Vol. 111: 249–257, 2014
doi: 10.3354/dao02780

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 16

Stability of the WSSV ORF94 VNTR genotype marker during passage in marine shrimp, freshwater crayfish and freshwater prawns

Nicholas Gudkovs[1], Murwantoko[2], Peter J. Walker[1,*]

[1]CSIRO Animal, Food and Health Sciences, Australian Animal Health Laboratory, 5 Portarlington Road, Geelong, Victoria 3220, Australia

[2]Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Jl. Flora Bulaksumu, Yogyakarta 55281, Indonesia

ABSTRACT: The white spot syndrome virus (WSSV) genome contains 3 variable number tandem repeat (VNTR) regions, located in open reading frame (ORF) 75, ORF94 and ORF125, which have been employed for molecular epizootiological studies. A previous report suggested that the ORF 94 VNTR is highly unstable, varying in the number of tandem repeats during single passages from shrimp to other crustaceans. As such rapid variations would have profound implications for the interpretation of molecular epizootiological data, we re-examined the stability of the ORF94 VNTR. Two WSSV isolates with different ORF94 VNTR genotypes (TRS5 and TRS7) were obtained from disease outbreaks in farmed black tiger shrimp *Penaeus monodon* in Indonesia. High titre stocks of each virus were produced by injection in specific pathogen-free (SPF) Pacific white shrimp *Litopenaeus vannamei* with filtered infected tissue extracts, and the genotypes were confirmed. Each stock (macerated tissue) was then used to feed SPF Pacific white shrimp, freshwater crayfish (*Cherax* sp.) and freshwater prawns *Macrobrachium rosenbergii* through 3 successive passages involving alternative hosts at each level. Taqman real-time PCR was conducted on samples from each group to confirm infection and quantify viral genetic loads. ORF94 VNTR genotype analysis conducted on samples from each of the 43 passage groups indicated no variations in the VNTR number in either genotype TRS5 or genotype TRS7. This finding is contrary to the previous report and suggests that ORF94 VNTR are stable during multiple passages in these crustaceans.

KEY WORDS: White spot syndrome virus · Variable number tandem repeat · Genotype · Crustacean · *Litopenaeus vannamei* · *Macrobrachium rosenbergii* · *Cherax* sp.

INTRODUCTION

White spot syndrome virus (WSSV) is an important pathogen of farmed penaeid shrimp. It is a large double-stranded DNA virus with a circular genome of approximately 300 kbp (van Hulten et al. 2001, Yang et al. 2001). Comparison of the complete genome sequences of WSSV isolates from China (WSSV-CN), Thailand (WSSV-TH) and Taiwan (WSSV-TW) has revealed >99% nucleotide sequence identity (Marks et al. 2004). However, although most genes are highly conserved, several loci have been identified in which there are variations including single nucleotide substitutions, large insertions/deletions and variation in the number of repeat sequence units within intergenic homologous regions (HRs) and in non-HR direct repetitive sequences, most of which occur within open reading frames (ORFs; Marks et al. 2004). The 3 non-HR direct repeat regions displaying the largest variation between the prototype isolates are located in ORF75, ORF94 and ORF125, and these loci have been employed for WSSV molecular epizootiological studies. Variable number tandem repeat (VNTR) genetic markers have been used to demonstrate variations in WSSV genotypes in moribund shrimp collected from different ponds during disease outbreaks

*Corresponding author: peter.walker@csiro.au

(Wongteerasupaya et al. 2003, Syed Musthaq et al. 2006, Hoa et al. 2012), distinguish WSSV genotypes in shrimp and wild crustaceans collected from the same pond (Hoa et al. 2005), identify new variant genotypes in specific geographic locations (Tang et al. 2012), identify multiple genotypes in individual diseased and healthy shrimp (Hoa et al. 2005) and detect evidence of transmission patterns amongst ponds (Walker et al. 2011a,b). Hoa et al. (2011) used a combination of all 3 VNTR markers in ORF75, ORF94 and ORF125 to study modes of transmission in semi-intensive and improved extensive farming systems and concluded that analysis based on the ORF94 VNTR only was a reasonable alternative to the triple marker approach. However, Waikhom et al. (2006) also reported that the number of repeat sequences in ORF94 can shift significantly during differential passaging through shrimp *Penaeus monodon*, crabs *Portunus sanguinolentus* and *P. pelagicus* and freshwater prawns *Macrobrachium rosenbergii*, and concluded that VNTR variations may result from host selection rather than geographic isolation. If confirmed, this could have significant implications for the interpretation of molecular epizootiological studies employing VNTR markers.

This study was undertaken to re-examine the stability of VNTR markers during sequential passage through different hosts. Two different WSSV genotypes derived from moribund black tiger shrimp *P. monodon* collected during disease outbreaks on farms in Indonesia were used to prepare a working stock of WSSV-infected tissues in specific pathogen-free (SPF) Pacific white shrimp *Litopenaeus vannamei*. These stocks were then used as a source of virus for sequential passage by feeding in Pacific white shrimp, freshwater crayfish and freshwater prawns.

MATERIALS AND METHODS

Sources of WSSV samples

Samples of moribund shrimp and swimming crabs were collected over a 2 mo period in late 2010 from suspect WSSV disease outbreaks in shrimp ponds in the area surrounding Kendal, Central Java, and Makassar, South Sulawesi, Indonesia (Table 1). The shrimp were collected whole onto ice and frozen at –80°C on arrival in the laboratory.

Table 1. Field samples collected for preparation of white spot syndrome virus (WSSV) stocks

No.	Source crustacean	Location	Comment
1	*Penaeus monodon*	Kendal, Central Java	Disease outbreak
2	*P. monodon*	Kendal, Central Java	Disease outbreak
3	*P. monodon*	Kendal, Central Java	Disease outbreak
4	*P. monodon*	Kendal, Central Java	Disease outbreak
5	*P. monodon*	Kendal, Central Java	Disease outbreak
6	*P. monodon*	Kendal, Central Java	Disease outbreak
7	*P. monodon*	Kendal, Central Java	Disease outbreak
8	*P. monodon*	Kendal, Central Java	Disease outbreak
9	*P. monodon*	Kendal, Central Java	Disease outbreak
10	*P. monodon*	Kendal, Central Java	Disease outbreak
11	*P. monodon*	Kendal, Central Java	Disease outbreak
12	Swimming crabs	Kendal, Central Java	Putrefied sample
13	*P. monodon*	Kendal, Central Java	Disease outbreak
14	*Cherax* sp.	Soekarno-Hatta, Java	Airport interception
15	*P. monodon*	Kendal, Central Java	Disease outbreak
16	*P. monodon*	Kendal, Central Java	Disease outbreak
17–19	*P. monodon*	Pangkep, South Sulawesi	Disease outbreak
20–22	*P. monodon*	Pangkep, South Sulawesi	Disease outbreak

PCR testing of WSSV samples

DNA was isolated from 20 to 30 mg of frozen tissues from pleopods and gills (shrimp) or walking legs (crabs) using Qiagen spin columns (DNeasy Blood and Tissue Kit). The suitability of DNA samples for amplification was confirmed using a conventional PCR for decapod 18S ribosomal RNA. The decapod PCR contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, with 0.2 µM of each primer (Deca-20a2: 5′-CTT CCC CCG GAA CCC AAA GAC T-3′ and Deca-20s9: 5′-GGG GGC ATT CGT ATT GCG A-3′) and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) and used 2 µl of DNA in a reaction volume of 20 µl. Cycle conditions were 1 cycle of 94°C for 2 min; 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final extension of 72°C for 5 min. The presence of WSSV in the field samples was confirmed by 1-step PCR under the same conditions using the WSSV-specific primers WSSV-1a16 (5′-TTC CAG ATA TCT GGA GAG GAA ATT CC-3′) and WSSV-1s5 (5′-CAC TCT GGC AGA ATC AGA CCA GAC CCC TGA C-3′). PCR products were analysed by electrophoresis in 1.5% (w/v) agarose gels (Fermentas) in 0.5× TBE buffer (Promega) using standard

conditions. Samples which did not yield the expected amplicon were diluted 1:10 with PCR-quality water and re-tested.

Quantitative real-time PCR

WSSV viral genetic loads in infected samples were determined by using a Taqman quantitative real-time PCR assay targeting WSSV ORF167 (Walker et al. 2011a). Primers used in this PCR were designed to target the same region as the OIE-recommended nested PCR (Lo et al. 1996, 1997) and have been validated against this assay.

Genotyping PCR

WSSV VNTR genotypes were determined by PCR amplification of the ORF94 tandem repeat region based on methods described by Walker et al. (2011a). Two primer sets were used with Qiagen HotStarTaq Master Mix. The master mixes and volumes were the same in each case, with 1.5 mM $MgCl_2$ and 0.2 µM of each primer in a total volume of 25 µl. The thermal cycling parameters were optimised for each primer pair and were as follows: Geno-WS-f1 (5′-TAT TGA CCC CGA CCA CCG CTG C-3′) and Geno-WS-r1 (5′-TCC GCC TCT GCC CAC GCA TTG A-3′) were subjected to 1 cycle of 94°C for 15 min; 40 cycles of 94°C for 20 s, 66°C for 20 s and 72°C for 90 s; and a final extension of 72°C for 10 min. The second primer set WS-94f (5′-CGA TGA CGA TGG AGG AAC T-3′) and WS-94r (5′-TCT TCA ACC ACC GTC ACT-3′) was cycled at 94°C for 15 min; followed by 40 cycles of 94°C for 20 s, 62°C for 20 s and 72°C for 75 s; with a final extension of 72°C for 10 min. Following electrophoresis, the number of tandem repeat sequences (TRSs) was calculated by estimating the size of PCR products relative to a 100 bp DNA ladder (Promega) and comparing these to the expected size of amplicons obtained with each primer pair as described by Walker et al. (2011a).

Preparation of WSSV inocula

Gill and muscle tissue dissected aseptically from selected shrimp was placed onto a Petri dish on ice and macerated using a sterile scalpel blade. Macerated tissue suspended 1:10 (w/v) in sterile phosphate-buffered saline was then clarified by centrifugation at 8000 × *g* (10 min) and passed through a 0.20 µm membrane filter (Sartorius). To infect SPF *Litopenaeus vannamei*, 50 µl of inoculum was injected into muscle at the second abdominal segment using a 1 ml disposable syringe fitted with a 25-gauge needle. Groups of 15 shrimp injected with each inoculum were then transferred to 20 l glass aquaria and monitored every 3 to 4 h for signs of disease. Dead or moribund animals were removed from the tank, chopped into small pieces and frozen in batches at −80°C.

WSSV passage in marine shrimp, freshwater crayfish and freshwater prawns

Clinically normal marine shrimp *L. vannamei*, freshwater crayfish (*Cherax* sp.) and freshwater prawns *Macrobrachium rosenbergii* were obtained from commercial producers in West Java and acclimated for 5 to 7 d in 100 l fibreglass tanks in the bio-secure aquarium facility in the Indonesian Centre for Fish Disease and Environment Laboratory, Serang. Tissue sampled from representative individuals of each species was tested by PCR to confirm freedom from WSSV.

The experimental design for sequential passage through different species is shown in Fig. 1. In order to provide sufficient material for subsequent feeding steps, 10 animals were used for Passage 1. For subsequent Passages 2 and 3, only 3 animals were used for each feeding group. When required, animals were transferred from the large holding tanks to 20 l glass aquaria for feeding. For animals found dead or moribund, or animals showing signs of disease, gills and pleopods were preserved in 80% (v/v) ethanol for PCR, with the remaining tissues used for feeding in the next passage step.

RESULTS

PCR testing of WSSV field samples and selection of genotypes

A total of 22 samples derived from 20 farmed shrimp (*Penaeus monodon*), 1 swimming crab and 1 freshwater crayfish (*Cherax* sp.) were tested for WSSV DNA by 1-step conventional PCR (Fig. 2). All samples displayed a strong band of approximately 200 bp after a single round of PCR amplification, suggesting that all contained high viral genetic loads. Sample 12 (swimming crab from Central Java) was found to be putrid when thawed for DNA isolation

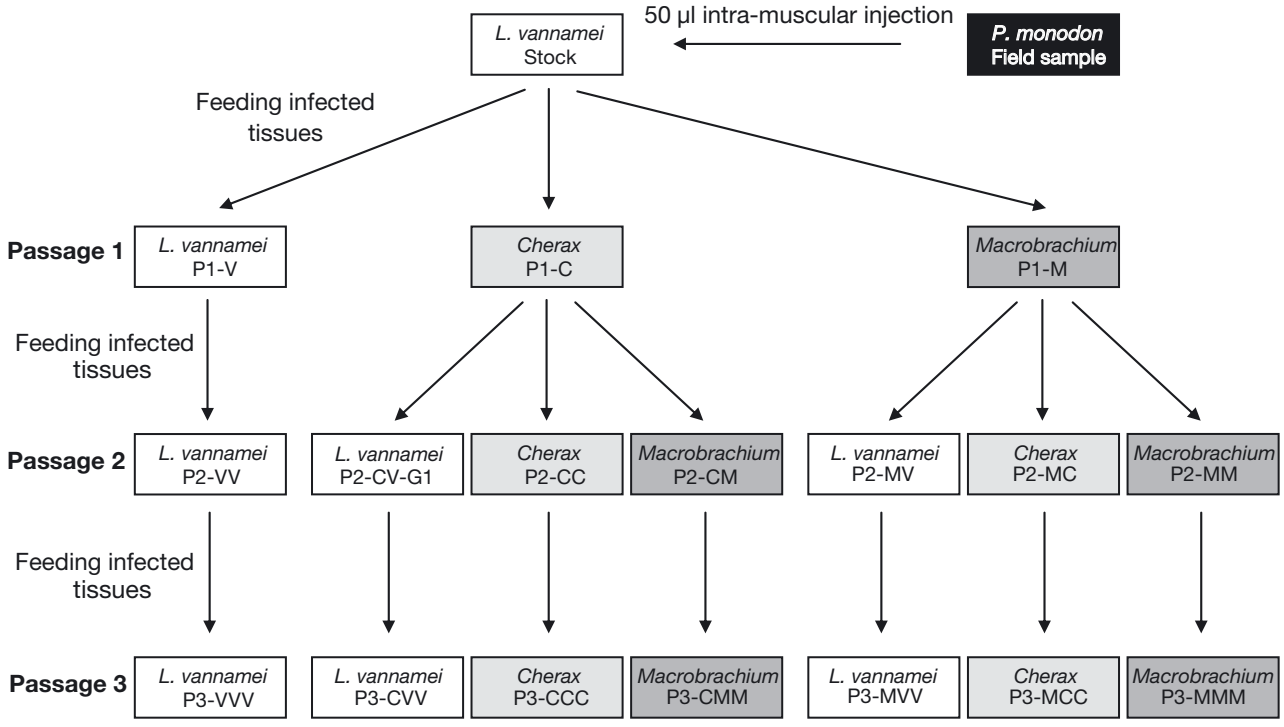

Fig. 1. Flow chart of the passage history of white spot syndrome virus isolates representing genotypes TRS5 and TRS7. Stock viruses were prepared in marine shrimp *Litopenaeus vannamei* by intra-muscular injection of the selected field samples. Subsequent passages in *L. vannamei* (V), crayfish *Cherax* sp. (C) or freshwater prawns *Macrobrachium rosenbergii* (M) were conducted by feeding on infected tissues

but still produced a clear positive result. Sufficient WSSV DNA was present to conduct WSSV ORF94 VNTR genotyping PCR analysis on all 22 samples (Fig. 3). Multiple bands were observed in most samples, suggesting mixed infections with several genotypes. Six samples were selected for further analysis using nested PCR primers WS-94f / WS-94r: samples 13 (*P. monodon*, Central Java) displaying genotype TRS7; samples 5 and 6 (*P. monodon*, Central Java) displaying genotype TRS8; sample 12 (swimming crab, Central Java) displaying genotype TRS11; and samples 17 and 18 (*P. monodon*, South Sulawesi) displaying genotype TRS5. Samples 12, 13 and 17 appeared to contain bands indicative of single genotypes (Fig. 4) and so these were selected for passaging experiments.

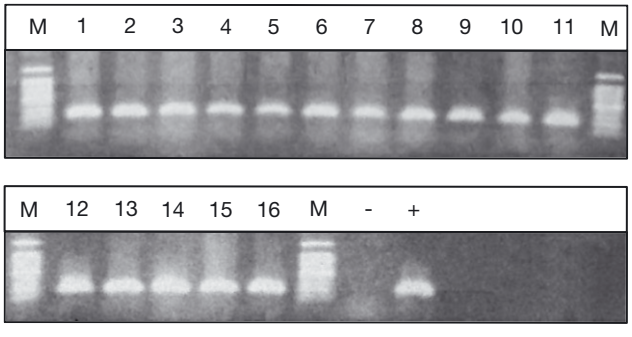

Fig. 2. Conventional 1-step (non-nested) white spot syndrome virus PCR testing of 16 of 22 shrimp, crab and freshwater crayfish samples collected from Indonesia. The samples are numbered as in Table 1. Lane M: 100 bp DNA ladder (Promega), (+) positive control, (–) negative control

Preparation of WSSV stocks

Three separate batches of 15 shrimp (*Litopenaeus vannamei*) were inoculated with clarified supernatants prepared from samples 12, 13 and 17. None of the shrimp in the group inoculated with sample 12 (TRS11; crab) showed any signs of disease, and all shrimp were euthanized 5 d post-inoculation. Sample 12 had undergone significant putrefaction before freezing, and it was concluded that the virus in this sample was no longer viable despite evidence of high WSSV genetic load by PCR. All shrimp inoculated with sample 13 (TRS7) were severely affected post-inoculation and all died or became moribund within 48 h post-inoculation. All shrimp in the group inocu-

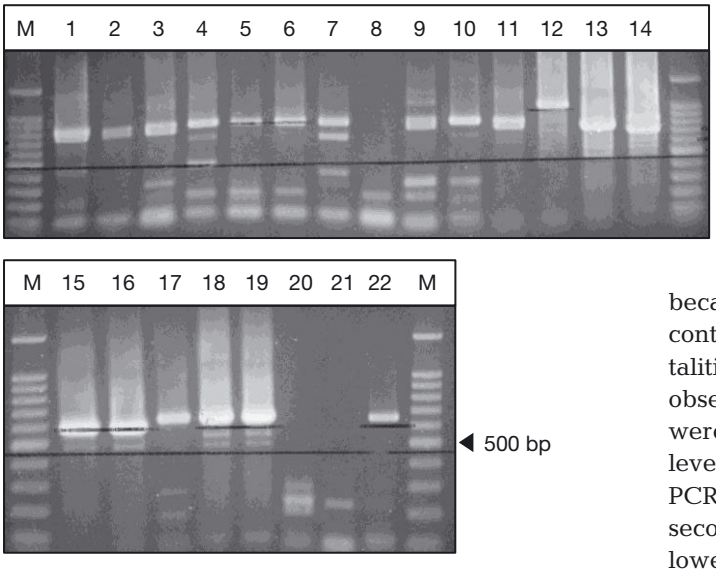

Fig. 3. One-step (non-nested) genotyping PCR (primers Geno-WS-f1 and Geno-WS-r1) testing of 22 white spot syndrome virus-infected shrimp, crab and freshwater crayfish samples collected from Indonesia. The samples are numbered as in Table 1. Lane M: 100 bp DNA ladder (Promega). Lines have been drawn on the image to facilitate estimation of tandem repeat sequence sizes with reference to the 100 bp DNA ladder

lated with sample 17 (TRS5) were similarly affected; although the onset of disease was slower, all animals were found dead or became moribund within 72 h post-inoculation. Shrimp tissues were collected from each of these 2 groups, cut into small pieces and pooled as stocks for the first transmission trial. Subsequent real-time PCR analysis verified high WSSV genetic loads in each of these stocks (Fig. 5).

WSSV passage in shrimp, freshwater crayfish and freshwater prawns

Sequential feeding of shrimp (*L. vannamei*), freshwater crayfish (*Cherax* sp.) and freshwater prawns (*Macrobrachium rosenbergii*) was conducted over 3 passages using the stocks of samples 13 and 17 prepared in *L. vannamei*. The passaging schedule is shown in Fig. 1. As additional animals were available, the WSSV TRS5 passage series commencing at Passage 1 in *M. rosenbergii* was performed in duplicate. Over the course of the trial, *L. vannamei* were most susceptible to WSSV infection. In all 3 passages by feeding with each genotype, all *L. vannamei* became moribund or died within 5 d of infection and contained high WSSV genetic loads. In contrast, mortalities in *Cherax* sp. and *M. rosenbergii* were only observed during the first passage when all animals were fed with the *L. vannamei* stocks containing high levels of WSSV. Although WSSV was detected by PCR in *Cherax* sp. and *M. rosenbergii* following the second and third passages, viral genetic loads were lower and few moribund animals were observed; most animals remained clinically normal. ORF94 VNTR genotype analysis was conducted on samples from each passage group using the 1-step genotyping PCR. As summarized in Fig. 5 and illustrated in Fig. 6, no variations in the VNTR number were observed in either genotype TRS5 or genotype TRS7 during sequential passage in the hosts examined.

DISCUSSION

VNTR regions of the WSSV genome are, by definition, variable, and it is this characteristic that makes them potentially useful as markers of virus strains for epidemiological studies. Conversely, use of VNTR markers to track virus strains is critically dependent upon their relative stability during the time frame of the intended study. Waikhom et al. (2006) suggested that the WSSV ORF94 VNTR is highly unstable, shifting upwards or downwards in the number of tandem repeats, during a single passage in shrimp (*Penaeus monodon*), or from shrimp to crabs (*Portunus pelagicus*) or freshwater prawns (*Macrobrachium rosenbergii*). If confirmed, this degree of instability would

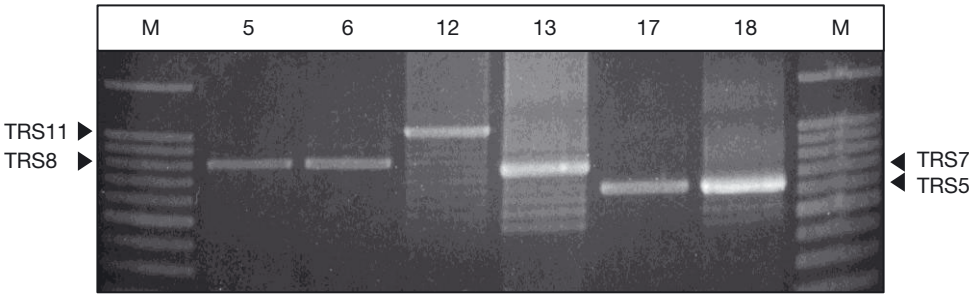

Fig. 4. Nested genotyping PCR testing of 6 white spot syndrome virus-infected shrimp and crab samples which appeared to contain a single genotype. The samples are numbered as in Table 1. Lane M: 100 bp DNA ladder (Promega). Tandem repeat sequence (TRS) genotypes based on the size of amplified bands relative to the DNA ladder are indicated

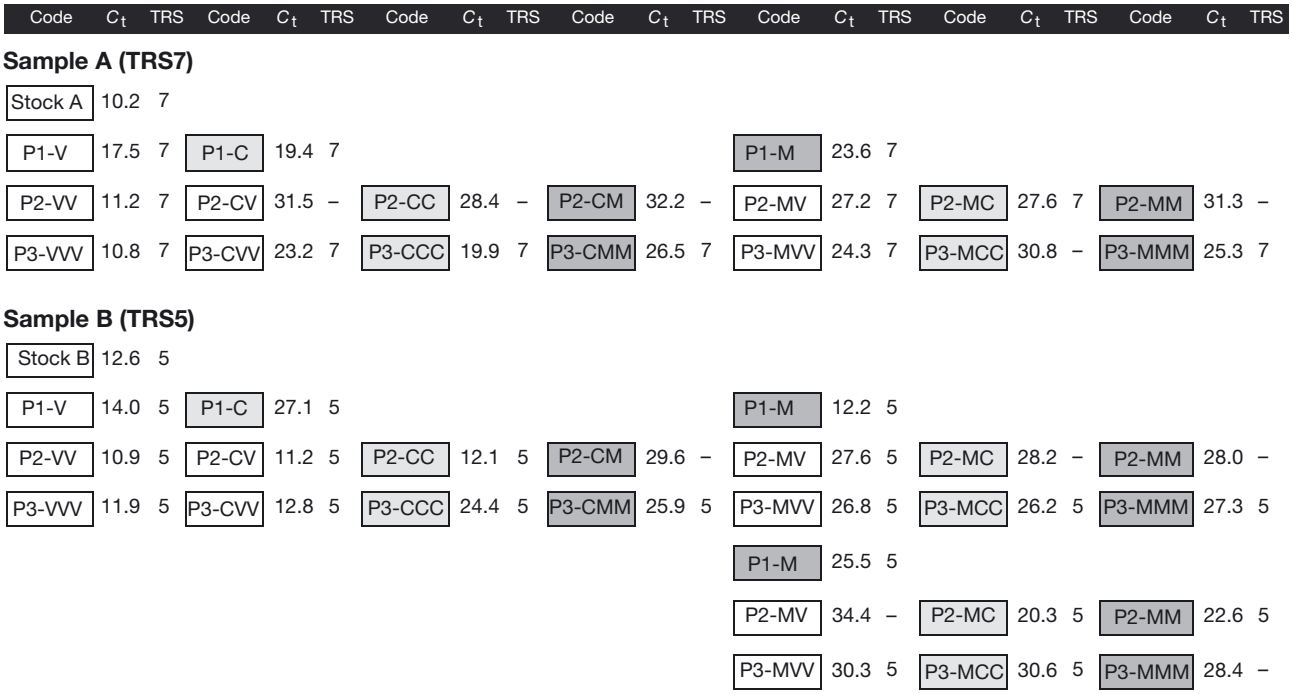

Fig. 5. Viral genetic loads (C_t values estimated by real-time PCR) and tandem repeat sequence (TRS) genotypes of virus stock A (TRS7) and stock B (TRS5), and samples of *Litopenaeus vannamei* shrimp (V), *Cherax* sp. freshwater crayfish (C) and *Macrobrachium rosenbergii* freshwater prawns (M) collected at each sequential passage level (P1, P2 and P3). Codes show the passage level and passage history. Dashes in the TRS columns: no amplification of a product

seriously affect the interpretation of VNTR-based epidemiological data, rendering them of little practical value for most purposes.

The study described in this paper has re-investigated the stability of the WSSV ORF94 VNTR over several passages in shrimp (*Litopenaeus vannamei*), crayfish (*Cherax* sp.) and freshwater prawns (*M. rosenbergii*). Using field isolates of WSSV from *P. monodon* collected from 2 locations in Indonesia, we prepared high titre stocks in *L. vannamei* which retained the ORF94 VNTR genotypes (TRS5 and TRS7) of the original virus isolates. Using these stocks, we conducted experimental infections by feeding macerated infected tissues and demonstrated no changes in the TRS genotypes during 3 sequential passages in *L. vannamei* or various combinations of alternate passage in *L. vannamei*, *Cherax* sp. or in *M. rosenbergii*. In total, 43 passages were performed from the original isolates with no observed variations in the TRS genotypes.

This result is clearly inconsistent with the TRS variability reported by Waikhom et al. (2006). Although the reasons for this are not immediately evident, we make the following observations. Firstly, in our study we used high titre stocks of WSSV (threshold cycle number [C_t] values of 10.2 and 12.6, respectively) as the source material for the first passage in each of the target hosts. During 3 sequential passages in *L. vannamei*, high viral genetic loads were maintained (C_t values between 10.8 and 17.5), and this was consistent with the observation that all animals were dead or moribund within 5 d of exposure to the virus. During alternate passaging of each stock in shrimp, crayfish or freshwater prawns, viral genetic loads were generally moderate to low (Fig. 5), and there were few mortalities in most groups. In contrast, Waikhom et al. (2006) commenced with 4 stocks of WSSV from *P. monodon* collected from various sites in India, of which 3 isolates (A117, G9 and G27) contained high levels of virus (generated PCR products using a 1-step PCR) and 1 isolate (A166) contained a relatively low level of virus (generated a product only by nested PCR). However, following passage in *P. monodon*, isolates A117, A166 and G9 were generally detected only by nested PCR despite high levels of mortality. Similarly, other crustaceans infected with these isolates developed infections that could be detected only by nested PCR and, in several cases, by only 1 of 2 nested PCR tests that were employed. Indeed, crabs (*P. pelagicus*) infected with isolate A117 were reported to have developed 100% mortality within 9 d, but WSSV could be detected only by

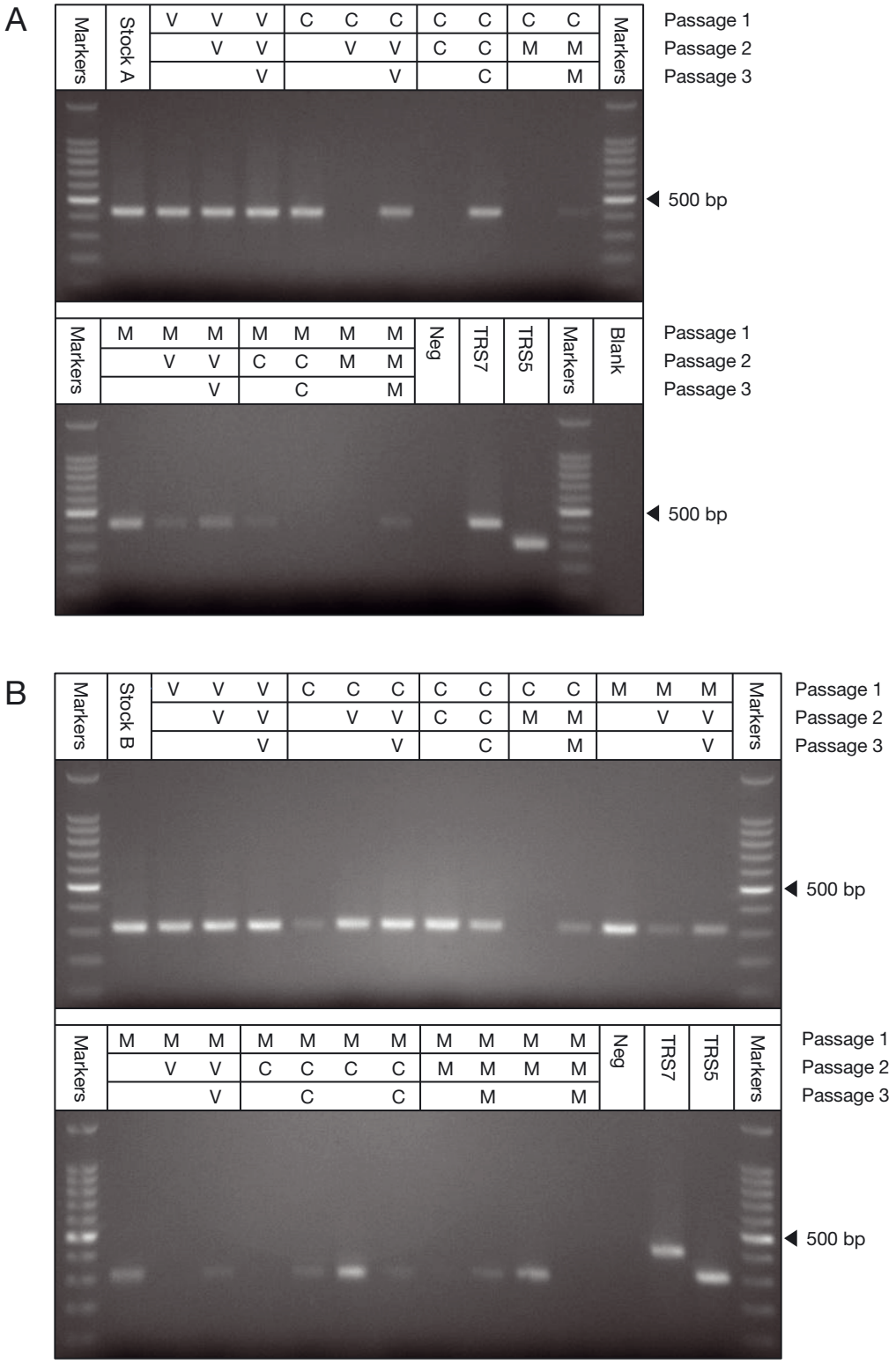

Fig. 6. One-step (non-nested) genotyping PCR (primers WS-94f / WS-94r) testing conducted on (A) virus stock A (TRS7) and (B) virus stock B (TRS5), and corresponding samples of *Litopenaeus vannamei* shrimp (V), *Cherax* sp. freshwater crayfish (C) and *Macrobrachium rosenbergii* freshwater prawns (M) collected at each sequential passage level. Codes show the passage level and passage history. Neg: negative control PCR; TRS7 and TRS5: positive controls; Markers: 100 bp DNA ladder (Promega), with 500 bp DNA marker indicated

nested PCR, and *P. monodon* subsequently infected with crab meat from these animals failed to develop mortalities. Mortalities due to white spot disease are generally associated with high levels of virus in the tissues of affected animals, and so the data presented by Waikhom et al. (2006) appear to be somewhat irregular with respect to the potency of the virus inocula and cause of mortalities. Furthermore, as low-dose WSSV infections do not necessarily result in disease and as the virus inocula used by Waikhom et al. (2006) were not quantified or standardised, interpretation with respect to changes in pathogenicity on passage should be considered cautiously.

This also raises some questions about the observations of genotype instability reported by Waikhom et al. (2006). Unlike our study in which a 1-step PCR was used for all genotyping, most of the analyses conducted by Waikhom et al. (2006) required a nested PCR due to the presence of very low levels of WSSV. Amongst the samples analysed, 4 variations in genotype were reported. Isolate A117 (TRS12) was reported to change to TRS18 when passaged once in shrimp (*P. monodon*) and to TRS8 after passage once in crabs (*P. pelagicus*). Surprisingly, the transformation of genotypes appears to have been complete in these animals as no samples displaying multiple genotypes are reported, implying that each shift in TRS number conferred significant selective advantage with the loss of the parent strain. As discussed previously, inoculum A117 displayed irregular characteristics, particularly when passaged in crabs, and the PCR amplicons representing each of these variable TRS genotypes appeared only as very weak bands, without any indication of the parental strains from which they were derived. Similarly, isolate G9 (TRS9) was reported to change to TRS12 when passaged in freshwater prawns (*M. rosenbergii*), and isolate G27 (TRS9) was reported to change to TRS8 when passaged in crabs (*P. pelagicus*), with each of these changes represented as very weak bands detected by nested PCR. As only random screening for pre-existing WSSV infections was reported, these data might be explained by pre-existing low-level WSSV infections in the animals used for experimental infections. Furthermore, as the PCR data indicated limited WSSV replication following experimental infections of these animals, apparent TRS variations may have been due to clonal selection in some animals of undetected secondary genotypes in the inocula. Mixed infection with 2 or more WSSV genotypes has been reported to occur commonly in shrimp and other crustaceans collected from farms in India and Vietnam (Hoa et al. 2005, Walker et al. 2011b) and was also evident amongst samples collected for this study from farms in Indonesia (Fig. 3).

The evolution of a new VNTR genotype during WSSV infection would require 2 independent events to occur. Firstly, a new genome containing a variant number of tandem repeats (in the ORF94 region) must be generated during replication. This must be a stochastic event that occurs as a consequence of duplication, deletion or recombination. Secondly, the virus with the variant genome must have a selective advantage over the existing virus in order to replace it as the dominant genotype. The observation that this can occur during a single passage in an alternative host, as reported by Waikhom et al. (2006), implies either that variant genotypes were already present at a low levels in the infecting virus population or that variants are generated with high frequency during replication and so are available for selection. The observation also implies that the VNTR region has a function that influences replication efficiency differentially in different host species, providing the variant virus with a selective advantage. Indeed, that the new genotype could completely replace the existing genotype in a single passage suggests that this selective advantage must be considerable. Alternatively, if new WSSV VNTR genotypes arise over much longer time frames, their selection may be driven not by the VNTR region itself, but by other mutations which arise in the genome and increase replication efficiency. Superficially at least, the latter possibility appears to be more plausible. However, we cannot exclude the possibility that different WSSV strains have higher rates of variation in the ORF94 VNTR region or that different host species, as employed in the experiments of Waikhom et al. (2006), offer a stronger selective advantage than others.

In conclusion, the data presented here indicate that the WSSV ORF94 VNTR can remain stable during sequential passages in shrimp and alternating passages in shrimp and other susceptible crustaceans. The reasons for the discrepancy with a previous observation that genotype variations occur rapidly through host species selection are yet to be clearly established.

Acknowledgements. This work was conducted as part of an international cooperative project sponsored by the Australian Centre for International Agricultural Research (ACIAR). We acknowledge the generous assistance of Dr. Isti Koesharyani, Research Centre for Aquaculture, Jakarta, Indonesia, the technical staff at the Indonesian Centre for Fish Disease and Environment Laboratory, Serang, and Dr. Richard Callinan, University of Sydney.

LITERATURE CITED

Hoa T, Hodgson R, Oanh D, Phuong N, Preston N, Walker P (2005) Genotypic variations in tandem repeat DNA segments between ribonucleotide reductase subunit genes of white spot syndrome virus (WSSV) isolates from Vietnam. In: Walker P, Lester R, Reantaso M (eds) Diseases in Asian aquaculture, Book V. Asian Fisheries Society, Manila, p 339–351

Hoa TTT, Zwart MP, Phuong NT, Vlak JM, de Jong MCM (2011) Transmission of white spot syndrome virus in improved-extensive and semi-intensive shrimp production systems: a molecular epidemiology study. Aquaculture 313:7–14

Hoa TTT, Zwart MP, Phuong NT, de Jong MCM, Vlak JM (2012) Low numbers of repeat units in variable number of tandem repeats (VNTR) regions of white spot syndrome virus are correlated with disease outbreaks. J Fish Dis 35:817–826

Lo CF, Leu JH, Ho CH, Chen CH and others (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25:133–141

Lo CF, Ho CH, Chen CH, Liu KF and others (1997) Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 30:53–72

Marks H, Goldbach RW, Vlak JM, van Hulten MC (2004) Genetic variation among isolates of white spot syndrome virus. Arch Virol 149:673–697

Syed Musthaq S, Sudhakaran R, Ishaq Ahmed VP, Balasubramanian G, Sahul Hameed AS (2006) Variability in the tandem repetitive DNA sequences of white spot syndrome virus (WSSV) genome and suitability of VP28 gene to detect different isolates of WSSV from India. Aquaculture 256:34–41

Tang KFJ, Navarro SA, Pantoja CR, Aranguren FL, Lightner DV (2012) New genotypes of white spot syndrome virus (WSSV) and Taura syndrome virus (TSV) from the Kingdom of Saudi Arabia. Dis Aquat Org 99:179–185

van Hulten MCW, Witteveldt J, Peters S, Kloosterboer N and others (2001) The white spot syndrome virus DNA genome sequence. Virology 286:7–22

Waikhom G, John KR, George MR, Jeyaseelan MJP (2006) Differential host passaging alters pathogenicity and induces genomic variation in white spot syndrome virus. Aquaculture 261:54–63

Walker PJ, Gudkovs N, Mohan CV, Stalin Raj V and others (2011a) Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. II. Multiple WSSV genotypes associated with disease outbreaks in ponds seeded with uninfected postlarvae. Aquaculture 319:18–24

Walker PJ, Gudkovs N, Pradeep B, Stalin Raj V and others (2011b) Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. III. A complex dynamic of WSSV infection and WSSV genotype distribution in farmed shrimp and wild crustaceans. Aquaculture 319:319–327

Wongteerasupaya C, Pungchai P, Withyachumnarnkul B, Boonsaeng V, Panyim S, Flegel TW, Walker PJ (2003) High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. Dis Aquat Org 54:253–257

Yang F, He J, Lin XH, Li Q, Pan D, Zhang XB, Xu X (2001) Complete genome sequence of the shrimp white spot bacilliform virus. J Virol 75:11811–11820

Editorial responsibility: Jeff Cowley, Brisbane, Queensland, Australia

Submitted: March 3, 2014; Accepted: June 16, 2014
Proofs received from author(s): September 29, 2014