Short Communication

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

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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 Tw2KPmTSV 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.

The genome of Taura syndrome virus (TSV; Jimenez, 1992; Lightner *et al.*, 1995) has been sequenced completely and consists of a linear, positive-sense, single-stranded RNA of 10 205 nt (GenBank accession no. AF277675; Mari *et al.*, 2002) and includes two large ORFs (ORF1 and ORF2). The predicted product of ORF1 contains sequence motifs of non-structural proteins, whereas ORF2 includes genes for the TSV capsid proteins VP1, VP2 and VP3 (Mari *et al.*, 2002). As recently as 1998, TSV was still considered to be a western-hemisphere virus (Lightner & Redman, 1998), but the international movement of *Litopenaeus vannamei* has led to an epizootic in cultured *L. vannamei* in Taiwan in recent years (Tu *et al.*, 1999; Yu & Song, 2000). Although differences have already been reported between TSV isolates sourced from *L. vannamei* and *Litopenaeus stylirostris* at different times and places (Erickson *et al.*, 2002; Robles-Sikisaka *et al.*, 2002), this study is the first to analyse the genetic and phenotypic variance of TSV in isolates from two other economically cultured shrimp species, *Penaeus monodon* and *Metapenaeus ensis*.

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

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The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AY355309–AY355311.

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

TSVF2 (5′-ACCCCAGAAATGTGAATAACC-3′) and TSVR2 (5′-GGAAAAGCAATGTCAATACCC-3′) served as the outer primer pair for the first PCR step and the primer TSVF3 (5′-ATACTTAGCACAGCGACCATA-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 Tw2KPmTSV or Tw2KMeTSV virus inoculum prepared from Tw2KPm and the Tw2KMe, respectively. Methods for inoculum preparation and injection challenge were as described by Lightner (1996), except that the Tw2KPmTSV 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 Tw2KMeTSV and Tw2KPmTSV, 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 Tw2KMe or Tw2KPm 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 Tw2KPm, Tw2KMe 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 10^4-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 Tw2KMe (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 Tw2KMe specimen (Fig. 1d). An adult *P. monodon* shrimp that was challenged with concentrated Tw2KPmTSV was positive for nested PCR in only two tested organs (Fig. 1e). Challenge with Tw2KMeTSV, 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 Tw2KMeTSV isolate was able to replicate much more freely than the Tw2KPmTSV isolate. In an additional, more rigorous demonstration of the difference in replication ability between Tw2KPmTSV and Tw2KMeTSV, challenge tests were performed on SPF *M. japonicus* juveniles with Tw2KMeTSV (Fig. 1g, lanes 1–4) virus loads (revealed by real-time RT-PCR) of $1{\cdot}2 \times 10^5$–$3{\cdot}7 \times 10^6$ copies in 0·1 μg total RNA. In the Tw2KPmTSV-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 Tw2KPmTSV, Tw2KMeTSV 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

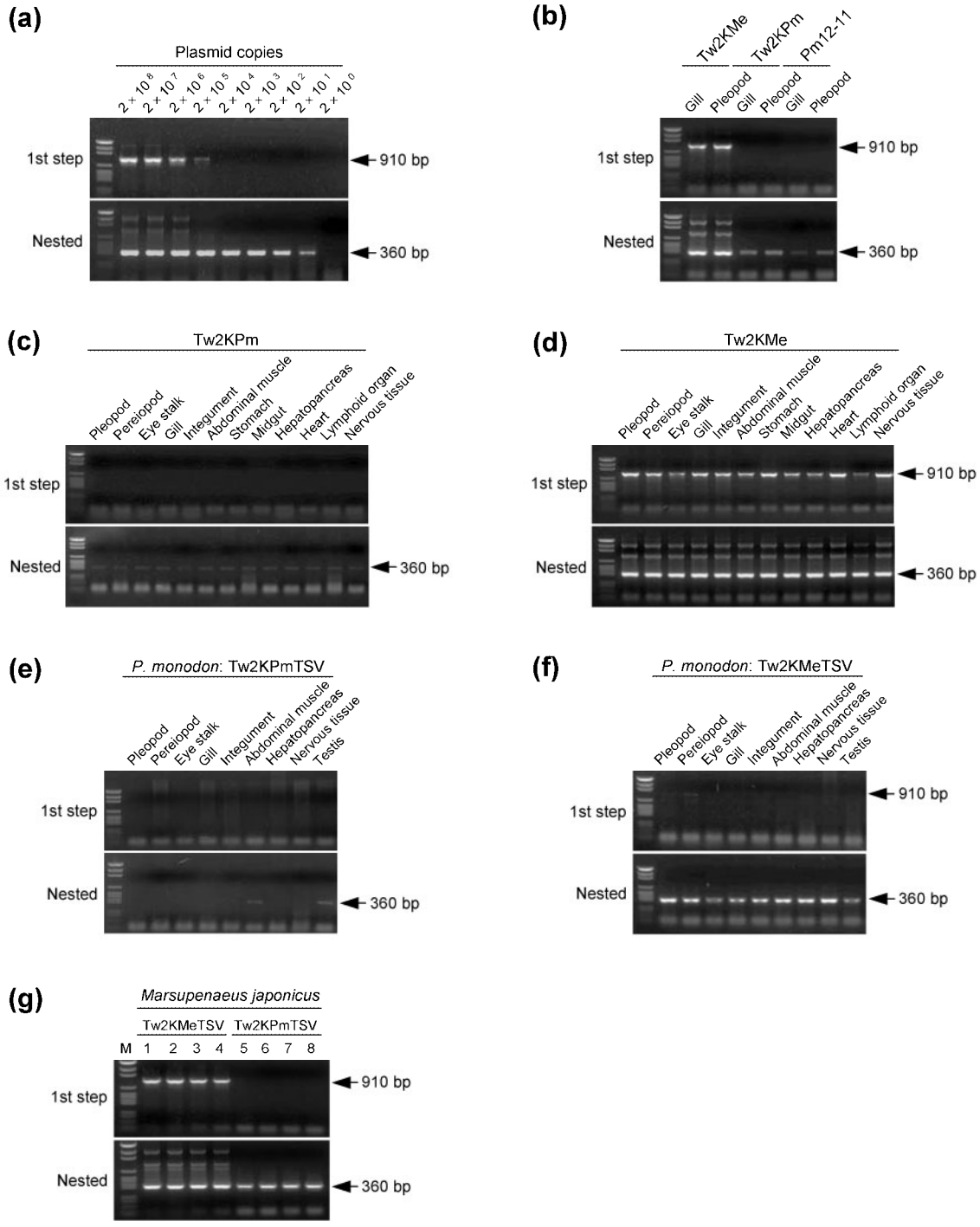

Fig. 1. TSV screening and tissue distribution as revealed by RT-PCR. (a) Calibration using serial dilutions of plasmid DNA containing the TSV partial *orf2* region cDNA sequence and showing the sensitivity of one-step (top panel) and nested (lower panel) TSV RT-PCR. (b) TSV screening results for the 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).

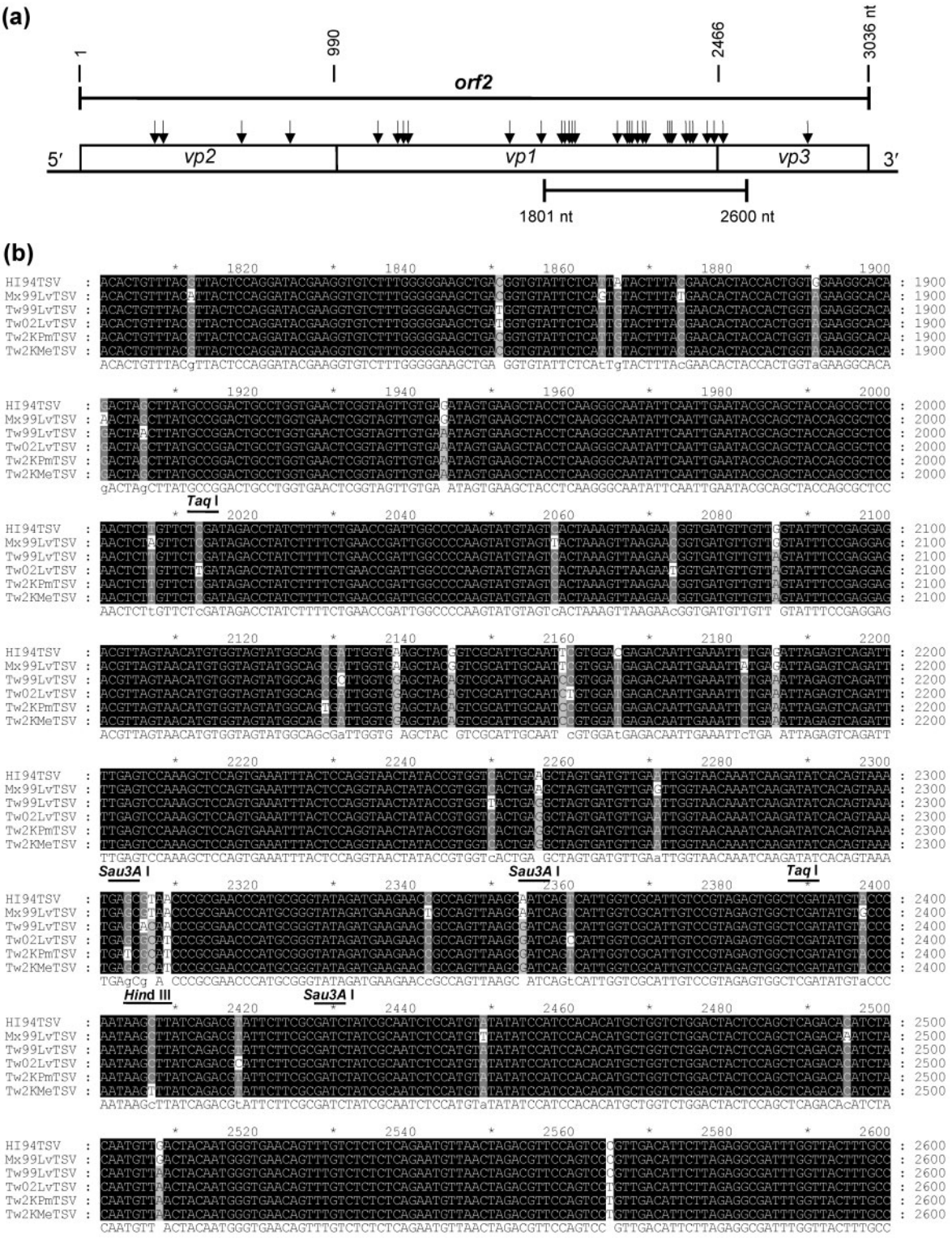

Fig. 2. (a) Schematic diagram of TSV showing the region sequenced in the present study (3036 nt). Vertical arrows indicate the relative positions of amino acids that differ from the Hawaiian isolate. The lower bar represents the region used here for multiple alignments (nt 1801–2600). (b) Nucleotide sequence alignment of the hypervariable region of the TSV *orf2* cDNA from different viral isolates. For reference, the *Hin*dIII, *Sau*3AI and *Taq*I restriction enzyme recognition sites are also shown.

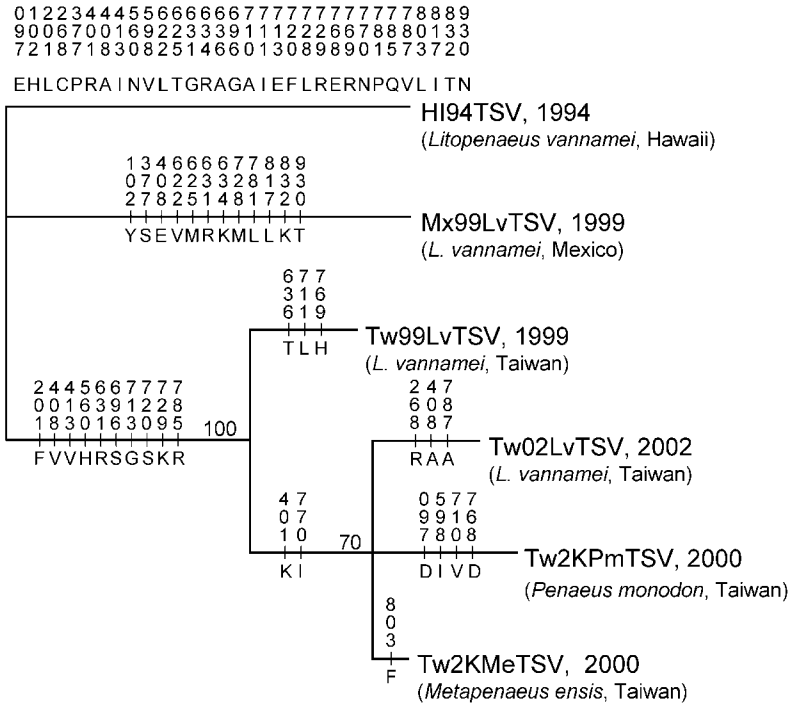

Fig. 3. Phylogenetic MP tree of the full-length (1011 aa) TSV ORF2 of six TSV isolates. The locations of the 32 variable amino acid positions are given with respect to the HI94TSV isolate, which also served as the tree's arbitrary root. Numbers on branches indicate bootstrap values (%) after 1000 replicates.

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.

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