Genetic and phenotypic variations of isolates of shrimp Taura syndrome virus found in *Penaeus monodon* and *Metapenaeus ensis* in Taiwan

Yun-Shiang Chang,1† Shao-En Peng,1† Hon-Tsen Yu,1 Feng-Chuan Liu,1 Chung-Hsiung Wang,2 Chu-Fang Lo1 and Guang-Hsiung Kou1

1Institute of Zoology, National Taiwan University, Taipei 106, Taiwan, Republic of China
2Department of Entomology, National Taiwan University, Taipei 106, Taiwan, Republic of China

Distinct Taura syndrome virus (TSV) isolates were found in *Metapenaeus ensis* (isolate Tw2KMeTSV), *Penaeus monodon* (isolate Tw2KPmTSV) and *Litopenaeus vannamei* (isolate Tw02LvTSV). Nucleotide sequence analysis of these three isolates revealed differences in the TSV structural protein (capsid protein precursor) gene orf2. TSV ORF2 amino acid sequence comparison and phylogenetic analysis suggested a comparatively close relationship between these three Taiwanese isolates and the Hawaiian isolate HI94TSV. In *P. monodon* specimens that were naturally and experimentally infected with the Tw2KpTSV isolate, the virus was contained and shrimps showed no clinical signs of infection. However, when *P. monodon* was challenged with the Tw2KMeTSV isolate, the virus replicated freely. The ORF2 amino acid sequence of the Tw2KMeTSV isolate differed from that of isolate Tw2KPmTSV in four positions and these differences may account for their phenotypic differences, at least in terms of their ability to replicate in specific hosts.

In this study, TSV-positive samples from three sources were collected. The first sample, a *P. monodon* brooder (Tw2KPm), was selected by screening the gills and pleopods of a batch of 24 brooders that were captured from southern Taiwan coastal waters in 2000. These brooders were frozen, transported to our laboratory and stored at −70°C. The sample brooder was used for TSV tissue-distribution analysis and to prepare a virus inoculum (see Supplementary Table, available in JGV Online, for the names and sources of the inocula used in this study). The second sample was from a batch of live, cultured *M. ensis* that were bought from a local fish market in 2000 and showed visible signs of Taura syndrome. The *M. ensis* specimen studied here (Tw2KMe) died 2 days after arrival in the laboratory, at which time its gills and two of its pleopods were excised and analysed for virus infection, while the rest of the body was stored at −70°C until used as above. For the third sample, two pleopods were taken from an *L. vannamei* shrimp (Tw02Lv) collected in 2002 from a culture pond in southern Taiwan in which there had recently been an outbreak of TSV. This sample was used for sequence comparison only.

Total RNA of tested specimens was extracted by using TRIzol reagent (Invitrogen). First-strand cDNAs of these RNAs were synthesized by using an oligo-dT primer (Roche) and Superscript II reverse transcriptase (Invitrogen). A nested (two-step) PCR using three TSV-specific primers for amplifying cDNA in a region of the TSV orf2 gene was used to confirm TSV infection. The primers for amplifying cDNA

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1These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AF277675; Mari et al., 2002

A table showing abbreviations and descriptions of TSV isolates used in this study is available as supplementary material in JGV Online.

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TSVF2 (5′-ACCCCAGAAATGTGAATATAAACC-3′) and TSVR2 (5′-GGAAAAAGCAATGTCAAATACC-3′) served as the outer primer pair for the first PCR step and the primer TSVF3 (5′-ATACTTAGCACAGGGCACCATA-3′) combined with TSVR2 served as the inner primers for the nested amplification. Amplicons resulting from TSVF2/TSVR2 and TSVF3/TSVR2 were 910 and 360 bp in length, respectively. The cDNA from 0.1 μg total RNA was subjected to PCR in a 50 μl reaction. Plasmids containing TSV orf2 cDNA were diluted serially to estimate the PCR sensitivities of one-step and nested amplification. cDNAs from the testis of TSV-challenged P. monodon and from the pleopods of the other tested individuals were also analysed by real-time RT-PCR following the method of Dhar et al. (2002).

Two cultured, TSV-free P. monodon shrimps (body mass approx. 40 g) were challenged experimentally by intramuscular injection with Tw2KpTSV or Tw2KmTSV virus inoculum prepared from Tw2Kp and the Tw2Km, respectively. Methods for inoculum preparation and injection challenge were as described by Lightner (1996), except that the Tw2KpTSV inoculum was concentrated 10-fold. At the end of the experimental period (51 h post-infection), various tissues/organs from the two challenged individuals were screened for TSV.

To demonstrate more rigorously the different replication abilities of Tw2KmTSV and Tw2KpTSV, a batch of specific pathogen-free (SPF) Marsupenaeus japonicus juveniles (body mass approx. 1–2 g) was used (kindly provided by Dr C.-M. Kuo, Marine Research Station, Academia Sinica, Taiwan). These shrimp were specifically free of TSV, white spot syndrome virus (WSSV), yellow head virus (YHV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV). Before challenge, the inoculum prepared from either Tw2Km or Tw2Kp was pre-treated with chloroform to exclude any possible low-level contamination of WSSV and then diluted to contain approximately 300 TSV genome copies in a volume of 30 μl as determined by real-time RT-PCR. Challenge was by intramuscular injection. Four shrimps were sampled from each group at 72 h post-infection and screened for TSV, WSSV, IHHNV and YHV.

For TSV genomic sequence analysis, cDNA products from the pleopods of Tw2Kp, Tw2Km and Tw02Lv were subjected to PCR using Pfu DNA polymerase (Promega). The entire orf2 gene of these three isolates was amplified and sequenced. Multiple nucleotide sequence alignments of the entire length of the TSV orf2 gene of these and three other TSV isolates (see Supplementary Table in JGV Online) were analysed by CLUSTAL X (Thompson et al., 1997) and GeneDoc (Nicholas et al., 1997). Multiple alignments of the ORF2 amino acid sequences of all six isolates were used for phylogenetic analysis based on the neighbour-joining and maximum-parsimony (MP) methods of the PAUP 4.0b1 program (Swofford, 1998). One thousand bootstrap replicates were generated to test the robustness of the trees.

Fig. 1(a) shows the calibration results for the nested TSV RT-PCR protocol that was used in this study. In this PCR test, a sample that is positive in the first amplification must have a viral load that is at least 10-fold greater than a sample that is positive only in the second (nested) step. The 24 P. monodon brooders that were screened had a low TSV prevalence rate, with only two specimens testing positive for TSV by nested PCR (Fig. 1b). This was comparable to the positive result for one-step PCR for Tw2Km (Fig. 1b) and all of the other randomly tested M. ensis specimens (data not shown). Tissue-distribution results for the naturally infected Tw2Kpm showed a low level of infection in all of the organs tested (Fig. 1c), compared with positive results for one-step PCR for each organ in the Tw2Km specimen (Fig. 1d). An adult P. monodon shrimp that was challenged with concentrated Tw2KpTSV was positive for nested PCR in only two tested organs (Fig. 1e). Challenge with Tw2KmTSV, however, produced positive results for nested PCR in all organs and positive results for one-step PCR in some (Fig. 1f). These preliminary data suggested that in P. monodon (a species in which TSV is normally found only at very low levels), the Tw2KmTSV isolate was able to replicate much more freely than the Tw2KpTSV isolate. In an additional, more rigorous demonstration of the difference in replication ability between Tw2KpTSV and Tw2KmTSV, challenge tests were performed on SPF M. japonicus juveniles with Tw2KmTSV (Fig. 1g, lanes 1–4) virus loads (revealed by real-time RT-PCR) of 1.2 × 10^6 copies in 0.1 μg total RNA. In the Tw2KpTSV-challenged group (Fig. 1g, lanes 5–8), virus loads were 33–100 copies in 0.1 μg total RNA. WSSV, YHV and IHHNV were not detected in any of these challenged M. japonicus (data not shown). The results reconfirmed that these two isolates replicate differently in intramuscularly challenged shrimp.

Having shown a difference in replication ability, we next proceeded to sequence the entire orf2 gene of the three isolates Tw2KpTSV, Tw2KmTSV and Tw02LvTSV. A multiple alignment of the hypervariable region (Erickson et al., 2002; Robles-Sikisaka et al., 2002) in orf2 is shown in Fig. 2. The entire ORF2 protein was used to construct the MP tree (Fig. 3), which revealed a well-supported clade of the four isolates from Taiwan, regardless of the host species. The Taiwanese clade itself formed a trichotomy with two isolates from L. vannamei – one from Hawaii and the other from Mexico. In Hawaii, L. vannamei is a cultured species that was introduced from its native habitats in Central and/or South America, whereas in Taiwan, L. vannamei was introduced not only from Hawaii and Ecuador, but probably also from other areas. Bearing this in mind, the existence of two subclades within the Taiwanese clade suggested that there may have been two separate introductions of TSV into Taiwan, presumably as a result of repeated restocking by the culture industry. An alternative
Fig. 1. TSV screening and tissue distribution as revealed by RT-PCR. (a) Calibration using serial dilutions of plasmid DNA containing the TSV partial orf2 region cDNA sequence and showing the sensitivity of one-step (top panel) and nested (lower panel) TSV RT-PCR. (b) TSV screening results for the gills and pleopods of the M. ensis (Tw2KMe) and two P. monodon (Tw2Kpm and Pm12-11) specimens. (c–f) TSV tissue distribution in naturally infected P. monodon shrimp (Tw2Kpm) (c), naturally infected M. ensis shrimp (Tw2KMe) (d), P. monodon shrimp challenged with Tw2KpmTSV (e) and P. monodon shrimp challenged with Tw2KMeTSV (f). (g) M. japonicus shrimp challenged with Tw2KMeTSV (lanes 1–4) and Tw2KpmTSV (lanes 5–8). M, pGEM DNA marker (Promega).
Fig. 2. (a) Schematic diagram of TSV showing the region sequenced in the present study (3036 nt). Vertical arrows indicate the relative positions of amino acids that differ from the Hawaiian isolate. The lower bar represents the region used here for multiple alignments (nt 1801–2600). (b) Nucleotide sequence alignment of the hypervariable region of the TSV orf2 cDNA from different viral isolates. For reference, the HindIII, Sau3AI and TaqI restriction enzyme recognition sites are also shown.
explanation may be that after being subjected to a new environment, rapid mutation in the virus produced some adaptive local substrains that facilitated infection of new hosts, such as *P. monodon* and *M. ensis*. The amino acid variations at positions 201, 408, 413, 560, 696, 713, 720, 729 and 785 may act as genetic markers of Taiwanese TSV isolates, particularly the variants at 201F and 560H, which have also been found in two other Taiwanese isolates (Tw1 and Tw2; Robles-Sikisaka *et al.*, 2002). As for the supposed host shift from *L. vannamei* to *P. monodon* and *M. ensis*, we also noted that changes at positions 97 (E→D), 598 (V→I), 710 (A→V), 768 (E→D) and 803 (L→F) occurred in the branches of the Tw2KpmTSV and Tw2KMeTSV isolates from Taiwan. Analysis of these sites may shed light on the causes of the shift between hosts. Meanwhile, our results have revealed two new natural TSV hosts, *P. monodon* and *M. ensis*, from which two distinct TSV isolates have been identified. These isolates differed both genetically and phenotypically.

**Acknowledgements**

This research was supported financially by the Council of Agriculture (grant nos 92AS-4.2.3-FA-F1[Z]-15 and 93AS-2.2.2-FA-F1-14). We are indebted to Mr Paul Barlow for his helpful criticism of the manuscript.

**References**


