1    **Genetic diversity of tilapia lake virus genome segment 1 from 2011 to 2019**

2    **and a newly validated semi-nested RT-PCR method**

3    Suwimon Taengphu[1,2], Pakkakul Sangsuriya [2,3], Kornsunee Phiwsaiya[1,2], Partho Pratim

4    Debnath[4], Jerome Delamare-Deboutteville[5], Chadag Vishnumurthy Mohan[5], Ha Thanh Dong[1,6*],

5    Saengchan Senapin[1,2*]

6    [1]Fish Health Platform, Center of Excellence for Shrimp Molecular Biology and Biotechnology

7    (Centex Shrimp), Faculty of Science, Mahidol University, Bangkok, Thailand

8    [2]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and

9    Technology Development Agency (NSTDA), Pathum Thani, Thailand

10   [3]Aquatic Molecular Genetics and Biotechnology Laboratory, BIOTEC, NSTDA, Pathum Thani,

11   Thailand

12   [4]WorldFish, Dhaka, Bangladesh

13   [5]WorldFish, Penang, Malaysia

14   [6]Faculty of Science and Technology, Suan Sunandha Rajabhat University, Bangkok, Thailand

15   

16   

17   **Running title:** Diversity of TiLV segment 1 and a new PCR protocol

18   *Corresponding authors:

19   S. Senapin (E-mail: saengchan@biotec.or.th)

20   H.T. Dong (Email: hathanh.do@ssru.ac.th)

## Abstract

The gene of RNA viruses, encoding RNA-directed RNA polymerase (RdRp) is relatively conserved due to its crucial function in viral genome replication and transcription making it a useful target for genetic diversity study and PCR detection. In this study, we investigated the genetic diversity of 21 tilapia lake virus (TiLV) genome segment 1 sequences predictively coding for RdRp subunit P1. Those sequences were obtained from infected fish samples collected in Ecuador, Israel, Peru, and Thailand between 2011 and 2019 (nine sequences from this study and 12 sequences from GenBank). Primers were then designed from the highly conserved regions among all 21 TiLV segment 1 sequences and used in semi-nested RT-PCR condition optimization. The result revealed that all 21 TiLV segment 1 sequences showed 95.00-99.94 and 99.00-100% nucleotide and amino acid sequence identity, respectively. These isolates were phylogenically clustered into three separate genetic clades, called i) Israeli-2011 clade (containing of TiLV isolates from Israel collected in 2011, Ecuador, and Peru isolates), ii) monophyletic Israel-2012 clade (containing only TiLV isolates collected from Israel in 2012), and iii) Thai clade (containing only sequences obtained from Thailand isolates). The newly established PCR protocol was 100 times more sensitive than our previous segment 3-based protocol when comparatively assayed with RNA extracted from infected fish. The assay was also shown to be specific when tested against negative control samples, i.e. RNA extracted from clinical healthy tilapia and from bacterial and viral pathogens (other than TiLV) commonly found in aquatic animals. Validation experiment with RNA extracted from naturally infected fish specimens collected in 2013-2019 yielded positive test results for all samples tested, confirming that our newly designed primers and detection protocol against TiLV segment 1, have a potential application for detection of all current genetic variants of TiLV.

**Keywords**: detection, semi-nested PCR, tilapia lake virus, TiLV, genome

## Introduction

A novel disease termed syncytial hepatitis of tilapia (SHT) associated with high mortality in farmed tilapia (Ferguson et al. 2014) caused by Tilapia lake virus (TiLV) (Eyngor et al. 2014). TiLV is a segmented RNA virus that has been taxonomically assigned to *Tilapia Tilapinevirus* (Bacharach et al. 2016; Adams et al. 2017). TiLV has been reported in 16 tilapia farming countries and this is likely to increase due to some underreporting (Jansen et al. 2018; Pulido et al. 2019). Rapid and accurate detection of the virus is crucial for selection of TiLV-free fish broodstock in view of the vertical transmission possibilities of the virus from parents to offspring (Yamkasem et al. 2019; Dong et al. 2020) and to prevent disease spread through movement of live fish for aquaculture (Dong et al. 2017a; Jansen et al. 2018). Following the characterization of the TiLV genomes (Eyngor et al. 2014; Bacharach et al. 2016), several PCR methods have been published, including RT-PCR (Eyngor et al. 2014), nested RT-PCR (Kembou Tsofack et al. 2017), semi-nested RT-PCR (Dong et al. 2017b), RT-qPCR (Kembou Tsofack et al. 2017; Tattiyapong et al. 2018; Waiyamitra et al. 2018), and RT-LAMP (Phusantisampan et al. 2019; Yin et al. 2019). Most of the aforementioned methods used genome segment 3 of TiLV as the target for primer design. There are insufficient information and understanding on genetic diversity and functional role of segment 3. Due to the nature of RNA viruses, we hypothesized that there was a certain level of sequence variation in the TiLV genomes. If so, there might be a possibility for mismatches of the designed primers and thus the current molecular detection methods may not be applicable for all genetic variants, if any, of TiLV.

So far, the functions of the putative protein products of the 10 genomic segments of TiLV have not been elucidated, except for the sequence of segment 1, that has weak percent identity (~17% amino acid identity, 37% sequence coverage) to the *PB1* gene of influenza C virus (Bacharach et al. 2016). Influenza virus *PB1* gene encodes for RNA-directed RNA polymerase (RdRp) catalytic subunit sometimes called polymerase basic protein 1 or RNA-directed RNA polymerase subunit P1. Generally, sequences encoding the *RdRp* genes of RNA viruses show a reasonably high level of conservation due to their crucial replication and transcription functions. *RdRp* gene was therefore considered a useful molecular marker for virus classification and detection (Koonin and Dolja 1993; Culley et al. 2003; Culley and Steward 2007; Senapin et al. 2007). Therefore, this study aims to i) investigate the genetic diversity of TiLV genome segment 1 from TiLV-infected fish samples and sequences available from GenBank and ii) to develop a new semi-nested RT-

76   PCR method based on primers designed from highly conserved regions of TiLV genome segment

77   1 for disease diagnosis and surveillance.

## Materials and methods

78

### Fish samples and RNA preparation

79

80   For amplification of TiLV genome segment 1, nine TiLV-infected specimens obtained from years

81   2013 to 2019 were used. They were archived specimens from our previous works (Dong et al.

82   2017a; Dong et al. 2017b) and a newly collected set (Table 1). Suspected TiLV-infected fish and

83   experimentally challenged fish samples (Table 2) were subjected to TiLV diagnosis using the

84   newly developed semi-nested RT-PCR described below. RNA was extracted from the fish tissues

85   using Trizol reagent (Invitrogen) following protocols recommended by the manufacturer. Quality

86   and quantity of the obtained RNA was measured by spectrophotometry at 260 and 280 nm. RNA

87   extracted from clinically healthy red and Nile tilapia tested negative for TiLV were used in

88   negative control PCR reactions.

### Amplification of TiLV genomic segment 1

89

90   Primers used for amplification of the putative open reading frame (ORF) of TiLV genomic

91   segment 1 were TiLV-S1-F; 5'-ATG TGG GCA TTT CAA GAA-3' and TiLV-S1-R; 5'-TTA

92   GCA CCC AGC GGT GGG CT-3' as previously described by Pulido et al. 2019. They were

93   designed based on the segment 1 sequence of the TiLV Israeli isolate TiL-4-2011 (accession

94   number KU751814, Eyngor et al, 2014, Bacharach et al, 2016). RNA extracted from 9 TiLV-

95   infected fish specimens obtained as described above were individually used as template. RT-PCR

96   reaction of 25 µl composed of 200 ng RNA template, 300 nM of each primer, 1 µl of SuperScript

97   III RT/Platinum Taq Mix (Invitrogen), and 1x supplied buffer (containing 0.2 mM of each dNTP,

98   2.6 mM $MgSO_4$, and stabilizers). Amplification profiles consisted of a reverse transcription step

99   at 50 °C for 30 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min; and a final

100  extension step at 72 °C for 5 min. After agarose gel electrophoresis, expected 1,560 bp amplicons

101  obtained from each of the nine reactions were removed from the gel, purified and cloned into

102  pGEM-T easy vector (Promega). Recombinant clones were then sent to Macrogen (South Korea)

103  for DNA sequencing using T7 promoter and SP6 promoter primers.

104

**DNA sequence analysis**

A total of 21 TiLV segment 1 sequences listed in Table 1 were used for comparison and phylogenetic relationship analysis. Nine sequences were obtained from the present study and other 12 sequences were retrieved from the GenBank database. Multiple sequence alignments were performed using MEGA 7 (Kumar et al. 2016) and a consensus sequence of 1,559 nucleotide residues out of the putative 1,560 nucleotide-ORF segment 1 were obtained. Deduced amino sequences of 519 nucleotide residues were translated from these consensus TiLV segment 1 using ExPASy translate tool and then used for sequence comparison. Putative PB domain of the translated sequences was predicted using InterPro database (https://www.ebi.ac.uk/interpro/). Phylogenetic tree based on TiLV segment 1 nucleotide sequences was constructed using Maximum-Likelihood with GTR+G (General Time Reversible model + Gamma distributed) method as suggested by a best model feature of the MEGA 7 program. An ORF coding for a putative *PB1* of influenza virus C isolate Ann Arbor 1950 (NC_006308) was used as outgroup. Pairwise distance analysis was also conducted using MEGA 7.

**Development of a new semi-nested RT-PCR method based on segment 1**

In this study, a new semi-nested RT-PCR detection of TiLV was developed. Primers were designed from the highly conserved regions from the multiple sequence alignments of the 21 sequences of TiLV segment 1. These sequences derived from TiLV isolates collected from the period from 2011 to 2019 (Table 1). Primer specificity was initially confirmed *in silico* with the NCBI primer-blast tool. Primers TiLV/nSeg1F; 5'- TCT GAT CTA TAG TGT CTG GGC C-3' and TiLV/nSeg1R; 5'- AGT CAT GCT CGC TTA CAT GGT-3' with an expected amplified product of 620 bp were used in the first round RT-PCR. Primers TiLV/nSeg1F and TiLV/nSeg1RN; 5'- CCA CTT GTG ACT CTG AAA CAG -3' with an expected product of 274 bp were employed in the second round PCR. Amplification conditions such as annealing temperature, number of cycles, buffer and primer concentration were evaluated to obtain optimized conditions. Consequently, the first RT-PCR reaction of 25 µl composed of 200 ng of RNA template, 400 nM of each primer, 0.5 µl of SuperScript III RT/Platinum Taq Mix (Invitrogen), and 1x of supplied buffer. Amplification profiles consisted of a reverse transcription step at 50 °C for 30 min; a denaturation step at 94 °C for 2 min, 30 PCR cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension step at 72 °C for 2 min. Positive control consisted of a reaction containing RNA extracted from

5

135   TiLV-infected red tilapia as template while no template added in the negative control. 5 µl of

136   product from the first round PCR was then used as template in the second round PCR reaction of

137   25 µl containing 500 nM of primer TiLV/nSeg1F, 600 nM of primer TiLV/nSeg1RN, 0.16 mM of

138   each dNTP, 0.8 mM $MgCl_2$, 1 unit of Platinum Taq DNA polymerase (Invitrogen), and 1.2x

139   supplied buffer. Thermocycling conditions consisted of a 5 min initial denaturation step at 94 °C

140   followed by 30 thermocycles and a final extension step described above. 10 µl of the amplified

141   products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide or

142   RedSafe DNA staining dye.

143   **Validation of TiLV segment 1 semi-nested RT-PCR assay**

144   Detection specificity of the assay was performed with RNA extracted from common viral and

145   bacterial pathogens found in aquatic animals as listed in Table 3. Viral infected fish tissues were

146   subjected to RNA extraction using Trizol reagent as mentioned above. For bacterial RNA isolation,

147   each bacterial strain was cultured in appropriated broth medium of 5 mL overnight at 30 °C with

148   shaking at 200 rpm. After centrifugation, bacterial cell pellets were washed once with nuclease-

149   free water and homogenized with Trizol reagent followed by the manufacturer's protocol.

150   Detection sensitivity of the TiLV segment 1 protocol (this study) was performed by comparison

151   with our previous semi-nested PCR protocol targeting TiLV segment 3 (Dong et al. 2017b). This

152   was done using serially diluted RNA template extracted from 3 individual TiLV-infected fish. Two

153   fish samples were assayed by Centex Shrimp in Thailand while another by WorldFish Khulna

154   laboratory, Bangladesh. The newly established semi-nested RT-PCR protocol was then employed

155   to screen for TiLV infection in both clinically healthy and clinically sick fish samples, including

156   representative from archived samples as well as naturally and experimentally infected fish (Tables

157   1 and 2).

158

159   **Results**

160   **Genome segment 1 based phylogenetic relationship of TiLV isolates**

161   After amplification of TiLV genome segment 1 from the nine TiLV-infected tilapia, their

162   sequences were compared to that of 12 sequences retrieved from the GenBank database.

163   Consequently, 21 TiLV segment 1 sequences obtained from samples collected in 2011 to 2019

6

164   were used for phylogenetic analysis. The results showed that the TiLV segment 1 sequences were

165   clustered into three separate clades namely Israeli-2011 clade, Israeli-2012 clade, and Thai clade

166   while the *RdRp PB1* sequence of influenza C virus used as an outgroup diverged into another

167   linage (Fig. 1). The Thai clade comprised all studied isolates from Thailand was closely related to

168   the monophyletic Israel-2012 clade, whereas Israeli-2011 clade composed of three TiLV isolates

169   (i.e. IL-2011-Til-2011, EC-2012, PE-2018-F3-4) from Israel, Ecuador, and Peru respectively.

170   Additionally, the phylogenetic tree revealed 2 subclades within the Thai clade (Fig. 1). Further

171   investigation using pairwise distance analyses, revealed that the three proposed clades diverged

172   among each other from 0.029 to 0.044 (i.e. 2.9-4.4%), with the lowest and highest values being

173   between Israeli-2012 & Thai clades and Israeli-2011 & Thai clades, respectively. Average

174   divergence within Israeli-2011 clade and within Thai clade were 0.033 and 0.035 (i.e. 3.3-3.5%),

175   respectively. The pairwise genetic distance is shown in Table S1.

176   **Conserved regions of TiLV segment 1 gene sequences and primer design**

177   Segment 1 gene sequences of the 21 TiLV isolates were compared using Clustal Omega and

178   MEGA 7 programs. Among the 21 sequences, there were 94.29-99.94 and 97.11-100% identity at

179   the nucleotide and amino acid sequence levels, respectively. Noted that among the 17 Thai isolates,

180   the respective percentages were 95.00-99.94 and 97.50-100%. PB domains and four RdRp motifs

181   were detected from all of the deduced amino acid sequences (Fig. S1). Based on prediction

182   published by Chu et al. 2012 for influenza virus and Bacharach et al. 2016 for TiLV, the four RdRp

183   motifs of TiLV containing motif I $^{48}$**TGD**L$^{51}$, motif II $^{260}$CPG**GM**L**MGMF**$^{269}$, motif III

184   $^{282}$DRFLSF**SDDF**ITS$^{294}$, and motif IV $^{312}$CH**NL**S**L**KKSYI**$^{322}$; where bold and underlined letters

185   represent residues matching to those of influenza virus sequences. Those specific residues were

186   found in all 21 TiLV isolates (Fig. S1).

187   The obtained multiple nucleotide sequence alignments displayed conserved regions along segment

188   1 gene sequences for the 21 TiLV isolates (Fig. 2). In this study, we aimed to design conserved

189   primers to be used in a semi-nested RT-PCR assay enabling amplification of all genetic variability

190   (known to date) of TiLV isolates if any. Primers TiLV/nSeg1F, TiLV/nSeg1R, and

191   TiLV/nSeg1RN are 21-22 nucleotides long and locate at nucleotide positions 717-737, 1315-1336,

192   and 1063-1083; all locate in the segment 1 ORF, showing 100% identity among all of the 21 TiLV

193   isolates analyzed (Fig. 2). Amplicon sizes of 620 and 274 bp were expected to be produced from

7

194    primers TiLV/nSeg1F and TiLV/nSeg1R; and TiLV/nSeg1F and TiLV/nSeg1RN, respectively

195    (Fig. 2). Additionally, these primer pairs were shown, *in silico,* to be TiLV specific using primer-

196    blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Thus, PCR condition optimization

197    for TiLV detection using the newly designed primers was conducted as described below.

198    **Development of a validated TiLV detection protocol**

199    The designed primer pairs described above were evaluated for their optimal annealing

200    temperatures which were found to be 60°C for both first and nested amplification (figure not

201    shown). Other parameters including numbers of cycles, buffer and primer concentration were also

202    evaluated and optimized. Finally, our new TiLV segment 1 detection protocol was shown to be

203    specific for only TiLV when tested against other 16 viral and bacterial pathogens (Fig. S2). The

204    detection sensitivity assay was conducted by comparing between the present newly established

205    TiLV segment 1 based protocol and our previous method which was based on segment 3 (Dong et

206    al. 2017b) using serially diluted RNA from three individual TiLV-infected fish specimens. Results

207    revealed that the new protocol described in this study was 100 times more sensitive than the

208    previous one (Fig. 3a). This assay was done in two different laboratories i.e. 2 samples (i.e. fish 1

209    and 2) performed in Thailand and fish 3 in WorldFish Khulna laboratory, Bangladesh. Note the

210    presence of a ~1.1 kb band predicted to be derived from cross hybridization of the amplified

211    products was visible at higher concentrated templates (Fig. 3a). Moreover, one set of specimens

212    from experimentally TiLV infected fish (Table 2, set 2, n=20) obtained from our previous study

213    (Dong et al. 2020) was assayed using both segment 1 and segment 3 based detection protocols (Fig

214    3b), also confirming higher sensitivity of the current semi-nested PCR detection targeting TiLV

215    segment 1 gene. This was evidenced by the numbers of positive tests obtained for segment 1 and

216    segment 3 protocols, which were 19 and 13 out of 20, respectively (Table 2). Representative test

217    results are shown in Fig. 3b. Comparison of TiLV infected fish samples collected from Thailand

218    (2013-2019) and from Peru in 2018 (Tables 1 and 2) confirmed that the new detection protocol

219    can be used to detect TiLV from all the samples tested representing Thai clade and Israeli-2011

220    clade in this study (Fig. 4). In addition, in one population, both clinically sick fish and apparently

221    healthy fish were all tested positive for TiLV segment 1 (Table 2, set 3). Remaining sets (Table 2,

222    sets 4-7) from field samples subjected to the test for TiLV were all negative.

## Discussion

223

224  Recently, Pulido et al. 2019 proposed two genetic clades of TiLV (Israeli and Thai clades) based

225  on multilocus sequence phylogenetic analysis (MLSA) of 8,305 nucleotides of 5 TiLV genomes.

226  Due to limited number of complete TiLV genomes available in the GenBank database, this study,

227  therefore, aimed to explore the genetic diversity of TiLV based on the open reading frame (ORF)

228  of segment 1 *PB1* gene (1,560 nucleotides) from 21 TiLV isolates which comprised of the isolates

229  from Israel, Peru, Ecuador, and Thailand. Although bootstrap values were not relatively high,

230  phylogenetic tree inferred from the segment 1 dataset indicates three separate clades (Israeli-2011,

231  Israeli-2012, and Thai clades). It is likely that the Israeli-2012 and Thai clades evolved from the

232  Israeli-2011 clade. However, to gain a better understanding on TiLV evolution and how the virus

233  emerged and widespread, larger number of complete TiLV genomes or sequences of segment 1 of

234  TiLV from a broader origin should be made available. Note that no correlation between sequence

235  variation or phylogenetic branches and disease severity was observed in this study.

236  Comparative nucleotide sequences revealed that TiLV segment 1 is relatively well conserved with

237  95.00-99.94% identity among all isolates used in this study, which is similar to that of

238  Orthomyxoviruses such as Salmon isavirus RNA polymerase PB1 (97.60-99.86%) and Influenza

239  C virus polymerase PB1 (97.12-99.34%) (BLAST search in this study). Taken together, the results

240  from this study, suggest that TiLV segment 1 is a promising gene candidate for the study of the

241  genetic diversity of TiLV globally.

242  Majority of tilapia farming countries are located in low and middle-income countries. In those

243  countries, conventional PCR remains the preferable technique for many laboratories (in terms of

244  costs and ease of use) compared to less accessible and more expensive quantitative PCR machine

245  (Charoenwai et al. 2019). The semi-nested PCR method in this study therefore was designed based

246  on conserved regions of genome segment 1 that was able to amplify representatives from both

247  Thai, Israeli-2011 and Israeli-2012 clades. For its higher sensitivity and specificity, this new

248  protocol, thus, might be able to reduce chance of false negative due to genetic variation if any.

249  Using serial dilutions of DNA plasmid containing insert of targeted TiLV segment 3 gene, our

250  previous semi-nested RT-PCR targeting segment 3 (Dong et al. 2017b) has a detection limit of 7.5

251  copies per reaction. Similar approaches have been used in previously reported TiLV detection

252  protocols (Kembou Tsofack et al. 2017; Tattiyapong et al. 2018). While detection limits are based

9

253    on serial dilutions of DNA plasmid containing target gene, diagnostic assays in reality started from

254    extracted RNA from infected fish, comprising both host and viral RNA. Thus, detection limit of

255    the PCR assay for clinical samples might not be comparable with the results obtained from

256    standard serial dilutions of DNA plasmid in the laboratory. This study, therefore, compared the

257    sensitivity of two detection protocols together instead of using plasmid template to find out which

258    protocol was the best for field detection purpose. Interestingly, inter-laboratory tests consistently

259    proved that segment 1 PCR method was 100 times more sensitive than our previous segment 3

260    PCR protocol, when assayed with RNA extracted from diseased fish samples. In this study,

261    segment 1 PCR detection method showed no cross-amplification against a panel of extracted RNA

262    from various bacterial and viral pathogens of freshwater fish. When applied to field samples, this

263    new segment 1 protocol was able to detect the virus not only from clinically sick fish but also from

264    asymptomatically infected fish, indicating its potential use for screening early infected fish

265    samples. Taken together, we recommend the use of this newly established segment 1 PCR method

266    as an alternative tool for TiLV diagnosis and in active surveillance programs.

271    **References**

272    Adams, M. J., E. L. Lefkowitz, A. M. Q. King, B. Harrach, R. L. Harrison, N. J. Kropinski, M.

273        Krupovic, J. H. Kuhn, A. R. Mushegian, M. Nibert, S. Sabanadzovic, H. Sanfacon, S. G.

274        Siddell, P. Simmonds, A. Varsani, F. M. Zerbini, A. E. Gorbalenya and A. J. Davison

275        (2017). Changes to taxonomy and the international code of virus classification and

276        nomenclature ratified by the international committee on taxonomy of viruses. Arch

277        Virology 162: 2505-2538.

278    Bacharach, E., N. Mishra, T. Briese, M. C. Zody, J. E. K. Tsofack, R. Zamostiano, N. C. Toussaint,

279        J. Ng, A. Nitido, A. Corvelo, S. C. Nielsen, M. Horning, J. Del Pozo, T. Bloom, H.

280        Ferguson, A. Eldar and W. Lipkin (2016). Characterization of a novel orthomyxo-like virus

281        causing mass die-offs of tilapia. MBio 7(2): e00431-00416.

282    Charoenwai, O., M. W., M. Sonthi, H. T. Dong and S. Senapin (2019). A validated semi-nested
283        PCR for rapid detection of scale drop disease virus (SDDV) in Asian sea bass (Lates
284        calcarifer). J Virol Methods 268: 37-41.

285    Chu, C., S. Fan, C. Li, C. Macken, J. H. Kim, M. Hatta, G. Neumann and Y. Kawaoka (2012).
286        Functional analysis of conserved motifs in influenza virus PB1 protein. PLoS ONE 7(5):
287        e36113.

288    Culley, A. I., A. S. Lang and C. A. Suttle (2003). High diversity of unknown picorna-like viruses
289        in the sea. Nature 424: 1054-1057.

290    Culley, A. I. and G. F. Steward (2007). New genera of RNA viruses in subtropical seawater,
291        inferred from polymerase gene sequences. Appl. Environ. Microbiol. 73: 5937-5944.

292    Dong, H. T., G. A. Ataguba, P. Khunrae, T. Rattanarojpong and S. Senapin (2017a). Evidence of
293        TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread.
294        Aquaculture 479: 579-583.

295    Dong, H. T., V. V. Nguyen, K. Phiwsaiya, W. Gangnonngiw, B. Withyachumnarnkul, C.
296        Rodkhum and S. Senapin (2015). Concurrent infections of *Flavobacterium columnare* and
297        *Edwardsiella ictaluri* in striped catfish, *Pangasianodon hypophthalmus* in Thailand.
298        Aquaculture 448: 142-150.

299    Dong, H. T., S. Senapin, W. Gangnonngiw, V. V. Nguyen, C. Rodkhum, P. P. Debnath, J. D.
300        Deboutteville and C. V. Mohan (2020). Experimental infection reveals transmission of
301        tilapia lake virus (TiLV) from tilapia broodstock to their reproductive organs and fertilized
302        eggs. Aquaculture 515: 734541.

303    Dong, H. T., S. Senapin, K. Phiwsaiya, C. Techatanakitarnan, K. Dokladda, P. Ruenwongsa and
304        B. Panijpan (2018). Histopathology and culturable bacteria associated with "big belly" and
305        "skin nodule" syndromes in ornamental Siamese fighting fish, *Betta splendens*. Microb
306        Pathog. 122: 46-52.

307    Dong, H. T., S. Siriroob, W. Meemetta, W. Santimanawong, W. Gangnonngiw, N. Pirarat, P.
308        Khunrae, T. Rattanarojpong, R. Vanichviriyakit and S. Senapin (2017b). Emergence of
309        tilapia lake virus in Thailand and an alternative semi-nested RT-PCR for detection.
310        Aquaculture 476: 111-118.

311    Dong, H. T., C. Techatanakitarnan, P. Jindakittikul, A. Thaiprayoon, S. Taengphu, W.
312        Charoensapsri, P. Khunrae, T. Rattanarojpong and S. Senapin (2017c). *Aeromonas jandaei*

313    and *Aeromonas veronii* caused disease and mortality in Nile tilapia, *Oreochromis niloticus*
314    (L.). J. Fish. Dis. 40: 1395-1403.

315    Eyngor, M., R. Zamostiano, J. E. K. Tsofack, A. Berkowitz, H. Bercovier, S. Tinman, M. Lev, A.
316    Huryitz, M. Galeotti, E. Bacharach and A. Eldar (2014). Identification of a novel RNA
317    virus lethal to tilapia. J Clin Microbiol. 52: 4137–4146.

318    Ferguson, H. W., R. Kabuusu, S. Beltran, E. Reyes, J. A. Lince and J. del Pozo (2014). Syncytial
319    hepatitis of farmed tilapia, *Oreochromis niloticus* (L.): a case report. J Fish Dis 37: 583-
320    589.

321    Jansen, M. D., H. T. Dong and C. V. Mohan (2018). Tilapia lake virus: a threat to the global tilapia
322    industry? Rev Aquacult doi:10.1111/raq.12254.

323    Kembou Tsofack, J. E., R. Zamostiano, S. Watted, A. Berkowitz, E. Rosenbluth, N. Mishra, T.
324    Briese, W. I. Lipkin, R. M. Kabuusu, H. Ferguson, J. del Pozo, A. Eldar and E. Bacharach
325    (2017). Detection of tilapia lake virus (TiLV) in clinical samples by culturing and nested
326    RT-PCR. J. Clin. Microbiol 55: 759–767.

327    Koonin, E. V. and V. V. Dolja (1993). Evolution and taxonomy of positive-strand RNA viruses:
328    implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol.
329    Biol. 28: 375-430.

330    Kumar, S., G. Stecher and K. Tamura (2016). MEGA7: Molecular Evolutionary Genetics Analysis
331    version 7.0 for bigger datasets. Mol Biol Evol. 33: 1870-1874.

332    Nguyen, V. V., H. T. Dong, S. Senapin, N. Pirarat and C. Rodkhum (2016). *Francisella*
333    *noatunensis* subsp. *orientalis*, an emerging bacterial pathogen affecting cultured red tilapia
334    (*Oreochromis* sp.) in Thailand. Aquac Res 47: 3697-3702.

335    Nguyen, V. V., C. Rodkhum, S. Senapin and H. T. Dong (2019). Retrospective diagnosis of
336    archived marine fish experienced unexplained mortality reveals dual infections of
337    *Nocardia seriolae* and *Streptococcus iniae*. Aquacult Int: doi.org/10.1007/s10499-10019-
338    00403-10494

339    Phusantisampan, T., P. Tattiyapong, P. Mutrakulcharoen, M. Sriariyanun and W. Surachetpong
340    (2019). Rapid detection of tilapia lake virus using a one-step reverse transcription loop-
341    mediated isothermal amplification assay. Aquaculture 507: 35-39.

342    Pulido, H. L. L., M. M. Chìo, A. L. H. Chaparro, H. T. Dong and S. Senapin (2019). Tilapia lake
343    virus (TiLV) from Peru is genetically close to the Israeli isolates. Aquaculture x: x.

344    Senapin, S., H. T. Dong, W. Meemetta, W. Gangnonngiw, P. Sangsuriya, R. Vanichviriyakit, M.
345        Sonthi and B. Nuangsaeng (2019). Mortality from scale drop disease in farmed *Lates*
346        *calcarifer* in Southeast Asia. J Fish Dis 42: 119-127.

347    Senapin, S., K. Phewsaiya, M. Briggs and T. W. Flegel (2007). Outbreaks of infectious
348        myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an
349        alternative RT-PCR detection method. Aquaculture 266: 32-38.

350    Subramaniam, K., H. W. Ferguson, R. Kabuusu and T. B. Waltzek (2019). Genome sequence of
351        tilapia lake virus associated with syncytial hepatitis of tilapia in an Ecuadorian aquaculture
352        facility.        Microbiol        Resour        Announc        8:        e00084-00019.
353        https://doi.org/00010.01128/MRA.00084-00019.

354    Surachetpong, W., T. Janetanakit, N. Nonthabenjawan, P. Tattiyapong, K. Sirikanchana and A.
355        Amonsin (2017). Outbreaks of tilapia lake virus infection, Thailand, 2015–2016. Emerg
356        Infect Dis. 23: 1031–1033.

357    Tajima, F. and M. Nei (1984). Estimation of evolutionary distance between nucleotide sequences.
358        Molecular Biology and Evolution 1: 269-285.

359    Tattiyapong, P., K. Sirikanchana and W. Surachetpong (2018). Development and validation of a
360        reverse transcription quantitative polymerase chain reaction for tilapia lake virus detection
361        in clinical samples and experimentally challenged fish. J Fish Dis 41: 255-261.

362    Waiyamitra, P., P. Tattiyapong, K. Sirikanchana, S. Mongkolsuk, P. Nicholson and W.
363        Surachetpong (2018). A TaqMan RT-qPCR assay for tilapia lake virus (TiLV) detection in
364        tilapia. Aquaculture 497: 184-188.

365    Wangman, P., P. Chaivisuthangkura, K. Sritunyalucksana, S. Taengchaiyaphum, S. Senapin, C.
366        Pengsuk, P. Sithigorngul and S. Longyant (2017). Development of monoclonal antibodies
367        specific to ToxA and ToxB of Vibrio parahaemolyticus that cause acute hepatopancreatic
368        necrosis disease (AHPND). Aquaculture 474: 75-81.

369    Yamkasem, J., Tattiyapong, P., Kamlangdee, A., Surachetpong, W. (2019) Evidence of potential
370        vertical transmission of tilapia lake virus. J Fish Dis 42: 1293-1300.

371    Yin, J., Q. Wang, Y. Wang, Y. Li, W. Zeng, J. Wu, Y. Ren, Y. Tang, C. Gao, H. Hu and S. M.
372        Bergmann (2019). Development of a simple and rapid reverse transcription–loopmediated
373        isothermal amplification (RT-LAMP) assay for sensitive detection of tilapia lake virus. J
374        Fish Dis 42: 817– 824.

375    **Tables and Figures**

376    **Table 1** Sources of TiLV sequences used to create the consensus segment 1 sequences in this
377    study

| No. | Code | Sample collection | Accession no. | Fish host | Fish health status | Ref. |
|---|---|---|---|---|---|---|
| 1 | TH-2013 | Jan-2013 | MN687685 | Nile tilapia fertilized eggs | Not specified | This study |
| 2 | TH-2014 | Aug-2014 | MN687695 | Nile tilapia fry | 90% mortality | This study |
| 3 | TH-2015 | Aug-2015 | MN687705 | Nile tilapia fingerling | 50% mortality | This study |
| 4 | TH-2016-CN | Dec-2016 | MN687725 | Red tilapia juvenile | 90% mortality | This study |
| 5 | TH-2016-CU | Dec-2016 | MN687715 | Tilapia fingerling | 20% mortality | This study |
| 6 | TH-2017 | 2017 | MN687735 | Nile tilapia | 90% mortality | This study |
| 7 | TH-2018-K | Aug-2018 | MN687755 | Nile tilapia juvenile | 50% mortality | This study |
| 8 | TH-2018-N | Jul-2018 | MN687745 | Red tilapia fingerling | 40-60% mortality | This study |
| 9 | TH-2019 | Feb-2019 | MN687765 | Nile tilapia fingerling | 30-50% mortality | This study |
| 10 | TH-2016-TV1 | Feb-2016 | KX631921 | Red tilapia | 20-90% mortality | Surachetpong et al. 2017 |
| 11 | TH-2015-TV2 | Dec-2015 | KX631931 | Red tilapia | | |
| 12 | TH-2016-TV3 | Jan-2016 | KX631932 | Red tilapia | | |
| 13 | TH-2016-TV4 | Jan-2016 | KX631933 | Nile tilapia | | |
| 14 | TH-2016-TV5 | Jan-2016 | KX631934 | Red tilapia | | |
| 15 | TH-2016-TV6 | May-2016 | KX631935 | Red tilapia | | |
| 16 | TH-2016-TV7 | May-2016 | KX631936 | Nile tilapia | | |
| 17 | TH-2018-WVL18053-01A | Apr-2018 | MH319378 | Nile tilapia | Not specified | NCBI |
| 18 | IL-2011- Til-4-2011 | 01-May-2011 | KU751814 | Tilapia | >80% mortality | Eyngor et al. 2014; Bacharach et al. 2016 |
| 19 | IL-2012-AD-2016 | 14-Aug-2012 | KU552131 | Hybrid tilapia | Not specified | NCBI |
| 20 | EC-2012 | 2012 | MK392372 | Nile tilapia | Up to 90% mortality | Subramaniam et al. 2019 |
| 21 | PE-2018-F3-4 | Feb-2016 | MK425010 | Nile tilapia | apparently healthy | Pulido et al. 2019 |

378    Country code: TH, Thailand, IT, Israel, EC, Ecuador, PE, Peru
379    Red tilapia (*Oreochromis* spp.); Nile tilapia (*Oreochromis niloticus*); Hybrid tilapia
380    (*Oreochromis niloticus x Oreochromis aureus*)

381 **Table 2** Sample sets subjected to TiLV detection using the present semi-nested RT-PCR

382 segment 1 protocol

| | Fish stage | Type of sample | Year collected | No. of +ve/tested sample |
|---|---|---|---|---|
| Set 1 | Nile and hybrid red tilapia, various stages | Archived extracted RNA from clinically sick fish | 2013-2019 | 9/9 |
| Set 2 | Nile tilapia, broodstock and eggs | Experimentally TiLV infected samples | 2019 | 19/20* |
| Set 3 | Hybrid red tilapia, fingerlings | Frozen specimens of clinically sick fish | 2019 | 4/4 |
| | | Frozen specimens of clinically healthy fish | 2019 | 4/4 |
| Set 4 | Nile tilapia, broodstock | Blood from apparently healthy fish preserved in Trizol | 2019 | 0/8 |
| Set 5 | Nile tilapia, broodstock | Blood from apparently healthy fish preserved in Trizol | 2019 | 0/6 |
| Set 6 | Nile tilapia, fingerlings | Frozen specimens of clinically healthy fish | 2019 | 0/3 |
| Set 7 | Nile tilapia, broodstock | Blood from apparently healthy fish preserved in Trizol | 2019 | 0/40 |
| | | Liver biopsy sample preserved in Trizol | 2019 | 0/40 |
| | | | Total | 36/134 |

383 *13/20 samples tested positive by TiLV segment 3 protocol

15

384 **Table 3** RNA samples used for specificity assay in this study

| Code* | Samples | Strain | Origin | References |
|---|---|---|---|---|
| S | SDDV-infected Asian sea bass | 2016 | Asian sea bass | Senapin et al. 2019 |
| V | NNV-infected tissue | G2019 | Grouper | Laboratory archived sample |
| I | ISKNV-infected tissue | SB2019 | Asian sea bass | Laboratory archived sample |
| N1, N2 | Clinical healthy Nile tilapia | NA | Nile tilapia | Laboratory archived sample |
| R1, R2 | Clinical healthy red tilapia | NA | red tilapia | Laboratory archived sample |
| Vo | *Vogesella* sp. | 1754 | Nile tilapia | Laboratory strain |
| Ps | *Plesiomonas shigelloides* | NA | Nile tilapia | Laboratory strain |
| Ac | *Aeromonas caviae* | 1926 | Betta fish | Dong et al. 2018 |
| Aj | *Aeromonas jandaei* | NT-01 | Nile tilapia | Dong et al. 2017c |
| Sa | *Streptococcus agalactiae* | NA | Nile tilapia | Laboratory strain |
| Ch | *Chryseobacterium* sp. | 2074 | Betta fish | Dong et al. 2018 |
| Et | *Edwardsiella tarda* | VMCU06 | Nile tilapia | Laboratory strain |
| Vc | *Vibrio cholerae* | NA | Nile tilapia | Laboratory strain |
| Si | *Streptococcus iniae* | VN2396 | Asian sea bass | Nguyen et al. 2019 |
| Ei | *Edwardsiella ictaluri* | T1-1 | Striped catfish | Dong et al. 2015 |
| Av | *Aeromonas veronii* | NT-03 | Nile tilapia | Dong et al. 2017c |
| Fc | *Flavobacterium columnare* | 2846 | Asian sea bass | Laboratory strain |
| Fn | *Francisella noatunensis* subsp. *orientalis* | VMCU-FNO131 | Red tilapia | Nguyen et al. 2016 |

385 *Code in Fig. S2

386 NA, not applicable

16
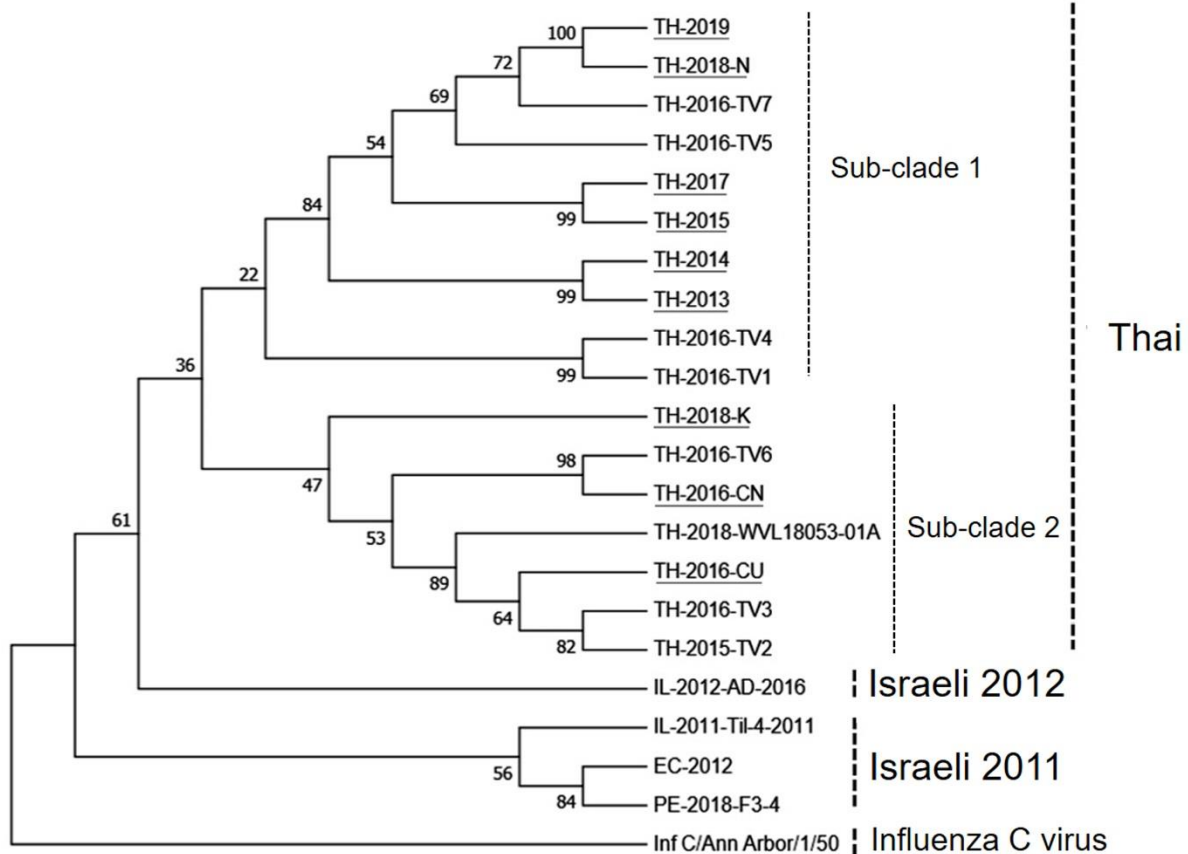
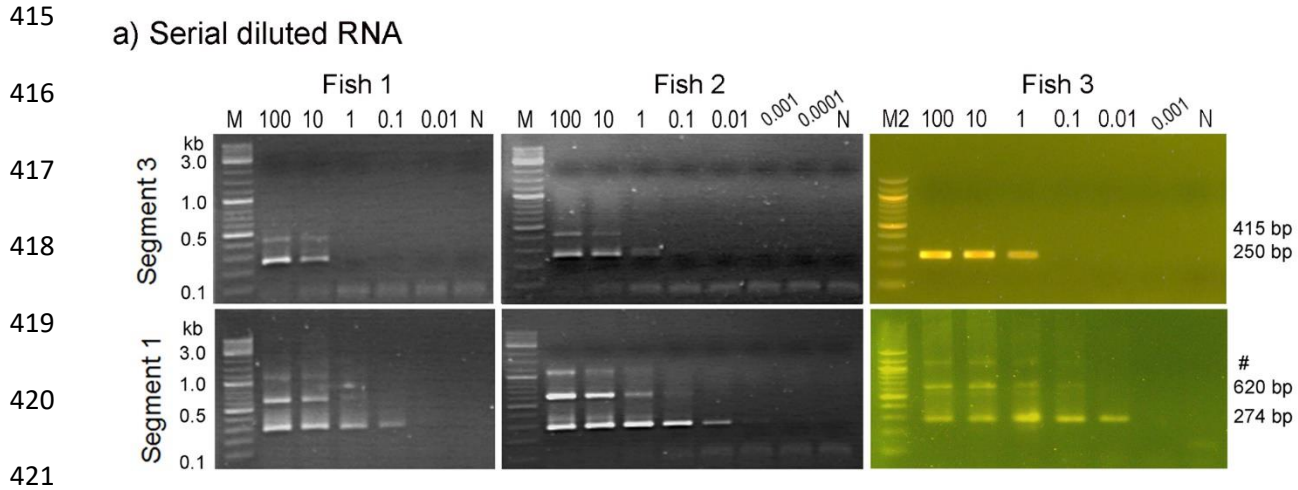**Figure 1** Maximum-likelihood phylogenetic trees based on TiLV genomic segment 1 *PB1* nucleotide sequences depicting TiLV being clustered into 3 clades. Isolates with and without underlines represent sequences obtained from this study and those from the GenBank database, respectively. Codes of the viral isolates are listed in Table 1. The influenza C virus sequence was used as outgroup. Percent bootstrap values from 1,000 replications are shown.
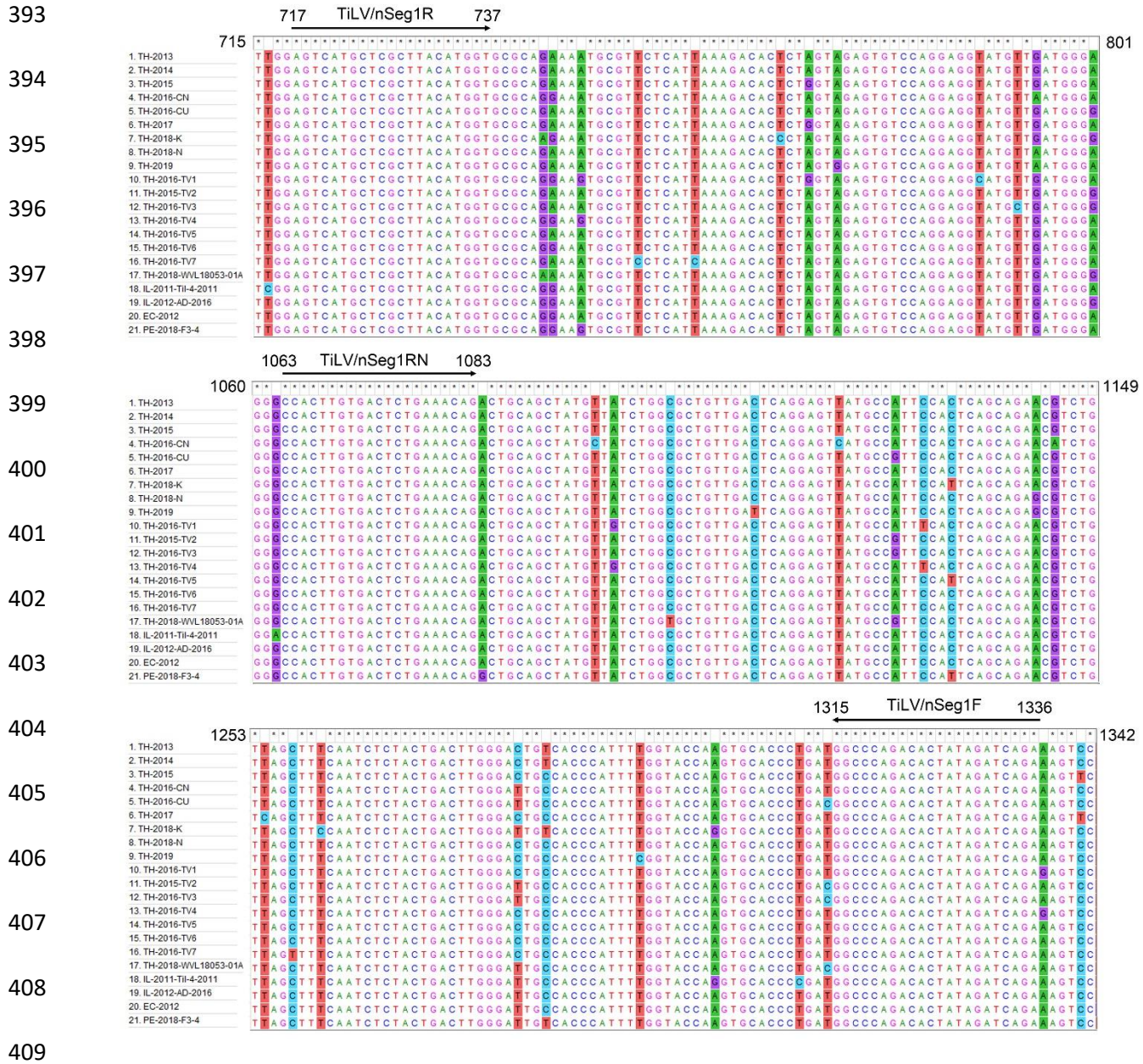
**Figure 2** Multiple nucleotide sequence alignments of TiLV segment 1 *PB1* genes of 21 viral isolates. A partial portion of an alignment is shown. Numbers represent nucleotide positions of the coding strand in the putative *PB1* ORF. Arrows indicate sites that were used to design primers for the semi-nested RT-PCR protocol developed in this study. * marks nucleotide identity. Columns with differences in colors indicate nucleotide variability.

**Figure 3** a) Sensitivity tests of two semi-nested RT-PCR protocols (segment 1 and 3) using serial dilutions of RNA from three TiLV-infected fish. A starting 100 ng of RNA from three individual TiLV-infected fish was 10-fold serially diluted and subjected to semi-nested RT-PCR protocols using primers targeting genome segment 3 (Dong et al. 2017b) and segment 1 (this study). M, 2 log DNA marker; N, no template control; #, cross hybridized PCR products. b) Representative comparison tests with 9 archived samples (1-9) obtained from Dong et al. (2020); P, positive control (infected tissue) and N, negative control (no RNA template).

434
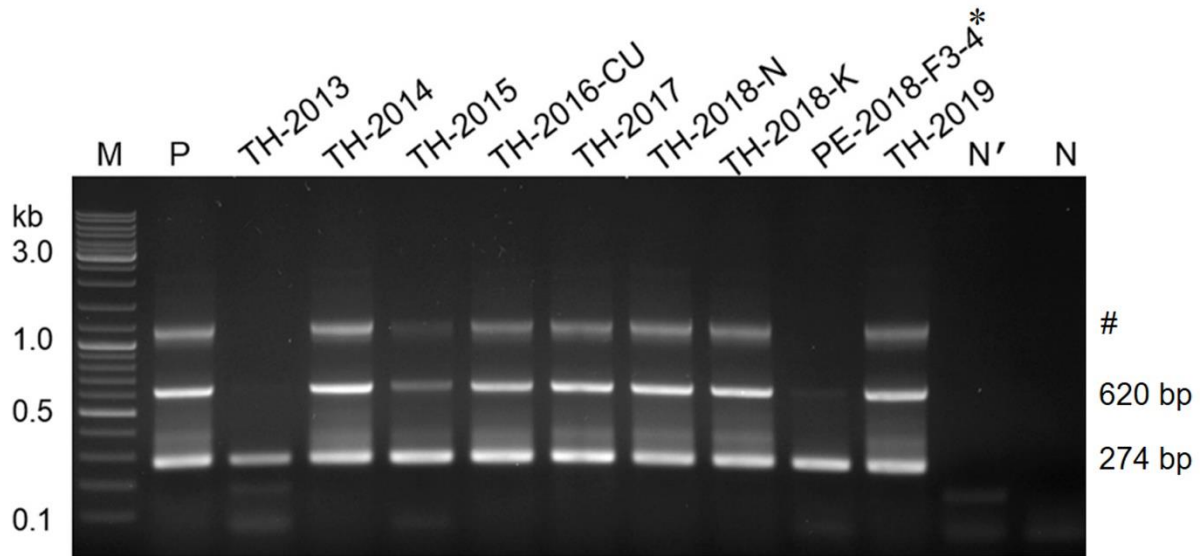


**Figure 4** Validation of the newly developed semi-nested RT-PCR assays in detecting TiLV segment 1 from archived samples obtained from years 2013 to 2019. *sample from Peru described in Pulido et al. (2019); M, 2 log DNA marker; P, positive control (infected tissue); TH, samples from Thailand; PE, sample from Peru; N', non-infected fish RNA; N, no template control; #, cross hybridized PCR products.

440 **Table S1** Pairwise genetic distance between TiLV isolates and influenza virus C as outgroup. (a)

441 Divergence between sequences (b) Mean divergence within clades (c) Divergence between clades.

442 The number of base substitutions per site from between sequences are shown below diagonal.

443 Standard error estimate(s) are shown above the diagonal. Analyses were conducted from 1,000

444 bootstrap replicates using the Tajima-Nei model in MEGA 7 (Tajima and Nei 1984; Kumar et al.

445 2016).
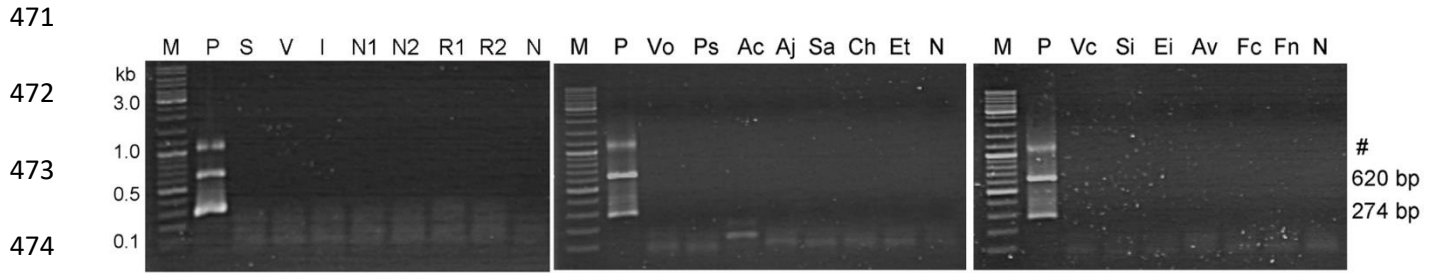
446 **a) Pairwise distance**

| | TiLV isolate & Outgroup | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TH-2016-TV7 | | 0.005 | 0.004 | 0.006 | 0.006 | 0.006 | 0.006 | 0.005 | 0.005 | 0.004 | 0.006 | 0.007 | 0.006 | 0.004 | 0.005 | 0.005 | 0.006 | 0.005 | 0.004 | 0.004 | 0.007 | 0.226 |
| 2 | TH-2016-TV6 | 0.035 | | 0.005 | 0.005 | 0.005 | 0.005 | 0.006 | 0.006 | 0.003 | 0.005 | 0.005 | 0.006 | 0.005 | 0.004 | 0.005 | 0.006 | 0.005 | 0.005 | 0.005 | 0.005 | 0.007 | 0.232 |
| 3 | TH-2016-TV5 | 0.025 | 0.032 | | 0.006 | 0.006 | 0.006 | 0.005 | 0.005 | 0.005 | 0.004 | 0.006 | 0.006 | 0.006 | 0.004 | 0.005 | 0.005 | 0.006 | 0.004 | 0.004 | 0.004 | 0.006 | 0.233 |
| 4 | TH-2016-TV4 | 0.039 | 0.036 | 0.041 | | 0.007 | 0.007 | 0.006 | 0.006 | 0.005 | 0.006 | 0.006 | 0.007 | 0.002 | 0.005 | 0.006 | 0.005 | 0.006 | 0.005 | 0.005 | 0.005 | 0.006 | 0.250 |
| 5 | TH-2016-TV3 | 0.043 | 0.033 | 0.037 | 0.044 | | 0.001 | 0.006 | 0.006 | 0.005 | 0.006 | 0.006 | 0.003 | 0.006 | 0.005 | 0.006 | 0.006 | 0.001 | 0.006 | 0.005 | 0.005 | 0.007 | 0.222 |
| 6 | TH-2015-TV2 | 0.044 | 0.034 | 0.038 | 0.044 | 0.002 | | 0.007 | 0.007 | 0.006 | 0.006 | 0.006 | 0.003 | 0.007 | 0.005 | 0.006 | 0.006 | 0.001 | 0.006 | 0.005 | 0.006 | 0.007 | 0.219 |
| 7 | EC-2012 | 0.042 | 0.041 | 0.039 | 0.041 | 0.044 | 0.045 | | 0.006 | 0.006 | 0.006 | 0.007 | 0.006 | 0.005 | 0.004 | 0.006 | 0.006 | 0.005 | 0.005 | 0.005 | 0.005 | 0.004 | 0.223 |
| 8 | TH-2019 | 0.029 | 0.044 | 0.031 | 0.050 | 0.047 | 0.048 | 0.049 | | 0.006 | 0.002 | 0.007 | 0.007 | 0.006 | 0.005 | 0.006 | 0.006 | 0.006 | 0.005 | 0.005 | 0.005 | 0.007 | 0.254 |
| 9 | TH-2016-CN | 0.043 | 0.018 | 0.041 | 0.044 | 0.042 | 0.043 | 0.047 | 0.053 | | 0.006 | 0.006 | 0.006 | 0.006 | 0.005 | 0.005 | 0.006 | 0.005 | 0.006 | 0.005 | 0.005 | 0.006 | 0.245 |
| 10 | TH-2018-N | 0.021 | 0.035 | 0.023 | 0.041 | 0.040 | 0.041 | 0.042 | 0.008 | 0.044 | | 0.006 | 0.007 | 0.006 | 0.005 | 0.006 | 0.005 | 0.006 | 0.004 | 0.004 | 0.004 | 0.007 | 0.247 |
| 11 | TH-2018-K | 0.042 | 0.035 | 0.036 | 0.045 | 0.041 | 0.042 | 0.045 | 0.049 | 0.043 | 0.041 | | 0.006 | 0.007 | 0.006 | 0.005 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | 0.007 | 0.224 |
| 12 | TH-2018-WVL18053-01A | 0.048 | 0.038 | 0.041 | 0.047 | 0.010 | 0.010 | 0.049 | 0.051 | 0.047 | 0.044 | 0.045 | | 0.007 | 0.006 | 0.007 | 0.007 | 0.002 | 0.006 | 0.006 | 0.006 | 0.008 | 0.231 |
| 13 | TH-2016-TV1 | 0.041 | 0.039 | 0.043 | 0.006 | 0.046 | 0.047 | 0.044 | 0.052 | 0.047 | 0.044 | 0.049 | 0.049 | | 0.005 | 0.006 | 0.005 | 0.006 | 0.005 | 0.005 | 0.005 | 0.006 | 0.251 |
| 14 | IL-2012-AD-2016 | 0.027 | 0.026 | 0.026 | 0.032 | 0.031 | 0.032 | 0.027 | 0.036 | 0.033 | 0.027 | 0.032 | 0.036 | 0.034 | | 0.004 | 0.005 | 0.005 | 0.004 | 0.004 | 0.004 | 0.006 | 0.220 |
| 15 | IL-2011-Til-4-2011 | 0.034 | 0.032 | 0.035 | 0.038 | 0.037 | 0.038 | 0.028 | 0.044 | 0.039 | 0.035 | 0.038 | 0.044 | 0.040 | 0.023 | | 0.005 | 0.006 | 0.004 | 0.004 | 0.004 | 0.006 | 0.231 |
| 16 | TH-2017 | 0.032 | 0.039 | 0.032 | 0.039 | 0.044 | 0.045 | 0.044 | 0.041 | 0.050 | 0.032 | 0.044 | 0.049 | 0.039 | 0.032 | 0.038 | | 0.006 | 0.002 | 0.005 | 0.005 | 0.007 | 0.231 |
| 17 | TH-2016-CU | 0.042 | 0.031 | 0.036 | 0.043 | 0.002 | 0.003 | 0.044 | 0.047 | 0.040 | 0.039 | 0.008 | 0.045 | 0.030 | 0.036 | 0.044 | 0.038 | | 0.006 | 0.005 | 0.005 | 0.007 | 0.223 |
| 18 | TH-2015 | 0.025 | 0.032 | 0.025 | 0.034 | 0.038 | 0.039 | 0.039 | 0.034 | 0.043 | 0.025 | 0.036 | 0.043 | 0.034 | 0.025 | 0.032 | 0.008 | 0.037 | | 0.004 | 0.004 | 0.006 | 0.231 |
| 19 | TH-2014 | 0.025 | 0.030 | 0.022 | 0.034 | 0.035 | 0.036 | 0.034 | 0.033 | 0.039 | 0.025 | 0.036 | 0.040 | 0.036 | 0.021 | 0.027 | 0.029 | 0.034 | 0.022 | | 0.001 | 0.006 | 0.227 |
| 20 | TH-2013 | 0.025 | 0.031 | 0.023 | 0.035 | 0.036 | 0.036 | 0.035 | 0.034 | 0.040 | 0.025 | 0.036 | 0.041 | 0.037 | 0.022 | 0.028 | 0.029 | 0.035 | 0.023 | 0.001 | | 0.006 | 0.230 |
| 21 | PE-2018-F3-4 | 0.055 | 0.056 | 0.053 | 0.052 | 0.055 | 0.056 | 0.029 | 0.082 | 0.061 | 0.056 | 0.056 | 0.061 | 0.054 | 0.043 | 0.044 | 0.053 | 0.055 | 0.047 | 0.047 | 0.047 | | 0.223 |
| 22 | Inf_C/Ann_Arbor/1/50 | 2.348 | 2.331 | 2.327 | 2.431 | 2.319 | 2.297 | 2.301 | 2.507 | 2.429 | 2.425 | 2.366 | 2.375 | 2.441 | 2.288 | 2.342 | 2.334 | 2.319 | 2.345 | 2.324 | 2.334 | 2.296 | |

**b) Within Clade Mean Distance**

| | Clade | d | SE |
|---|---|---|---|
| 1 | Israeli-2011 clade | 0.033 | 0.004 |
| 2 | Israeli-2012 clade | NA | NA |
| 3 | Thai clade | 0.035 | 0.004 |
| 4 | Outgroup | NA | NA |

**c) Between Clade Mean Distance**

| | Clade | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| 1 | Israeli-2011 clade | | 0.004 | 0.004 | 0.075 |
| 2 | Israeli-2012 clade | 0.03 | | 0.003 | 0.075 |
| 3 | Thai clade | 0.044 | 0.029 | | 0.078 |
| 4 | Outgroup | 1.147 | 1.138 | 1.171 | |

**Figure S1** Multiple alignment of the deduced amino acid sequences of TiLV segment 1 PB1 coding genes of 21 viral isolates. Length of amino acid sequence is indicated on the right margin. *marks amino acid identity. The shaded amino acid sequences represent putative PB domains while the 4 predicted RdRp motifs are marked by boxes (I, II, II, and IV). Arrows indicate positions and orientation of the primers used in the newly developed TiLV detection assay.

22

471



**Figure S2** Specificity of the semi-nested TiLV detection assays. M, 2-log DNA marker (New England Biolabs); P, positive control using RNA extracted from TiLV-infected fish tissue as template; N, no template control; S, Scale Drop Disease Virus (SDDV); V, Nervous Necrosis Virus (NNV); I, Infectious Spleen and Kidney Necrosis Virus (ISKNV); N1/N2, clinically healthy Nile tilapia; R1/R2, clinically healthy red tilapia; Vo, *Vogesella* sp.; Ps, *Pleisiomonas shigelloides;* Ac, *Aeromonas caviae;* Aj, *Aeromonas jandaei;* Sa, *Streptococcus agalactiae*; Ch, *Chryseobacterium* sp.; Et, *Edwardsiella tarda*; Vc, *Vibrio cholerae*; Si, *Streptococcus iniae*; Ei, *E. ictaluri*; Av, *Aeromonas veronii*; Fc, *Flavobacterium columnare*; Fn, *Francisella noatunensis* subsp. *orientalis*. Details of bacterial isolates are shown in Table 2.