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RESEARCH ARTICLE

Phylogenomic characterization of ranaviruses isolated from cultured fish and amphibians in Thailand

Preeyanan Sriwanayos[ab], Kuttichantran Subramaniam[a], Natalie K. Stilwell[ac], Kamonchai Imnoi[a], Vsevolod L. Popov[d], Somkiat Kanchanakhan[be], Jaree Polchana[b], and Thomas B. Waltzek[a]*

[a]Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA; [b]Aquatic Animal Health Research and Development Division, Department of Fisheries, Bangkok 10900, Thailand; [c]Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA; [d]Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA; [e]Department of Fisheries, Chonburi Provincial Fishery Office, Chonburi 20000, Thailand

*tbwaltzek@ufl.edu

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Abstract

Ranaviruses are emerging pathogens associated with worldwide epizootics in farmed and wild ectothermic vertebrates. In this study, we determined the full genomes of eight ranaviruses isolated from marbled sleeper goby (*Oxyeleotris marmorata*), goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*), tiger frog (*Hoplobatrachus tigerinus*), Asian grass frog (*Fejervarya limnocharis*), and East Asian bullfrog (*H. rugulosus*) cultured or imported into Thailand. These ranaviral isolates induced the same cytopathic effects (i.e., progression of coalescing round plaques) in *epithelioma papulosum cyprini* (EPC) cell cultures. Transmission electron microscopy of infected EPC cells revealed cytoplasmic viral particles with ultrastructural features typical for ranaviruses. Pairwise genetic comparisons of the complete major capsid protein coding sequences from the Thai ranaviruses displayed the highest identity (99.8%–100%) to a ranavirus (tiger frog virus; TFV) isolated from diseased tiger frogs cultured in China, a slightly lower identity (99.3%–99.4%) to a ranavirus (Wamena virus; WV) isolated from diseased green tree pythons (*Morelia viridis*) illegally exported from Papua New Guinea, and a lower identity to 35 other ranaviruses (93.7%–98.6%). Phylogenomic analyses supported the eight Thai ranaviruses, Chinese TFV, and WV as a subclade within a larger frog virus 3 clade. Our findings confirm the spread of TFV among cultured fish and amphibians in Asia and likely in reptiles in Oceania. Biosecurity measures are needed to ensure TFV does not continue to spread throughout Southeast Asia and to other parts of the world via international trade.

Key words: iridovirus, phylogenomics, ranavirus, Thailand, tiger frog virus

Introduction

Globally, aquaculture is an essential food-producing sector and it has played a crucial role in the Thai economy for decades (Food and Agriculture Organization of the United Nations 2020). Between 1998 and 2017, Thailand's average annual aquaculture production was approximately 1.1 million tons per year, with freshwater aquaculture production contributing nearly half of the annual production (Department of Fisheries Thailand 2019a). Of the total Thai freshwater aquaculture production in 2017, 4000 tons were contributed from ranaculture products including live frogs, chilled and frozen

frog legs, and whole frogs (Department of Fisheries Thailand 2019b). However, diseases including those caused by ranaviruses have emerged as an impediment to the continued growth of ranaculture around the world including Asia (Zhang et al. 2001; Weng et al. 2002), North America (Miller et al. 2007), and South America (Mazzoni et al. 2009).

Members of the genus *Ranavirus* (family *Iridoviridae*; subfamily *Alphairidovirinae*) possess an enveloped nucleocapsid (150–200 nm in diameter) that encloses a double-stranded DNA genome (103–220 kbp) (Chinchar et al. 2017). The genus includes the following species recognized by the International Committee on Taxonomy of Viruses: *Frog virus 3* (FV3), *Ambystoma tigrinum virus*, *Common midwife toad virus*, *Epizootic haematopoietic necrosis virus* (EHNV), *Santee-Cooper ranavirus*, and *Singapore grouper iridovirus* (International Committee on Taxonomy of Viruses 2020). Ranaviruses are emerging pathogens infecting at least 175 species and 52 families of ectothermic vertebrates (fish, amphibians, and reptiles) across six continents (Duffus et al. 2015). FV3 has the widest-known host and geographic range of all ranaviruses and includes Asian strains isolated from diseased fish, amphibians, and reptiles reared for food in China and Thailand. FV3 strains have negatively impacted the culture of tiger frogs (*Hoplobatrachus tigerinus*) and East Asian bullfrogs (*H. rugulosus*) in Thailand and China (Kanchanakhan et al. 1998, 1999; Weng et al. 2002), Chinese softshell turtles (*Pelodiscus sinensis*) in China (Chen et al. 1999), pig frogs (*Lithobates grylio*) in China (Zhang et al. 2001), and marbled sleeper goby (*Oxyeleotris marmorata*) in Thailand (Prasankok et al. 2005). Ranaviruses have been identified as a global threat to free-ranging amphibian populations as they have the potential to cause population declines and extinctions (Duffus and Cunningham 2010; Teacher et al. 2010; Miller et al. 2011; Earl and Gray 2014; Price et al. 2014; Peace et al. 2019). Ranaviral infections in amphibians and EHNV infection in rainbow trout (*Oncorhynchus mykiss*) and redfin perch (*Perca fluviatilis*) are notifiable to the World Organization for Animal Health (2019).

Over the last two decades, strains of FV3 have repeatedly been isolated during mortality events in ranaculture and aquaculture facilities in Thailand. From 1998 to 2002, ranaculture facilities in nine provinces in the central part of Thailand experienced moderate (20%–50%) to high (95%) mortalities in cultured tadpoles and juvenile and adult tiger frogs (Kanchanakhan 1998, 2011; Kanchanakhan et al. 1999). Diseased frogs displayed ulcerative lesions on the rostrum (lips and mouth) and dorsal part of the body and legs. Bacteria were not isolated from the liver, kidney, and spleen in the early stages of the disease; however, bacteria were cultured from frogs that experienced chronic high mortality infections. Skin and internal tissue homogenates from diseased frogs and tadpoles were processed for viral isolation in *epithelioma papulosum cyprini* (EPC) cells and resulted in the recovery of passageable agents in 65% (70/107) of the individuals tested from 1998 to 2002 (Kanchanakhan 2011). Physicochemical and ultrastructural features of the isolates supported them as members of the family *Iridoviridae*. In 2004, high mortality (>50%) in Asian grass frogs (*Fejervarya limnocharis*) was reported on a private fish farm in Northeast Thailand following importation from Cambodia (Kanchanakhan et al. 2004). The diseased frogs showed skin ulcerations on the rostrum and legs. A replicating agent was isolated in EPC cultures and determined to be an iridovirus based on its physicochemical and ultrastructural features. PCR amplification and sequencing of the iridoviral partial major capsid protein (MCP) and ATPase genes demonstrated it was nearly identical (98%–99%) to a strain of FV3 known as tiger frog virus (TFV), previously isolated from diseased tiger frog tadpoles cultured in China in 2000 (Weng et al. 2002) and hereafter referred to as Chinese TFV. In 2000, high mortalities in cultured marbled sleeper goby were reported in the Nakonpathom province of Central Thailand (Prasankok et al. 2005). Fish displayed ulcerative lesions on the body and around the mouth. No consistent parasites or bacteria were observed or cultured from the diseased fish. A replicating agent was isolated in EPC cultures and again determined to be an iridovirus based on its physicochemical and ultrastructural features.

PCR amplification and sequencing of the iridoviral partial MCP gene demonstrated the isolate was nearly identical (98%–99%) to the aforementioned Chinese TFV and Thai ranaviruses isolated from imported Asian grass frogs and cultured tiger frogs.

Herein, we describe the in vitro growth (i.e., observed cytopathic effects), ultrastructural, and phylogenomic characteristics of ranaviruses isolated from fish and amphibians cultured in Thailand for food or ornamental purposes. The determined complete genome sequences are the first to be publicly available for ranaviruses isolated from Thai ectothermic vertebrates. This genomic data was used in phylogenomic analyses that revealed TFV, a strain of FV3, has spread among facilities in Asia rearing fish and amphibians, and perhaps also in free-ranging reptiles in Oceania.

Materials and methods

Clinical history

From 1998 to 2017, a series of ranaviruses was isolated from diseased fish, tadpoles, and frogs submitted by Thai aquaculture and ranaculture facilities to the Aquatic Animal Health Research and Development Division (AAHRDD), Department of Fisheries, Bangkok, Thailand. Details of AAHRDD diagnostic evaluations (e.g., bacteriology, virology, transmission electron microscopy, and conventional PCR) that resulted in ranaviral isolates from tiger frogs (isolate AV9803) in 1998, marbled sleeper goby in 2000 (isolate D2008) (Fig. 1A), and imported Asian grass frogs in 2004

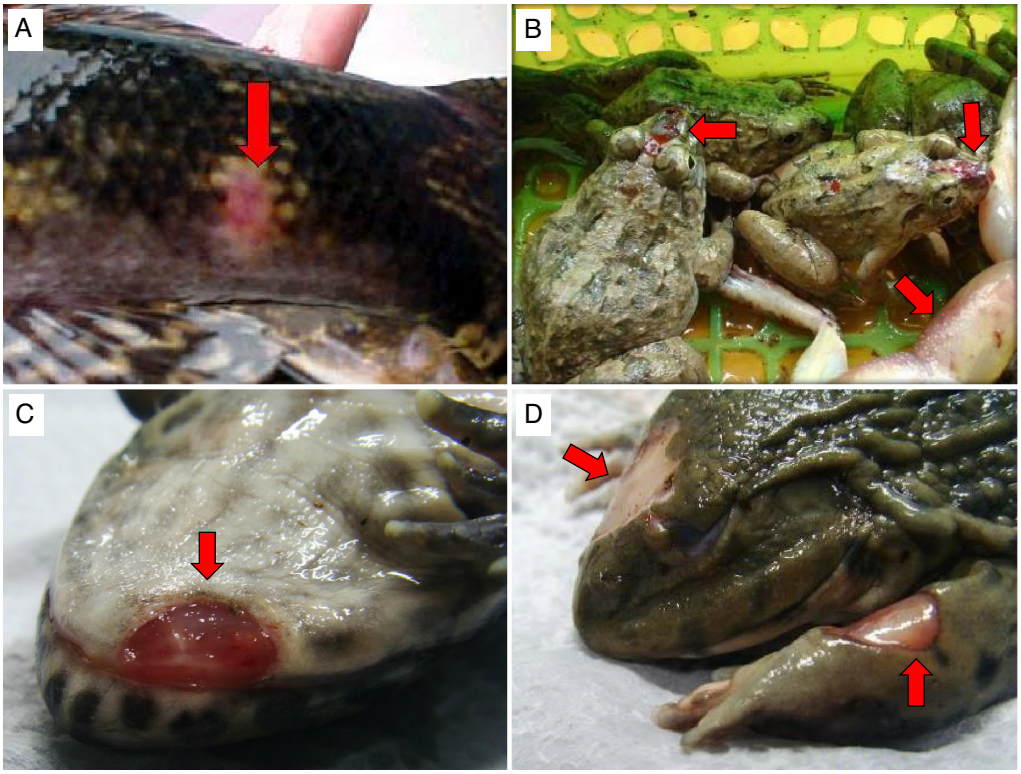

Fig. 1. Gross lesions in TFV-infected fish and frogs. (A) Marbled sleeper goby (*O. marmorata*) displaying cutaneous ulceration of the caudal peduncle (arrow). (B) Asian grass frogs (*F. limnocharis*) and (C, D) East Asian bullfrogs (*H. rugulosus*) displaying cutaneous ulcerations on the rostrum (lips and mouth) and dorsum of the head, body, and legs (arrows).

Table 1. Transmission electron microscopy results for the seven Thai ranaviral isolates (excluding VD-17-007).

Viral name (abbreviation)	Isolate	Host	Naked virion apex–apex (nm), mean (SD)	Naked virion side-side (nm), mean (SD)	Enveloped virion apex–apex (nm), mean (SD)	Enveloped virion side–side (nm), mean (SD)
Tiger frog virus (TFV-1998)	AV9803	Tiger frog (*Hoplobatrachus tigerinus*)	157.3 (2.1)	132.5 (3.2)	196.5 (4.9)	187.3 (5.7)
Oxyeleotris marmorata ranavirus (OMRV)	D2008	Marbled sleeper goby (*Oxyeleotris marmorata*)	158.8 (1.2)	127.6 (1.6)	201.3 (2.8)	186.3 (7.6)
Poecilia reticulata ranavirus (PPRV)	F2112	Guppy (*Poecilia reticulata*)	158.2 (1.6)	123.5 (2.3)	203.7 (4.0)	187.3 (3.7)
Goldfish ranavirus (GFRV)	F0207	Goldfish (*Carassius auratus*)	150.2 (1.2)	125.5 (1.6)	185.5 (1.5)	164.3 (3.1)
Asian grass frog ranavirus (AGFRV)	D03-034	Asian grass frog (*Fejervarya limnocharis*)	158.7 (1.0)	128.9 (1.4)	201.7 (2.1)	185.3 (3.8)
East Asian bullfrog ranavirus (EABRV-2011)	D11-067	East Asian bullfrog (*H. rugulosus*)	158.8 (1.3)	130.4 (1.6)	194.9 (4.5)	177.6 (5.6)
East Asian bullfrog ranavirus (EABRV-2016)	VD-16-006	East Asian bullfrog (*H. rugulosus*)	158.9 (1.7)	129.8 (2.7)	206.0 (3.3)	185.8 (4.0)
East Asian bullfrog ranavirus (EABRV-2017)	VD-17-007	East Asian bullfrog (*H. rugulosus*)	NO	NO	NO	NO

Note: Means (±standard deviation, SD) are based on the measurement of 20 unenveloped virions and 3–16 enveloped virions per isolate. NO, not observed.

(isolate D03-034) (Fig. 1B) have previously appeared (Kanchanakhan 1998, 2011; Kanchanakhan et al. 1999, 2004; Prasankok et al. 2005). Ranaviruses isolated by AAHRDD during a health assessment of overtly healthy guppies (*Poecilia reticulata*) in 2001 (isolate F2112) and goldfish (*Carassius auratus*) in 2002 (isolate F0207) were also included in this study (S. Kanchanakhan, personal observation, 2001, 2002). In 2011 and 2016, Thai facilities rearing adult East Asian bullfrogs reported elevated mortality with moribund frogs displaying ulcerative lesions on the dorsal part of the body and legs (Figs. 1C and 1D). In 2017, a Thai facility rearing East Asian bullfrog tadpoles reported elevated mortality with moribund tadpoles displaying cutaneous ulcerations and edema (J. Polchana, personal observation, 2017). Samples from these East Asian bullfrog tadpoles and frogs were submitted to AAHRDD for virology and resulted in three additional ranaviral isolates (D11-067, VD16-006, and VD17-007) used in this study. The Thai ranaviral isolates analyzed in this study and their respective host and temporospatial details are provided in Table 1.

In vitro propagation of ranaviruses

Eight presumed ranaviral isolates recovered at AAHRDD from cultured frogs and fish from 1998 through 2017 were shipped to the University of Florida Wildlife and Aquatic Veterinary Disease Laboratory (WAVDL), Gainesville, Florida, USA (Table 1). At WAVDL, 1 mL of each isolate was inoculated onto a confluent monolayer of EPC cells within 75 cm^2 flasks maintained in modified Eagle's medium with 10% fetal bovine serum. Inoculated EPC cultures were maintained at 25 °C and observed daily for cytopathic effects (CPE).

Transmission electron microscopy (TEM)

Seven of the isolates (VD-17-007 was not submitted for TEM) were individually propagated in 75 cm^2 flasks of EPC cells until CPE was observed. The cells were fixed in 15 mL of modified Karnovsky's fixative (2P + 2G, 2% formaldehyde prepared from paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L cacodylate buffer pH 7.4) at room temperature for 1 h. Then the monolayer was washed in cacodylate buffer, scraped off the flask, and pelleted (3000*g* at 4 °C for 10 min). The pellet was shipped in phosphate-buffered saline overnight on ice packs to the University of Texas Medical Branch Department of Pathology Electron Microscopy Laboratory (UTMB-EML). At UTMB-EML, the cell pellet was washed in cacodylate buffer and left in 2P + 2G fixative overnight at 4 °C. The next day the cell pellet was washed twice in cacodylate buffer, postfixed in 1% OsO_4 in 0.1 mol/L cacodylate buffer pH 7.4, en bloc stained with 2% aqueous uranyl acetate, dehydrated in ascending concentrations of ethanol, processed through propylene oxide, and embedded in Poly/Bed 812 epoxy plastic (Polysciences). Ultrathin sections were cut on a Leica ULTRACUT EM UC7 ultramicrotome (Leica Microsystems), stained with 0.4% lead citrate, and examined in a JEM-1400 electron microscope (JEOL USA) at 80 kV.

DNA extraction, whole genome sequencing and assembly

Each viral isolate was inoculated into two 75 cm^2 flasks of EPC cells and harvested when extensive CPE was observed (48–72 h postinfection). Then, the cells were subjected to three rounds of freezing–thawing prior to clarification of the supernatant by centrifugation at 5520*g* for 20 min at 4 °C. The clarified supernatant was then centrifuged at 100 000*g* for 1.15 h at 4 °C to obtain a viral pellet. Pelleted virus was then resuspended in 360 µL of animal tissue lysis (ATL) buffer prior to extraction of viral genomic DNA using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instruction. DNA sequencing libraries were generated using a TruSeq DNA PCR-Free Library Preparation kit (Illumina) and sequencing was performed using a v3 chemistry 600 cycle kit on a MiSeq sequencer (Illumina). De novo assembly of the paired-end reads was performed in SPAdes 3.5.0 (Bankevich et al. 2012). The quality of the genome assembly was verified by mapping the reads back to the consensus sequence in Bowtie2 2.1.0 (Langmead and Salzberg 2012) and visually inspecting the alignment in Tablet 1.14.10.20 (Milne et al. 2013).

Genome annotation and phylogenomic analyses

The genomes of the Thai ranaviruses were annotated using the Genome Annotation Transfer Utility (Tcherepanov et al. 2006) with TFV (GenBank Accession No. AF389451.1) used as the reference genome. Putative open reading frames (ORFs) were added or removed by comparison to the FV3 genome annotation (Tan et al. 2004) and using GenemarkS (Besemer et al. 2001) and CLC Genomics Workbench 12.0 based on the following criteria: (*i*) only ORFs larger than 120 nucleotides were considered; (*ii*) ORFs were not allowed to overlap with neighboring ORFs by more than 25%; and (*iii*) in the event of overlapping, only the larger ORF was annotated or overlapping ORFs were permitted provided they represent existing ranaviral orthologs (Bennett et al. 2017; Koda et al. 2018). Gene function was predicted based on BLASTP searches against the National Center for Biotechnology Information GenBank nonredundant protein sequence database. The eight Thai ranaviral genomes and 36 fully sequenced ranaviral genomes retrieved from GenBank (**Table 2**) were aligned using Mauve 2.4 software (Darling et al. 2004) to visualize genomic inversions and obtain the locally collinear block (LCB) alignments. The LCB alignments were then concatenated in Geneious v.10.0.2 (Kearse et al. 2012) and a Maximum Likelihood (ML) analysis was performed in IQ-TREE (http://iqtree.cibiv.univie.ac.at/) with the Bayesian information criterion used to determine the best model fit and 1000 nonparametric bootstraps to determine node support (Nguyen et al. 2015). To elucidate the relationship of Thai ranaviruses to the Wamena virus (WV), for which only

Table 2. Viral names, abbreviations, and GenBank accession numbers of the ranaviruses used in the phylogenetic and genetic analyses.

Viral name	Viral abbreviation	GenBank Accession No.
Frog virus 3	FV3	AY548484
Tiger frog virus	TFV	AF389451
Rana grylio iridovirus	RGV	JQ654586
Soft-shelled turtle iridovirus	STIV	EU627010
Bohle iridovirus	BIV	KX185156
German gecko ranavirus	GGRV	KP266742
Ambystoma tigrinum virus	ATV	AY150217
Epizootic haematopoietic necrosis virus	EHNV	FJ433873
European sheatfish virus	ESV	JQ724856
Common midwife toad virus	CMTV-E	JQ231222
Common midwife toad virus	CMTV-NL	KP056312
Testudo hermanni ranavirus	THRV-CH8/96	KP266741
Tortoise ranavirus isolate 1	ToRV1	KP266743
Frog virus 3 isolate SSME	SSME	KJ175144
Andrias davidianus ranavirus	ADRV	KC865735
European catfish virus	ECV	KT989885
Short-finned eel ranavirus	SERV	KX353311
Ranavirus maximus	Rmax	KX574343
Cod iridovirus	CoIV	KX574342
Pike-perch iridovirus	PPIV	KX574341
Lumpfish ranavirus isolate F140-16	LMRV-F140-16	MH665359
Lumpfish ranavirus isolate F24-15	LMRV-F24-15	MH665358
Lumpfish ranavirus isolate V4955	LMRV-V4955	MH665360
Andrias davidianus ranavirus	ADRV-2010SX	KF033124
Chinese giant salamander iridovirus	CGSIV-HN1104	KF512820
Common midwife toad virus	CMTV-Lv/2015	MF004272
Common midwife toad virus	CMTV-Pe/2015	MF125269
Common midwife toad virus	CMTV-Pe/2016	MF125270
Pelophylax esculentus virus	PEV	MF538627
Rana catesbeiana virus isolate RC-Z	RCV-Z	MF187210
Rana esculenta virus	REV	MF538628
Trioceros melleri ranavirus 1	TMRV1	MG953519
Trioceros melleri ranavirus 2	TMRV2	MG953520
Terrapene carolina carolina ranavirus	TCCRV	MG953518

(*continued*)

Table 2. (*concluded*)

Viral name	Viral abbreviation	GenBank Accession No.
Frog virus 3	FV3-Op/2015	MF360246
Rana nigromaculata ranavirus isolate MWH421017	RNRV-MWH421017	MG791866
Zoo ranavirus isolate 040414	ZRV	MK227779
Wamena virus	WV	MT507284

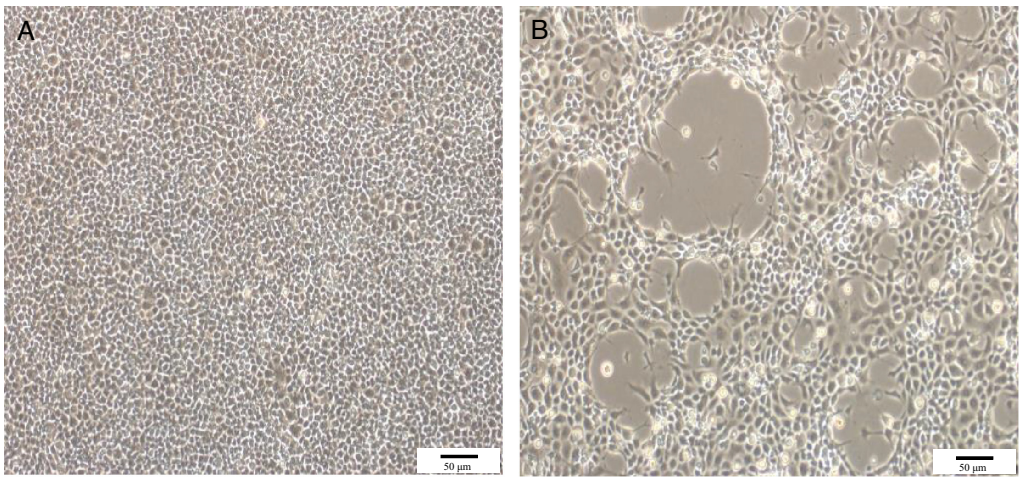

Fig. 2. Cytopathic effects observed following inoculation of *epithelium papulosum cyprini* (EPC) cells with a Thai TFV isolate (D11-067). (A) Uninfected control. (B) Cytopathic effects characterized by enlarged, refractile EPC cells and the formation of plaques. Scale bar = 50 µm.

the MCP gene sequence has been determined (Hyatt et al. 2002; Marsh et al. 2002), an additional MCP gene tree was constructed as described above (Table 2). The genetic divergence between the eight Thai ranaviruses and other ranaviruses was determined by aligning their MCP nucleotide sequences using the Sequence Demarcation Tool Version 1.2 (Muhire et al. 2014) with the MAFFT option implemented.

Results

In vitro propagation of ranaviruses

The EPC cultures inoculated with the eight Thai ranaviral isolates all displayed the same CPE characterized by enlarged, refractile cells that lysed resulting in plaques within 24 h postinoculation (Fig. 2). Complete destruction of the monolayer was observed within 48–72 h postinoculation.

TEM

On TEM, all seven EPC cultures displayed infected cells containing abundant hexagonal nucleocapsids, each with electron-dense nucleic acid cores surrounded by a translucent zone and an outer nucleocapsid layer (Fig. 3). Mean apex–apex measurements for unenveloped and enveloped viral particles ranged from 150.2 to 158.9 nm and from 196.5 to 206.0 nm, respectively (Table 1). All isolates formed paracrystalline arrays within the cytoplasm of infected EPC cells (Figs. 3A and 3B). In all EPC

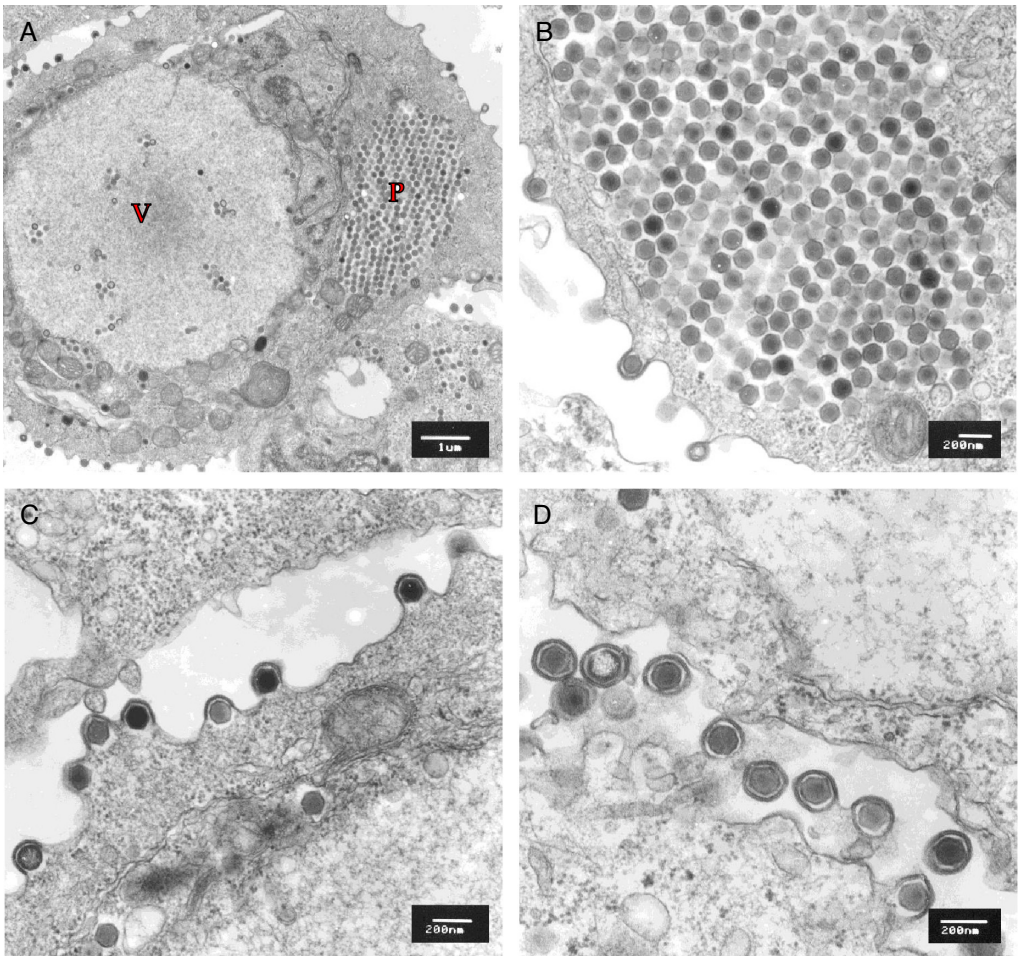

Fig. 3. Ultrastructural features of a Thai TFV isolate (D11-067) developing in *epithelium papulosum cyprini* (EPC) cells. (A) Ranaviral particles arranged in a paracrystalline array (P) next to a viral assembly site (V). Scale bar = 1 µm. (B) Higher magnification demonstrating naked viral particles with an electron-dense nucleic acid core surrounded by a translucent zone and an outer nucleocapsid layer. Scale bar = 200 nm. (C) Viral particles observed budding out from the plasma membrane. Scale bar = 200 nm. (D) Extracellular enveloped virions. Scale bar = 200 nm.

cultures, viral particles were observed obtaining an envelope via budding through the plasma membrane (Figs. 3C and 3D).

Genome annotation and phylogenomic analyses

De novo assemblies of the paired-end reads generated from each of the eight sequencing libraries resulted in contiguous consensus sequences ranging in size from 105 022 to 106 226 bp with G + C contents ranging from 53.82% to 55.73% and average coverages ranging from 8341 to 17 435 reads per nucleotide (Table 3). Comparative genomic analyses predicted 100 ORFs in OMRV; 101 ORFs in AGFRV; 103 ORFS in TFV-1998, GPRV, GFRV, EABRV-11, EABRV-16, and EABRV-17; and 104 ORFS in TFV-China (Table 3). The Mauve 2.4 analysis revealed that the Chinese TFV and the eight Thai ranaviruses possessed a FV3-like ranaviral genome arrangement (Claytor et al. 2017)

Table 3. Summary of genomic features of the tiger frog viruses (TFVs).

Viral name (abbreviation)	Isolate designation	Host	Country of origin (province)	Year of isolation	Size (kb)	No. of ORFs	% G + C	Mean coverage (reads/nt)	GenBank Accession No.
TFV-1998	AV9803	Tiger frog (*H. tigerinus*)	Thailand (Bangkok)	1998	105 022	103	55.41	10 007	MT512504
TFV-China	TFV-China	Tiger frog (*H. tigerinus*)	China (Guangdong)	2000	105 057	104	55	ND	AF389451
Oxyeleotris marmorata ranavirus (OMRV)	D2008	Marbled sleeper goby (*Oxyeleotris marmorata*)	Thailand (Nakhon Pathom)	2000	105 405	100	55.73	10 634	MT512502
Poecilia reticulata ranavirus (PPRV)	F2112	Guppy (*Poecilia reticulata*)	Thailand (Samut Sakhon)	2001	105 249	103	55.47	8888	MT512503
Goldfish ranavirus (GFRV)	F0207	Goldfish (*Carassius auratus*)	Thailand (Bangkok)	2002	106 226	103	53.82	8341	MT512501
Asian grass frog ranavirus (AGFRV)	D03-034	Asian grass frog (*Fejervarya limnocharis*)	Thailand[a] (Sa Kaeo)	2004	105 529	101	54.86	17 435	MT512497
East Asian bullfrog ranavirus-2011 (EABRV-2011)	D11-067	East Asian bullfrog (*H. rugulosus*)	Thailand (Phattalung)	2011	105 418	103	55.03	9536	MT512498
East Asian bullfrog ranavirus-2016 (EABRV-2016)	VD-16-006	East Asian bullfrog (*H. rugulosus*)	Thailand (Ratchaburi)	2016	105 206	103	55.22	9053	MT512499
East Asian bullfrog ranavirus-2017 (EABRV-2017)	VD-17-007	East Asian bullfrog (*H. rugulosus*)	Thailand (Rayong)	2017	105 114	103	54.09	15 188	MT512500

[a]Frogs were recently imported from Cambodia.

(Fig. S1). TFV ORFs 26 and 63, encoding hypothetical proteins, were present in the Chinese TFV and all Thai ranaviruses except OMRV (Table S2). TFV ORFs 6.5 and 59 were present in the Chinese TFV and all Thai ranaviruses except OMRV and AFGRV (Table S2). TFV ORF 54 encoding a putative nuclear calmodulin-binding protein was only present in the Chinese TFV, OMRV, and AFGRV (Table S2). TFV ORF 97, encoding a hypothetical protein, was present in the Chinese TFV and all Thai ranaviruses except AGFRV (Table S2). The Chinese TFV and Thai ranaviruses possessed a putative gene (TFV ORF 28) that was predicted to encode a protein of unknown function and was absent in all other ranaviruses (Table S2). The Chinese TFV and Thai ranaviruses were all missing FV3 ORF 30R (GenBank Accession No. NC005946) that was present in all other ranaviruses (Table S2). In addition, a single nucleotide deletion resulted in the loss of a stop codon and the merger of two ORFs (equivalent to ORFs 49L and 50L in FV3 and ORFs 51R and 52L in the Chinese TFV) in all Thai TFVs (TFV ORF 50).

The ML analysis, based on the concatenated LCB alignment, generated a resolved and supported tree with a topology congruent to a recent analysis (Fig. 4; Stagg et al. 2020). The ML tree supported the Chinese TFV and the eight Thai ranaviruses as a unique subclade (hereafter referred to as the TFV subclade) within the larger FV3 clade. The OMRV and AGFRV isolates branched off at the base of

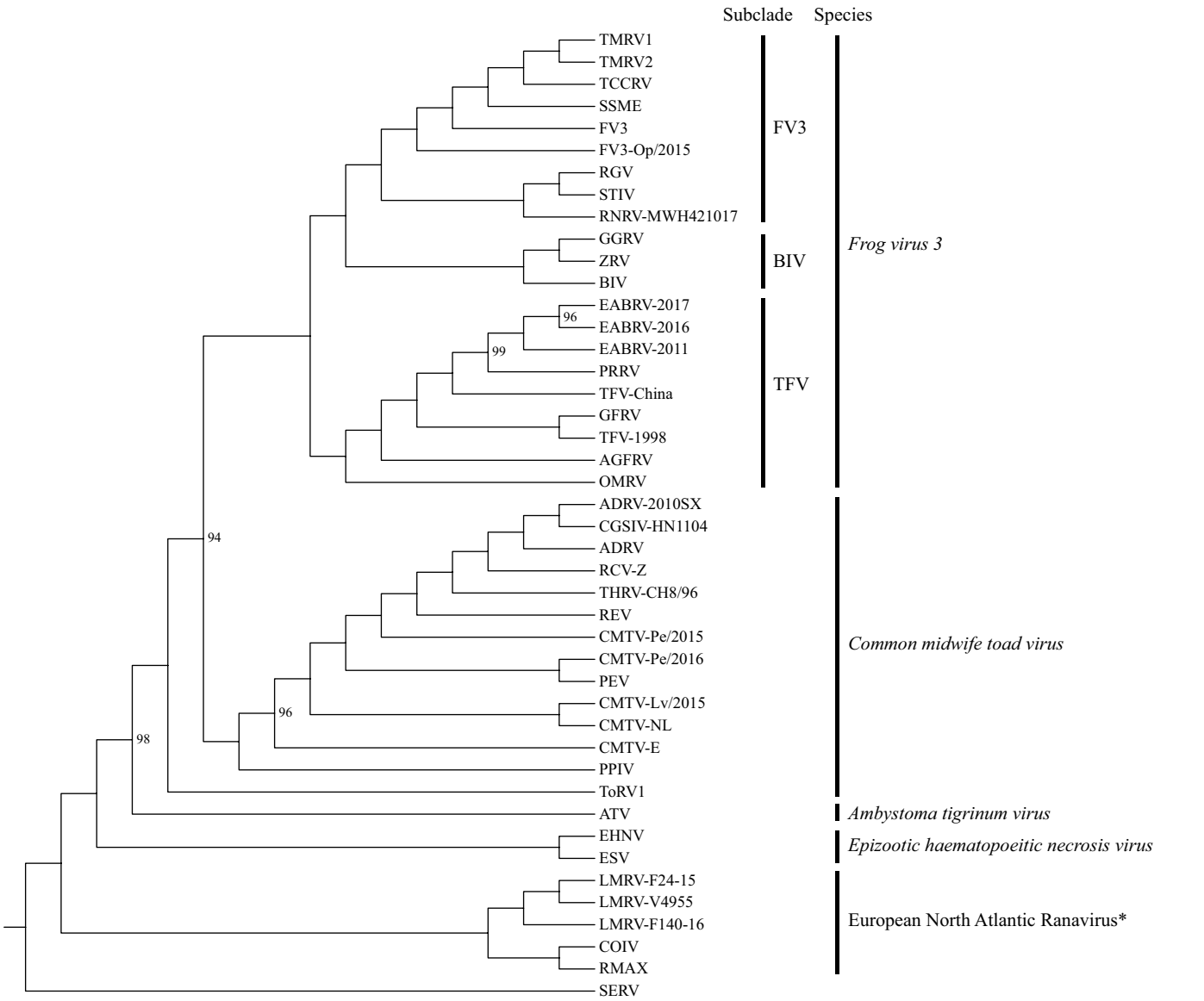

Fig. 4. Cladogram depicting the relationship of the Thai TFVs to other members of the genus *Ranavirus* based on the concatenated locally collinear blocks alignments. All nodes are supported by bootstrap values of 100% from the Maximum Likelihood analysis except the nodes labelled with bootstrap values. See **Tables 1** and **2** for viral abbreviations. *Note: European North Atlantic Ranavirus has not been approved as a ranaviral species by the International Committee on Taxonomy of Viruses.

the TFV subclade. The ML analysis, based on the MCP gene alignment, generated a similar overall topology to the tree built from the full genome (LCB) alignment; however, the resolution of this tree and its node support were significantly reduced (**Fig. S3**). Importantly, the MCP gene tree again supported the TFVs as a monophyletic group with the WV as the most basal branch. The MCP gene nucleotide identity of the Thai TFVs ranged from 99.8% to 100% as compared with each other and the Chinese TFV, 99.3%–99.4% when compared with the WV, and 93.7%–98.6% when compared with 35 other ranaviruses (**Fig. S4**).

Discussion

In this investigation, we report the complete genome sequences of eight ranaviruses isolated from cultured fish and amphibians in Thailand. The growth of the Thai ranaviruses in EPC cells facilitated their ultrastructural and phylogenomic characterization. The induction of CPE in EPC cultures (i.e., progression of coalescing round plaques) and the observed ultrastructural features of the viral particles (i.e., hexagonal viral particles within the cytoplasm and enveloped extracellular virions) were congruous with a previous report of TFV in tiger frogs in China (Weng et al. 2002) and expectations for ranaviruses (International Committee on Taxonomy of Viruses 2020). Phylogenomic analyses supported the Thai ranaviruses as a unique subclade (hereafter referred to as the TFV subclade) within the larger FV3 clade. To our knowledge, this study represents the only genetic characterization of ranaviruses in Thailand and adds to a growing body of literature on the expanding host range and potential threat ranaviruses pose to cultured fish and amphibians.

Our phylogenomic analyses strongly supported the TFV subclade as the sister group to a clade composed of three separate subclades: the Bohle iridovirus subclade (e.g., BIV, GGRV, ZRV), a second subclade with isolates from Chinese frogs (RGV, RNRV) and turtles (STIV), and a third subclade with strains from North American reptiles (TMRV1, TMRV2, TCCRV) and amphibians (FV3, SSME) and a European frog (FV3-Op). Further support for TFVs as strains of FV3 was evident by the fact that they all possess the previously defined FV3-like genome arrangement (Claytor et al. 2017). A shared derived feature (i.e., synapomorphy) that delineates TFVs from other ranaviruses, including other FV3 strains, is the acquisition of a putative gene (TFV ORF 28) that is predicted to encode a protein of unknown function. A second synapomorphy is the absence of FV3 ORF 30R (GenBank Accession No. NC005946) in TFV isolates. Our sequencing of eight Thai TFVs revealed a single nucleotide deletion resulting in the merger of two ORFs (FV3 ORFs 49L and 50L) as previously reported in the following FV3 strains: spotted salamander Maine (SSME), Rana grylio virus (RGV), and soft-shelled turtle iridovirus (STIV) (Morrison et al. 2014). In contrast, the Chinese TFV and FV3 lacked this deletion and resulting gene merger. The biological significance of these TFV gene gains, losses, and merger events remain to be determined.

The ranaviral-infected marbled sleeper goby, tadpoles, and frogs from farms in Thailand displayed cutaneous ulcers, edema (tadpoles), and no observable internal gross lesions (Kanchanakhan 1998, 2011; Kanchanakhan et al. 1999, 2004; Prasankok et al. 2005; J. Polchana, personal observation, 2017) (**Fig. 1**). In contrast, cutaneous ulcerative lesions were not observed in East Asian bullfrog tadpoles or frogs during TFV outbreaks on farms in China (Weng et al. 2002). The disease in cultured tadpoles in China was named "abdominal distension disease" as a result of the observed enlargement of the liver, kidney, and spleen. Petechial hemorrhages were also noted on the serosal surfaces of the enlarged internal organs. Although differences in gross lesions have been reported in cultured tadpoles and frogs on farms in China versus Thailand, the cutaneous ulceration (Thailand) and petechial hemorrhages along the serosal surfaces of enlarged internal organs (China) are all considered classic gross lesions associated with ranaviral-infected tadpoles and frogs (Miller et al. 2015). Furthermore, the tissue sections from diseased Chinese tadpoles and frogs displayed microscopic lesions consistent with ranaviral infections in amphibians including diffuse hepatocellular necrosis and focal necrosis in the kidney (Weng et al. 2002; Miller et al. 2015). Although histopathological examination of Thai TFV-infected tadpole and frog tissues has not been reported, the observation of cutaneous ulcerative lesions paired with the high prevalence of ranaviruses isolated from diseased tadpoles and frogs suggests TFV is likely a significant source of mortality in cultured amphibians in Thailand (Kanchanakhan 1998, 2011; Kanchanakhan et al. 1999, 2004; Prasankok et al. 2005). Controlled laboratory studies are needed to elucidate the role Thai TFV strains play in disease of cultured frog and fish species.

In this study, the TFV isolates from goldfish (F0207) and guppy (F2112) were recovered from overtly healthy fish during routine health surveys conducted at AAHRDD (S. Kanchanakhan, personal observation, 2001, 2002). Similarly, strains of FV3 have previously been reported from outwardly healthy fathead minnow (*Pimephales promelas*) and northern pike (*Esox lucius*) during surveillance efforts targeting viral haemorrhagic septicemia virus in the United States (Waltzek et al. 2014). Experimental challenge studies have resulted in little to no mortality in black bullhead (*Ameiurus melas*), bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), fathead minnow, goldfish (*Carassius auratus*), mosquito fish (*Gambusia affinis*), Nile tilapia (*Oreochromis niloticus*), northern pike (*Esox Lucius*), and pike-perch (*Sander lucioperca*) following exposure to FV3 isolates (Bang Jensen et al. 2009, 2011a, 2011b, Gobbo et al. 2010; Brenes et al. 2014a, 2014b). Similarly, experimental challenge studies resulted in no mortality in grass carp (*Ctenopharyngodon Idella*), goldfish, and mandarin fish (*Siniperca chautsi*) following injection of a Chinese TFV isolate (Weng et al. 2002). In contrast, farm-reared sturgeon species, including the highly endangered pallid sturgeon (*Scaphirhynchus albus*), appear highly susceptible to FV3 (Waltzek et al. 2014). Although few fish species appear highly susceptible to strains of FV3, at least some fish become infected and can transmit the virus to frog tadpoles under laboratory conditions (Brenes et al. 2014a).

Tiger frog virus was originally characterized from a disease episode in cultured tiger frog tadpoles and frogs in China in 2000 (He et al. 2002; Weng et al. 2002). The present study confirms that strains of TFV have been circulating in cultured fish and amphibian populations in Thailand from 1998 to 2017. Our analysis of the major capsid protein of the WV, isolated from diseased green pythons confiscated during their attempted importation into Australia from Papua New Guinea in 1998, suggests that TFVs may be circulating more widely, including in wild squamate reptiles in Oceania (Hyatt et al. 2002). Like other strains of FV3, TFVs are promiscuous pathogens capable of infecting three classes of vertebrates including bony fish, amphibians, and reptiles (Chinchar and Waltzek 2014). Experimental studies have demonstrated interclass transmission can occur by cohabitation of FV3-infected Cope's treefrog tadpoles (*Hyla chrysoscelis*) with naïve red-eared sliders (*Trachemys scripta elegans*) and cohabitation of FV3-infected mosquitofish (*Gambusia affinis*) or FV3-infected red-eared sliders with naïve Cope's treefrog tadpoles (Brenes et al. 2014a). In Thailand, opportunities for interclass transmission of TFVs may occur at large pet markets where tadpoles and frogs are housed in open containers next to ornamental fishes. The aquaculture practice of feeding juvenile frogs to increase the growth of large predatory ornamental fishes may have also contributed to the spread of TFVs in Thailand (S. Kanchanakhan, personal observation, 2016).

Continued TFV surveillance efforts are needed to monitor any impact these ranaviruses may have on Thai aquatic animal industries. Improved biosecurity programs should be implemented (e.g., discontinuing the practice of feeding live juvenile frogs to predatory fishes) to reduce the impact of infectious diseases on Thai farms and pet markets. Although TFVs have only been detected in Asia and likely in Oceania, their promiscuous nature coupled with the largely unregulated international trade in live animals raises concerns that they might emerge in more distant ranaculture and aquaculture markets or wildlife populations (Picco and Collins 2008; Schloegel et al. 2009; Kolby et al. 2014).

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Author contributions

PS, KS, VLP, and TBW conceived and designed the study. PS, KS, NKS, and KI performed the experiments/collected the data. PS, KS, VLP, and TBW analyzed and interpreted the data. SK, JP, and TBW contributed resources. PS, KS, NKS, KI, VLP, SK, JP, and TBW drafted or revised the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Data availability statement

All relevant data are within the paper and in the Supplementary Material.

Supplementary materials

The following Supplementary Material is available with the article through the journal website at doi:10.1139/facets-2020-0043.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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