs; ORF125: 4, 5, 6 and 7 RUs) were also regularly found in non-outbreak shrimp ponds. The genotypes found in single-genotype infections in outbreak ponds were also regularly found in mixed-genotype infections in non-outbreak ponds.

There was much variation in the frequency at which mixed-genotype infection were detected for each locus (Table 1). However, if one molecular marker indicated a mixed infection, then would the other two markers be more likely to indicate a mixed infection also? I.e., are probabilities for detecting a mixed-genotype infections independent for each marker? To test whether this was the case, we considered the observed rate of mixed infection at all three loci, $P_{obs}(A \cap B \cap C)$, and compared it to the predicted rate of mixed infection at all three loci under the assumption that the outcome of PCR genotyping (mixed-genotype infection or single-genotype infection) is independent: $P(A \cap B \cap C) = P(A) \cdot P(B) \cdot P(C)$. $P(A)$, $P(B)$ and $P(C)$ are the probabilities that a mixed infection is found for ORF75, ORF94 and ORF125, respectively, which we assume is the observed frequency. The observed rate of occurrence ($P_{obs}(A \cap B \cap C) = 0.256$) was significantly higher than the predicted rate ($P(A \cap B \cap C) = 0.028 \cdot 0.106 \cdot 0.188 = 0.0008$), when compared with an exact binomial test ($P < 0.001$; R2.7.0) indicating there is dependence between test outcomes and therefore supporting the idea that the mixed-infection results for the different markers overlap. However, there is no correlation among WSSV strains detected in a single sample (e.g., a sample containing ORF94-4 and ORF94-6 was

detected in some cases, but a combination of ORF94-4 and ORF94-9 could be also isolated in other cases).

Experimental work has demonstrated that mixed-genotype virus populations are in many instances more virulent (i.e., causing lethal infection) than single genotype populations (Hodgson *et al.*, 2004; Símon *et al.*, 2006; Vignuzzi *et al.*, 2006). Even defective viruses, which often reduce the virulence of the virus population (e.g. Muñoz *et al.*, 1998; Zwart *et al.*, 2008), in some particular instances increase the virulence of the population when co-infecting with a helper virus (Lopez-Ferber *et al.*, 2003; Lauzon *et al.*, 2005). Here, however, we have identified a case were genetic heterogeneity is inversely correlated with disease outbreaks. The underlying causal relationship between mixed-genotype infection and disease outbreaks still needs to be unraveled, however.

There are three such causal relationships between mixed-genotype infections and pond disease statuses possible. First, WSSV genotypic composition could determine outbreak status. Under this explanation, interference between different genotypes within the host (Chao *et al.*, 2000) may explain why the virus exhibits less virulence and there are fewer outbreaks for mixed-genotype infections. Host immune responses (Venegas *et al.*, 2000; Wu *et al.*, 2002) may be the mechanism mediating this interference. The fact that the same VNTR variants were found in both outbreak and non-outbreak ponds may contradict this explanation. On the other hand, linkage between the VNTR variants and virulence loci – if these exist in the WSSV genome – may be low, as VNTR sequences are highly variable (e.g., Pradeep *et al.*, 2008a; Dieu *et al.*, 2010a). Possible virulence loci include the ORF14/15 and ORF23/24 variable regions, where deletion size may be inversely related to virulence (Marks *et al.*, 2005; Zwart *et al.*, 2010a). Second, outbreak status could determine WSSV genotypic composition. For example, there may be strong selection for certain WSSV genotypes during an outbreak, and this selection leads to the displacement of other genotypes from the virus population. Third, another factor determines both WSSV genotypic composition and outbreak status. Such a factor could be water quality, which could in principle lower the immunocompetence of shrimp while reducing the probability of water-borne WSSV transmission, and therefore the occurrence of mixed-genotype infections. Another factor that could influence both WSSV genotypic composition and

outbreak status is the prevalence of other shrimp pathogens, such as species of Vibrio bacteria (Phuoc *et al*., 2008).

To better understand how mixed-genotype infections and outbreaks are causally related will require both epidemiological field work and experimental approaches. Longitudinal epidemiological studies could estimate at what time point – prior to an outbreak – mixed-genotype infections at the individual-shrimp level are lost, and what factors are associated with this loss (e.g., water quality, the presence of other pathogens). Experimental work could address what the biological properties of mixed-genotype WSSV infections are, and whether there is interference between WSSV genotypes within the host. Mixed-genotype WSSV infections have been studied in the laboratory (Marks *et al.*, 2005; Pradeep *et al.*, 2009). Quantitative analyses of the biological properties of mixed-genotype WSSV inoculums have not been reported (e.g., Símon *et al.*, 2006; Zwart *et al.*, 2010b), and such experiments could help if there is a relationship between mixed-genotypes and virulence.

This study shows the presence of multiple WSSV genotypes in single infected shrimp, and provides evidence that mixed-genotype infections are correlated with low virulence, whereas single genotypes dominate in outbreak ponds. It is not clear how the transition from non-outbreak to outbreak ponds occurs and which environmental and viral factors influence this transition. Nevertheless, this observation provides an extra criterion to help establish the health status of a pond at an early stage and to take appropriate measures prior to an anticipated outbreak, such as boosting the defense of the shrimp by using probiotics or using vaccines (Witteveldt *et al.*, 2004).

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339 **Table 1.** Number of shrimp with mixed-genotype WSSV infections, based on disease status of sampled ponds. The total number of shrimp
340 sampled is given in parentheses, and the percentage of mixed infections is also given (% mixed).

Disease status	Locus							
	ORF75		ORF94		ORF125		All	
	Mixed (Total)	% mixed	Mixed (Total)	% mixed	Mixed (Total)	% mixed	Mixed (Total)	% mixed
non-outbreak	14 (321)	4.4	55 (321)	17.1	86 (321)	26.8	120 (321)	37.4
100% disease outbreak	2 (252)	0.8	6 (252)	2.4	22 (252)	8.7	27 (252)	10.7
Total	16 (573)	2.8	61 (573)	10.6	108 (573)	18.8	147 (573)	25.7

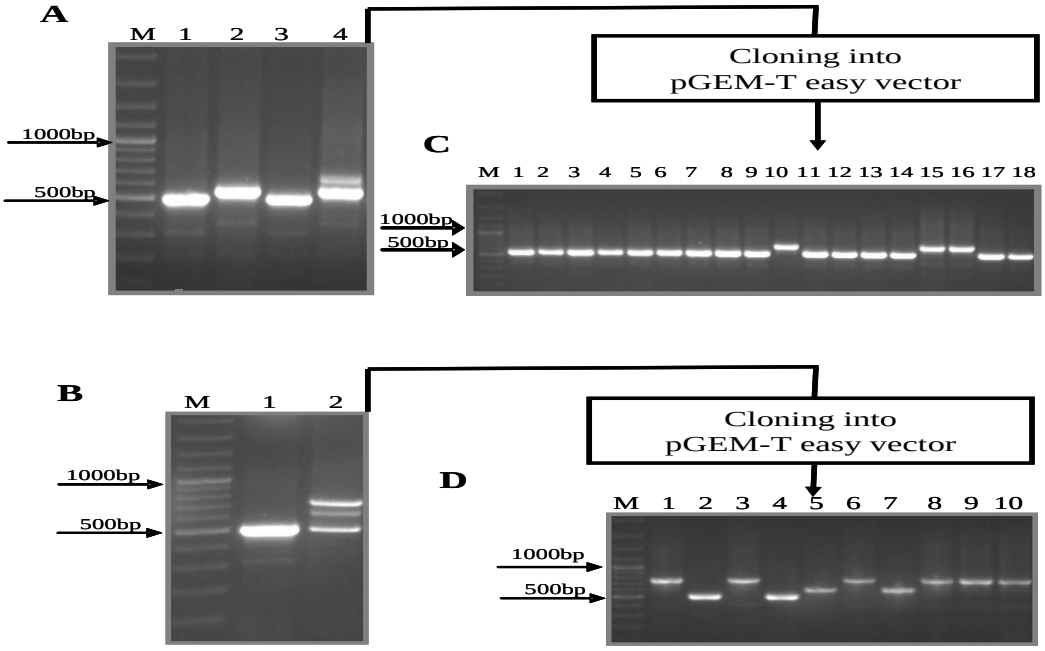
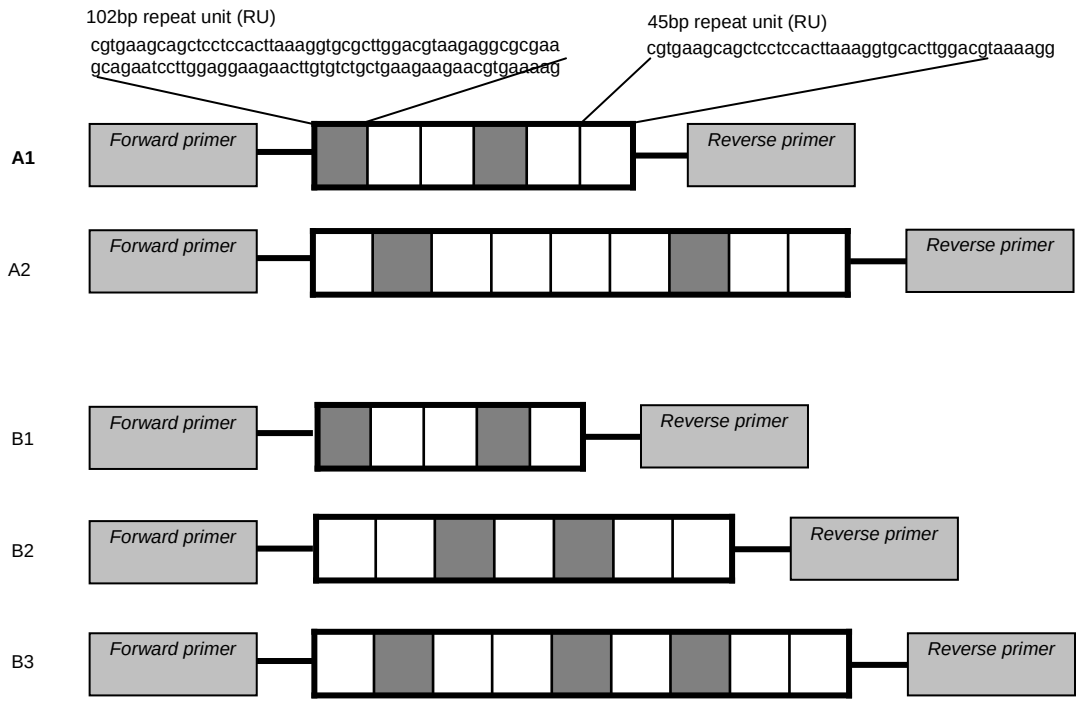


Figure 1. Electrophoresis of PCR-genotyping (ORF75). Two ORF75 PCR products were ligated into the pGEM-T easy vector (Promega) and transformed into competent *Escherichia coli* DH5α by electroporation. White colonies were selected for plasmid isolation and colony PCR with ORF75 primer. (A) PCR products (PCR genotyping of repeat sequences at ORF75) of DNA shrimp samples (lane 4) containing 2 WSSV genotypes at approximately 500bp and 650bp. (B) PCR products (PCR genotyping of repeat sequences at ORF75) of DNA shrimp samples (lane 2) containing 3 WSSV genotypes at approximately 500bp, 600bp and 700bp. (C) PCR products (PCR genotyping of repeat sequences at ORF75) of 18 colonies from cloning of PCR product contained 500bp and 650bp in PGEMT easy vector which showed individual band at approximately 500bp (lanes 1-9,11,12-14,17,18) and 650bp (lanes 10, 15, 16). (D) PCR products (PCR genotyping of repeat sequences at ORF75) of 10 colonies from cloning of PCR product contained 500bp, 600bp and 700bp in PGEMT easy vector which showed individual band at approximately 500bp (lanes 2,4), 600bp (lanes 5,7) and 700bp (lanes 1, 3, 6, 8-10).



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375 **Figure 2.** Schematic representation of difference in the number of repeat units among
376 clones A1, A2 (derived from PCR product of sample A) and clones B1, B2, B3
377 (derived from PCR product of sample B). Plasmid clones of sample A and B,
378 containing inserts of the correct size, were subjected for sequencing using universal
379 T7 and/or Sp6 primers.

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382 **Online supplementary material:**

383

384 **Table S1. Sequences of PCR primers used for WSSV genotype analysis**

Site	Primer name	Sequence (5'- 3')	Annealing temperature / Elongation time	References
ORF75	ORF75-flank-F	GAAGCAGTATCTCTAACAC	49^0C/80sec	Dieu *et al.*, 2004
	ORF75-flank-R	CAACAGGTGCGTAAAAGAAG		
ORF94	ORF94 - F	TCTACTCGAGGAGGTGACGAC	52^0C/60sec	Wongteerasupaya *et al.*, 2003
	ORF94 - R	AGCAGGTGTGTACACATTTCATG		
ORF125	Geno125 - F	ACAGTGACCACACGATAATACCA	60^0C/60sec	This study
	Geno125 - R	TCGTTCACCATATCCATTGCCCT		

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