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Research Note

Phylogenetic Analysis of Tilapia Lake Virus (TiLV) Isolates from the Philippines Based on Partial Genome Segment 3 Sequences

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This study aimed to expand current knowledge on TiLV genetic diversity by sequence analysis of a portion of genome segment 3 of TiLV detected from the Philippines. This includes a recently deposited sequence in GenBank (Accession No. LC504279) and six new sequences from cases of infection reported from 2017 to 2020. Phylogenetic analysis of 179 bp fragment of segment 3 showed that the seven TiLV isolates from the Philippines can be divided into three phylogenetic groups. When comparing all sequences, unique nucleotide substitutions and amino acids were noted among these groups. Variation in mortality rates in naturally infected samples was also observed; however, poor environmental conditions during the disease outbreak may also contribute to the mortalities. These suggest the presence of at least three phylogenetic groups of TiLV in the Philippines which has significant implications for the future development of a vaccine, diagnostic kits, and genetic selection programs.

Keywords: phylogenetics, Tilapia Lake Virus, segment 3, Philippines

Abbreviations: cDNA—complementary DNA, ML—maximum likelihood, OIE—World Organisation for Animal Health, PCR—polymerase chain reaction, TiLV—tilapia lake virus

INTRODUCTION

Tilapia lake Virus (TiLV) is recognized as a newly emerging pathogen causing mortalities in wild (*Sarotherodon galilaeus, Tilapia zilli, Oreochromis aureus, Tristamellasimonis intermedia*) (Eyngor et al. 2014) and cultured tilapias (*Oreochromis* spp.) (Eyngor et al. 2014; Ferguson et al. 2014; Dong et al. 2017; Surachetpong et al. 2017; Nicholson et al. 2017; Amal et al. 2018; Behera et al. 2018; Koesharyani et al. 2018; Ahasan et al. 2020) and as such, it poses a threat to the global tilapia industry (Surachetpong et al. 2020). Clinical signs of the disease include loss of appetite, pale color (Dong et al. 2017), ocular alterations, skin erosions, congestion of spleen and kidney (Eyngor et al. 2014), lethargy, bilateral exopthalmia, and abdominal swelling (Behera et al. 2018). In terms of histopathology, swollen and dissociated hepatocytes, congested blood vessel in the brain and syncytial giant cells can be observed (Dong et al. 2017; Behera et al. 2018). Furthermore, a brightly eosinophilic or brown lippoproteinaceous material ranging from 1–2 mm up to 5–6 mm can be found in the hepatocytes and syncytial cells of infected fish (Ferguson et al. 2014). In the Philippines, two TiLV disease reports were submitted in 2017 and 2019 to the World Organisation for Animal Health (OIE). The first outbreak occurred in Bulacan, Central Luzon, the Philippines where an estimated mortality of 34% in the tilapia fingerlings was noted. Clinical signs in diseased fish included distended abdomen and bulging eyes (OIE 2017). The second report

covered 3 cases in Pampanga, also in Central Luzon and Batangas, a province in Southern Luzon where TiLV was detected both in fingerlings and adult fish. No mortalities nor clinical signs were observed in the infected fish in Pampanga. However, in Batangas, bulging of eyes, distended abdomen, and erratic swimming behavior were noted (OIE 2019).

A challenging area in TiLV genetics and epidemiology is the lack of genetic information particularly for isolates detected in countries with reported cases of infection. TiLV has a negative-sense RNA genome consisting of 10 segments and the genome size is 10,323 nucleotides (Bacharach et al. 2016). Segment 1 which is the largest has a weak sequence homology to the polymerase subunit (PB1) of influenza C virus while the other segments showed no homology to other viruses (Kembou Tsofack et al. 2017). Segment 3 is 1,371 bp with an open reading frame (ORF) of 419 amino acids (Acharya et al. 2019). In this study, the amplification targets are located within the ORF of segment 3 using primers described by Dong et al. (2017). Genomic information will give important insights into the genetic relationship between isolates and the variation in virulence which may elucidate the differences observed in clinical signs and host susceptibilities (Surachetpong et al. 2020). In the Philippines, so far there is only one partial TiLV gene sequence (Accession No. LC504279) deposited in GenBank and there has been no in-depth molecular analysis on the same. In this research, we investigated the partial segment 3 sequences of seven TiLV isolates from the Philippines. This includes the recently deposited sequence in GenBank and six new sequences from cases of infection reported from 2017 to 2020. The main aim of our study is to expand the current knowledge on TiLV genetic diversity.

MATERIALS AND METHODS

Seven positive samples were collected from various provinces in the Philippines (Fig. 1). These were obtained from diagnostic investigations of tilapia disease (Agusan del Sur, Angat Dam - Isolate 1, Angat Dam – Isolate 2, Pulilan), screening for health certificate or prior to stocking (Calauan, Nueva Ecija), and wild fish monitoring (Laguna de Bay). All of these were sampled and sequenced by the researchers except Pulilan in which the sequence and some of the epidemiological information were retrieved from from GenBank and OIE (2017), respectively.

The epidemiological data on the isolates are summarized in Table 1. Samples from Angat Dam, Calauan, and Agusan del Sur were initially tested positive for TiLV using insulated isothermal PCR (iiPCR) (GeneReach Biotechnology Corp.). Total nucleic acids were isolated using PetNAD Nucleic Acid Co-prep kit (GeneReach Biotechnology Corp.) and were further processed for reverse transcription (SensiFAST cDNA Synthesis Kit, Bioline Reagents Ltd.) followed by semi-nested PCR using Vivantis Technologies Sdn. Bhd. (Selangor Darul Ehsan, Malaysia) according to the method described by Dong et al. (2017). For the samples from Laguna de Bay and Nueva Ecija, nucleic acids were extracted using TRIzol reagent (Thermo Fisher Scientific) and tested using the same PCR protocol. Amplicons (250 bp) were submitted to 1st BASE DNA Sequencing Services (1st Base Laboratories, Kuala Lumpur, Malaysia) for gel purification and unidirectional Sanger sequencing. The chromatogram was viewed, edited, and checked for miscalled nucleotides using Sequence Scanner Version 2 (Applied Biosystems). Sequences (179 bp) from this study were aligned with the sequences of isolates from other countries (as downloaded from GenBank; www.ncbi.nlm.nih.gov/genbank) using ClustalW in BioEdit (Hall 1999). Ends of multiple sequence alignment were further trimmed using MEGA 6 (Tamura et al. 2013) and visualized employing BOXSHADE 3.21 (https://embnet.vital-it.ch/software/BOX_form.html). A Maximum Likelihood (ML) phylogenetic tree was constructed using PhyML (Guindon and Gascuel 2003) based on the best-fit

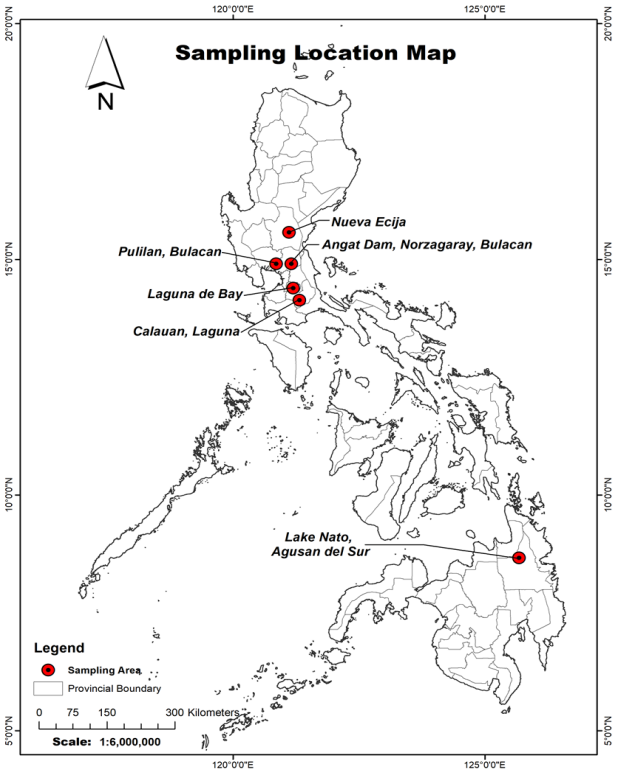

Fig. 1. Map of the Philippines highlighting the sampling sites for TiLV isolates used in this study.

Table 1. Epidemiological data of TiLV isolates from the Philippines used in this study.

Sample Name (Code)	Location	Region	Stage of Infected Fish	Culture Type	Source of Breeders	Clinical Signs	Histopathology	Estimated Mortality	Detection Method/s	GenBank Accession Number*
Agusan del Sur (AGS)	Lake Nato, Agusan del Sur	CARAGA (Region XIII)	Adult	Cage culture	Nueva Ecija	Daily mortalities observed after stocking Erratic swimming behavior, haemorrhages, exopthalmia, abdominal distention, ulcerations, scale loss and fin rot Parasitic infestation (*Trichodina spp.; gill flukes)* Subclinical	Not done	50-70%	Insulated isothermal PCR (iiPCR) (GeneReach Biotech. Corp.) Semi-nested PCR (Dong et al., 2017)	OK274112
Nueva Ecija (NE)	Nueva Ecija	Central Luzon (Region III)	Fry	Hatchery	Nueva Ecija	Parasitic infestation (*Trichodina spp. in mucus only)*	Not done	None	Semi-nested PCR (Dong et al., 2017)	OK274110
Laguna de Bay (LDB)	Laguna de Bay	CALABARZON (Region IV-A) NCR (National Capital Region)	Adult	Wild	Laguna de Bay, Nueva Ecija	Subclinical	Suspected inclusion bodies in the liver	None	Semi-nested PCR (Dong et al., 2017)	OK274111
Calauan (CAL)	Calauan, Laguna	CALABARZON (Region IV-A)	Fingerlings	Hatchery	Nueva Ecija	Subclinical	Not done	None	iiPCR Semi-nested PCR (Dong et al., 2017)	OK274115
Pulilan** (PUL)	Pulilan, Bulacan	Central Luzon (Region III)	Fingerlings	Nursery	Nueva Ecija	Elevated mortality after 15 days of stocking reaching approximately 25% Bulging of eyes and distended abdomen	Eosinophilic intracytoplasmic inclusion bodies in liver and spleen	34%	iiPCR Semi-nested PCR (Dong et al., 2017)	LC504279
Angat Dam Isolate 1 (ANG1) Angat Dam Isolate 2 (ANG2)	Norzagaray, Bulacan	Central Luzon (Region III)	Juvenile & adult	Wild	Nueva Ecija, Laguna, Bulacan	Bulging of eyes and distended abdomen	Eosinophilic intracytoplasmic inclusion bodies in the liver	28-33%	iiPCR Semi-nested PCR (Dong et al., 2017)	OK274113 OK274114

*GenBank accession numbers of partial segment 3 sequences amplified from TiLV samples detected in this study

**In this sample, sequence and epidemiological information except histopathological findings and source of breeders were retrieved from GenBank and OIE (2017), respectively.

substitution model determined by FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel .html) and confidence in the nodes was assessed by bootstrapping (1,000 reiterations). The ML tree was viewed using FigTree v1.4.4.exe (Rambaut 2018) and rerooted using the outgroup (Influenza A virus; GenBank MT318434.1). ExPaSy translate tool (http://expasy.org/tools/dna.html) was used to deduce amino acid sequences. All new sequences were deposited in the GenBank under accession numbers OK274110 to OK274115.

RESULTS AND DISCUSSION

Results showed that the seven TiLV isolates from the Philippines can be divided into three phylogenetic groups based on the ML tree generated using a 179 bp fragment of segment 3 (Fig. 2). Group 1 composed of Laguna de Bay, Agusan del Sur, and Nueva Ecija isolates while Group 2 comprised of Pulilan and Calauan isolates. The Angat Dam isolates formed a separate clade under Group 3. Group 3 isolates had the highest number of nucleotide substitutions (Fig. 3-A).

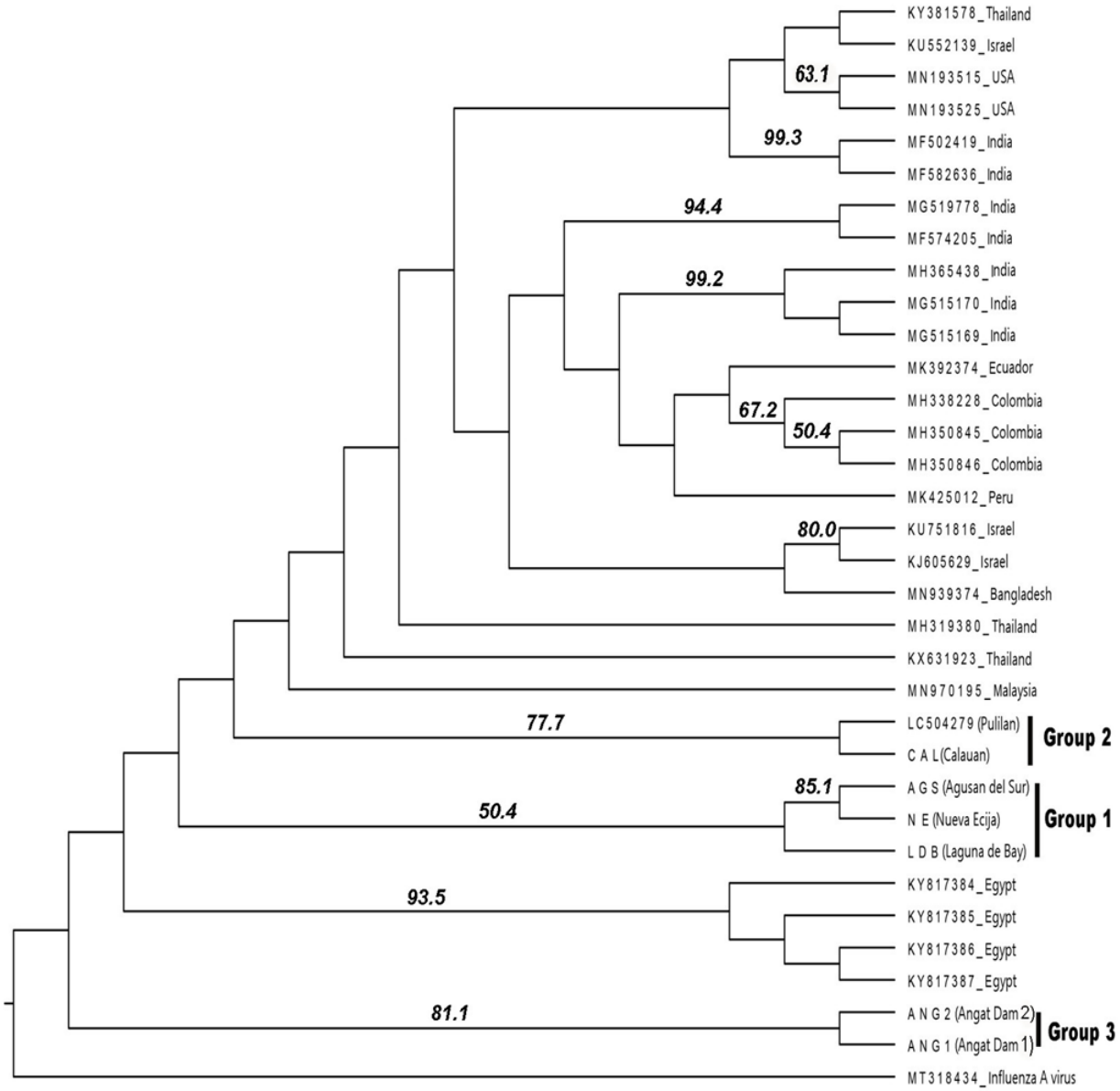

Fig. 2. Maximum Likelihood phylogenetic tree generated from 179 bp sequences within segment 3. ML tree was constructed using PhyML based on Kimura 2- parameter model plus gamma (discrete gamma model; 4 categories; gamma shape parameter = 0.240). The numbers on the branches represent bootstrap values expressed in percentage out of 1,000 replicates. Values with > 50% were shown.

All eight mutations (positions 16, 25, 40, 52, 76, 79, 116, 139) were unique to the group although only one of these was non-synonymous. Group 1 and 2 had two (positions 4, 55) and three (positions 11, 85, 130) nucleotide substitutions that were unique to the group, respectively. There were amino acid residues unique for Group 2 and 3 namely arginine (position 4) and serine (position 39), respectively (Fig. 3-B). The specific bootstrap support values for Group 1, Group 2, and Group 3 clades were 50.4%, 77.7%, and 81.1%, respectively. Moreover, it can be inferred from the ML tree that Groups 1 and 3 had a closer genetic relationship with Egyptian isolates.

We also studied the possible relationship between the phylogenetic groups and epidemiological data, specifically the geographic location and mortality rates observed in the naturally infected samples. Isolates classified under Groups 1 and 2 were scattered in various provinces in the country. In Group 1, for instance, Nueva Ecija and Agusan del Sur clustered together in one clade with a bootstrap support value of 85.1%. Nueva Ecija is a province located in the northern part of the Philippines while the latter is situated in the southernmost region (Fig. 1). According to BFAR, there were transports of tilapia from Nueva Ecija to Agusan del Sur. Variation in

Figure 3 - A

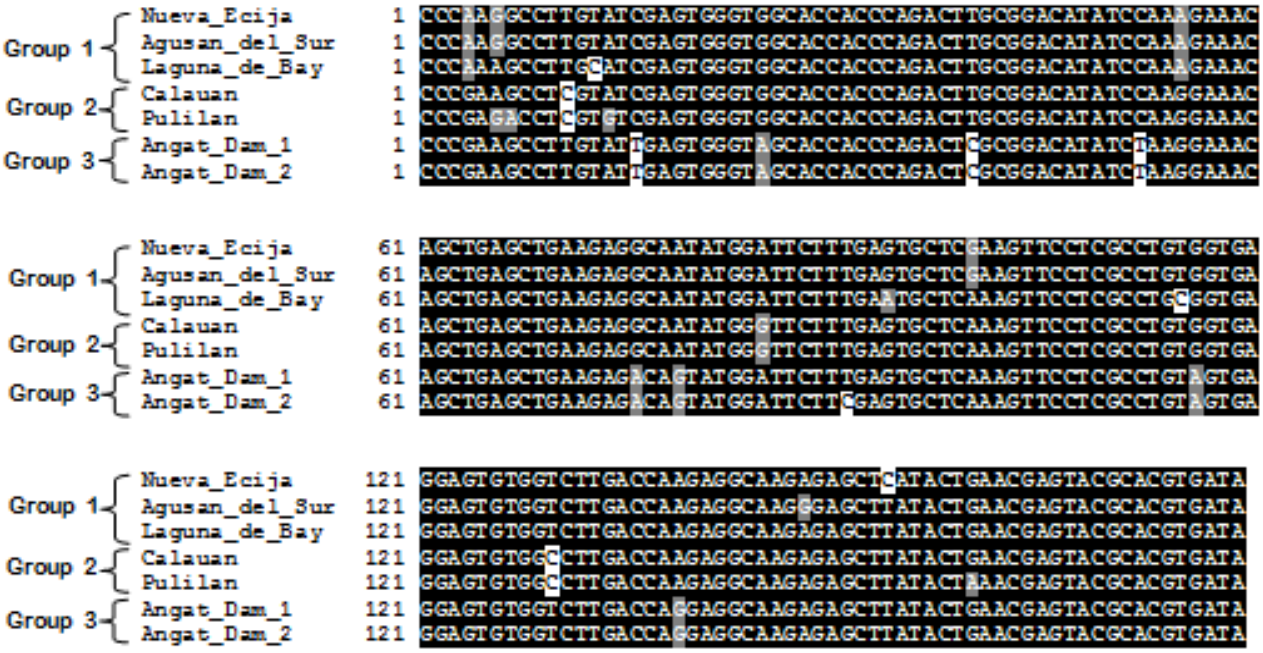

Figure 3 - B

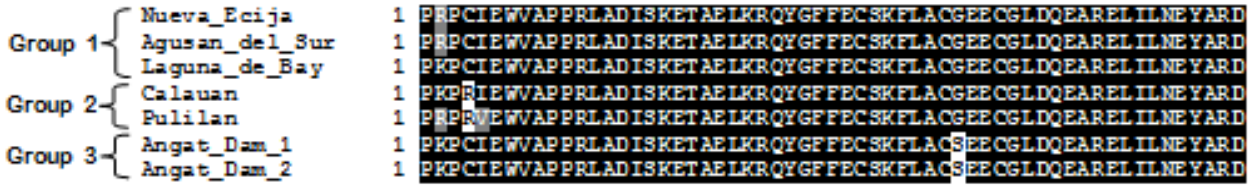

Fig. 3. Multiple sequence alignment of nucleotide (3-A) and deduced amino acid sequences (3-B) among TiLV isolates from the Philippines based on the 179 bp sequences within segment 3. The alignment was produced with ClustalW and edited with Boxshade. The conserved regions are highlighted in black whereas changes in nucleotides and amino acids are highlighted in white and grey.

mortality rates was observed for different phylogenetic groups. Groups 1 and 2 were detected from diseases with estimated mortality of 50-70% (Agusan del Sur) and 34% (Pulilan), respectively; whereas, Angat Dam recorded an estimated mortality of 28-33%. Variation in fish mortality has been reported for TiLV (Surachetpong et al. 2020). This is maybe due to the genetic diversity of the virus isolates, host susceptibility, environmental factors, co-infection, or a combination of these (Jansen et al. 2019). In this study, poor environmental conditions contributed to the fish mortality as extremely warm weather, episodes of rainfall, turbid, and low water levels were noted in the case history for the disease outbreak area. These conditions may induce a stress response making tilapia more susceptible to the virus. Tilapia's age and genotype may also influence the observed mortality. Similarly, TiLV was detected in Egypt and Israel during the hot season in which mass mortalities of tilapia were observed (Eyngor et al. 2014; Nicholson et al. 2017). Bangladesh also noted that the majority of the TiLV outbreaks occurred during months with wide temperature fluctuations brought about by sudden heavy rainfall

(Debnath et al. 2020). Although, other factors such as tilapia strains (Ferguson et al. 2014), co-infection with bacteria (Nicholson et al. 2017; Amal et al. 2018; Nicholson et al. 2020) and other pathogens could play a role in morbidity and mortality.

TiLV genome was detected in subclinical fish (Table 1). It has been noted that TiLV was detected in fish obtained from a tilapia breeding center. This may suggest the possibility of TiLV pathway transmission from a breeding center to a farm with uninfected fish stocks. Hence, it is strongly recommended that comprehensive protocols for screening tilapia stocks such as the use of a highly sensitive diagnostic test, certification of tilapia hatcheries, active surveillance, and biosecurity measures be strictly implemented. Furthermore, tilapia hatcheries should ensure that their potential and current breeding stocks are tested for TiLV since previous studies demonstrated vertical transmission of the virus (Dong et al. 2020; Yamkasem et al. 2019). This is to prevent the production and dissemination of infected fry for use by small-scale farmers.

CONCLUSION

Studying genetic diversity of TiLV isolates in the country is imperative for the prevention and control of the disease. The identification of the three phylogenetic groups of the virus has significant implications for the future development of diagnostic kits, vaccines, and genetic improvement programs selecting for disease resistance. Further work must also focus on the investigation of genetic variation, origin, and transmission of TiLV in wild and domesticated tilapias in the Philippines based on whole-genome sequencing.

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