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Ranavirus phylogenomics: Signatures of recombination and inversions among bullfrog ranaculture isolates

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ABSTRACT

Ranaviruses are emerging pathogens of fish, amphibians, and reptiles that threaten aquatic animal industries and wildlife worldwide. Our objective was to genetically characterize ranaviruses isolated during separate bullfrog *Lithobates catesbeianus* die-offs that occurred eight years apart on the same North American farm. The earlier outbreak was due to a highly pathogenic strain of common midwife toad virus (CMTV) previously known only from Europe and China. The later outbreak was due to a chimeric ranavirus that displayed a novel genome arrangement and a DNA backbone typical for *Frog virus 3* (FV3) strains except for interspersed fragments acquired through recombination with the CMTV isolated earlier. Both bullfrog ranaviruses are more pathogenic than wild-type FV3 suggesting recombination may have resulted in the increased pathogenicity observed in the ranavirus isolated in the later outbreak. Our study underscores the role international trade in farmed bullfrogs may have played in the global dissemination of highly pathogenic ranaviruses.

1. Introduction

Members of the genus *Ranavirus* belong to the family *Iridoviridae* and possess linear, terminally redundant, double-stranded DNA genomes packaged within enveloped icosahedral nucleocapsids (Chinchar et al., 2017). There are seven recognized *Ranavirus* species, three of which, *Ambystoma tigrinum virus* (ATV), *Bohle iridovirus* (BIV), and *Frog virus 3* (FV3), infect predominantly amphibians (Granoff et al., 1965, Speare et al., 1992, Jancovich et al., 1997, Chinchar et al., 2017). However, both BIV and FV3 are capable of infecting not only the amphibians from which they were originally isolated, but other amphibian species as well as fish and reptile species (Moody and Owens, 1994; Mao et al., 1997; Allender et al., 2013; Waltzek et al., 2014). Ranaviruses infect no fewer than 175 species across 53 families of ectothermic vertebrates on every continent except Antarctica (Duffus et al., 2015). They are considered globally emerging pathogens given they appear to be increasing in geographic distribution, disease prevalence, and host range (Duffus et al., 2015; Gray and Chinchar, 2015).

Recent studies have revealed that strains from two ranavirus groups, FV3- and common midwife toad virus (CMTV)-like ranaviruses, pose a significant threat to global ectothermic vertebrate biodiversity including species of conservation concern such as the pallid sturgeon *Scaphirhynchus albus* and Chinese giant salamander *Andrias davidianus* (Geng et al., 2011; Waltzek et al., 2014; Duffus et al., 2015; Gray and Chinchar, 2015). The risk to amphibians is especially alarming as 43% of amphibian populations are in decline and 32% are considered threatened (Stuart et al., 2004). For example, declines in wild populations of the common frog *Rana temporaria* in England have been attributed to a strain of FV3 that may have been introduced by the movement of infected animals from North America (Hyatt et al., 2000; Teacher et al., 2010). Likewise, CMTV has been responsible for wild amphibian epizootics in Spain (Balseiro et al., 2009, 2010; Price et al., 2014), Belgium (Sharifian-Fard et al., 2011), and The Netherlands (Kik et al., 2011; Van Beurden et al., 2014). Genomic sequencing of isolates from the threatened Hermann's tortoise *Testudo hermanni* and critically endangered Egyptian tortoise

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Testudo kleinmanni demonstrated CMTV-like ranaviruses also circulate among managed chelonian populations in Europe (Stöhr et al., 2015).

The FV3- and CMTV-like ranaviruses also threaten global ranaculture and aquaculture industries (Waltzek et al., 2014; Duffus et al., 2015; Gray and Chinchar, 2015). The cultivation of the North American pig frog *Lithobates grylio* for food in China began in the 1980s and since 1995 a FV3-like ranavirus (Rana grylio virus, RGV) has negatively impacted culture efforts (Zhang et al., 2001). Similarly, FV3-like ranaviruses have negatively impacted the cultivation of tiger frogs *Hoplobatrachus tigerinus* (Tiger frog virus, TFV) and soft-shelled turtles *Pelodiscus sinesis* (Soft-shelled turtle iridovirus, STIV) in China (He et al., 2002; Huang et al., 2009), tiger frogs and sleeper goby *Oxyeleotris marmoratus* in Thailand (Kanchanakhan et al., 2002; Prasankok et al., 2005), bullfrogs *L. catesbeianus* and pallid sturgeon in the United States (Majji et al., 2006; Miller et al., 2007; Waltzek et al., 2014), and bullfrogs in Brazil (Mazzoni et al., 2009) and Korea (Kim et al., 2011). Since 2010, a lethal CMTV-like ranavirus (referred to either as the Chinese giant salamander iridovirus; CGSIV or Andrias davidianus ranavirus; ADRV) has negatively impacted farms rearing the critically endangered Chinese giant salamander for research, food/medicinal, and conservation purposes (Dong et al., 2011; Geng et al., 2011; Jiang et al., 2011; Chen et al., 2013; Wang et al., 2014; Cunningham et al., 2016). Genomic sequencing of the pike-perch iridovirus (PPIV), isolated from asymptomatic cage-cultured pike-perch *Sander lucioperca* in Finland in 1995, demonstrated it represents the earliest detection of a CMTV-like ranavirus and the only report in fish (Holopainen et al., 2016).

One clade of ranaviruses with collinear genomes from North America (FV3 isolated from leopard frog *L. pipiens* and SSME isolated from spotted salamander *A. maculatum*), Asia (RGV/STIV/TFV), and Australia (BIV isolated from ornate burrowing frog *Limnodynastes orantus*) have been referred to as the FV3-like ranaviruses (Tan et al., 2004; Jancovich et al., 2015; Morrison et al., 2014; Hick et al., 2016). Another clade of ranaviruses with collinear genomes isolated from European or Chinese fish, amphibians, and reptiles have been referred to as the CMTV/ADRV-like ranaviruses and will be referred to as the CMTV-like ranaviruses hereafter (Mavian et al., 2012a; Chen et al., 2013; Van Beurden et al., 2014; Jancovich et al., 2015; Stöhr et al., 2015; Holopainen et al., 2016). ATV isolated from tiger salamander *Ambystoma tigrinum* and the fish ranviruses at the base of the ranavirus tree isolated from redfin perch *Perca fluviatilis* (*Epizootic haematopoietic necrosis virus*, EHNV), sheatfish *Silurus glanis* (European sheatfish virus, ESV), turbot *Scopthalamus maximus* (Ranavirus maximus, Rmax), Atlantic cod *Gadus morhua* (Cod iridovirus, CoIV), and short-finned eel *Anguilla australis* (short-finned eel ranavirus, SERV) are collinear except for a small inversion reported in the Rmax/CoIV clade (Jancovich et al., 2003, 2010; Ariel et al., 2016; Subramaniam et al., 2016). Collectively, these ranaviruses have been referred to as the EHNV/ATV-like ranaviruses and will be referred to as the EHNV-like ranaviruses hereafter (Jancovich et al., 2015). The detection of closely related FV3-, CMTV-, and EHNV-like ranaviruses around the world is strong evidence of their anthropogenic spread via trade in live animals for human consumption, bait, pets, zoological exhibition, and laboratory research (Picco et al., 2010; Hick et al., 2016).

Despite a number of ranavirus epizootics at ranaculture (Majji et al., 2006; Miller et al., 2007) and aquaculture (Waltzek et al., 2014) facilities in the United States, no genomic sequencing efforts have been conducted to elucidate molecular epidemiological and evolutionary patterns. In this investigation, we sequenced the complete genomes of two American bullfrog ranavirus isolates from epizootics in 1998 (Majji et al., 2006) and 2006 (Miller et al., 2007) at the same ranaculture facility. Subsequently, we performed phylogenomic analyses by comparing them to 19 other ranavirus sequences to determine the identity of the agents responsible for these outbreaks. As detailed below, our data suggest that the initial outbreak was caused by a CMTV-like ranavirus, whereas the latter was due to a FV3 x CMTV recombinant ranavirus.

2. Methods

2.1. Isolate collection

Two ranaviral isolates were obtained from separate American bullfrog epizootics in 1998 (Majji et al., 2006) and 2006 (Miller et al., 2007) at the same aquaculture facility. Reflecting the order in which they were isolated, the 1998 isolate was designated RCV-Z (Majji et al., 2006), whereas the 2006 isolate was designated RCV-Z2 (Miller et al., 2007). RCV-Z was obtained from visceral tissue from a moribund bullfrog tadpole (Majji et al., 2006). RCV-Z2 was isolated from visceral tissue in a bullfrog that had recently undergone metamorphosis (Miller et al., 2007).

2.2. Cell culture, virus purification, and DNA extraction

The isolates were propagated in epithelioma papulosum cells (EPC) grown to confluence in four 175 cm^2 flasks at 20 °C in Eagle's Minimal Essential Medium (MEM) with Earle's buffered salt solution containing 10% fetal bovine serum (FBS), 50 IU penicillin ml^{-1}, 50 μg streptomycin ml^{-1}, and 2 mM L-glutamine. The two isolates were grown in separate months to reduce the risk of cross contamination. Following a 60 min virus adsorption period, 50 ml of MEM with 2% FBS was added and the cells held at 23 °C until complete cytopathic effect was observed. Cells were harvested using a cell scraper and the cell suspension frozen at −80 °C. Virion purification was performed as described by Majji et al. (2006) with minor modifications. Briefly, cell suspensions (approximately 200 ml per isolate) were frozen-thawed three times to release cell-associated virus and cell debris were removed by centrifugation at 5509×g at 4 °C for 20 min using a Beckman JA-14 fixed angle rotor. Virus particles were pelleted from the clarified supernatant by ultracentrifugation at 100,000×*g* at 4 °C for 60 min in a Beckman Type 50.2 Ti rotor. The resulting pellet was resuspended in 3 ml resuspension buffer (RSB; 10 mM Tris-HCl, pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$). To remove adventitiously associated cellular DNA, concentrated virions (3 ml) were treated with DNAse (200 μg ml^{-1}, Sigma) in the presence of 10 mM $MgCl_2$ for 60 min at 37 °C. After 1 h the reaction was stopped by adding EDTA to a final concentration of 50 mM, and the virions were layered over a 20% (w/w) sucrose-RSB cushion and centrifuged at 150 000×*g* for 90 min at 4 °C in a Beckman Type 50.2 Ti rotor. The overlay was removed by aspiration and the pelleted virions were resuspended in RSB prior to extraction using a Qiagen DNeasy extraction kit as specified by the manufacturer. The resulting DNA concentration was measured by fluorometry using a Qubit® dsDNA BR Assay Kit.

2.3. Library preparation, next generation sequencing, assembly, genome annotation, and BLASTP analyses

A viral DNA library was prepared using a TruSeq Dual Index HT DNA PCR-free Library Preparation Kit (Illumina) as specified by the manufacturer. Sequencing was performed using v3 chemistry on an Illumina MiSeq sequencer. *De novo* assembly of paired end reads was performed with the SPAdes 3.5.0 genome assembly algorithm (Bankevich et al., 2012) using default settings. The quality of the assembly was verified by mapping reads against the final genome consensus using Bowtie 2 2.1.0 (Langmead and Salzberg, 2012) and visualized in Tablet 1.14.10.20 (Milne et al., 2010). The genomes of the two bullfrog isolates were annotated using the Genome Annotation Transfer Utility (Tcherepanov et al., 2006) with FV3 (RefSeq acc. NC_005946) as the reference genome. Additional putative open reading frames (ORF) were identified using GeneMarkS (Besemer et al., 2001)

Table 1
Virus and abbreviation, host common and scientific names, year and country of isolation, reference, accession number, and genome size of 21 ranavirus isolates used in the phylogenomic analyses.

Ranavirus (abbreviation)	Host Common Name	Host Scientific Name	Year of Isolation	Country of Isolation	Reference	Accession No.	Genome size (bps)
Common midwife toad virus (CMTV-E)	Alpine newt	*Mesotriton alpestris cyreni*	2008	Spain[W]	Mavian et al. (2012a)	JQ231222	106,878
Rana catesbeiana virus (RCV-Z)	Bullfrog	*Lithobates catesbeianus*	1998	USA[M]	Majji et al. (2006)	MF187210	106,890
Rana catesbeiana virus (RCV-Z2)	Bullfrog	*Lithobates catesbeianus*	2006	USA[M]	Miller et al. (2007)	MF187209	104,968
Andrias davidianus ranavirus (ADRV 2010SX)	Chinese giant salamander	*Andrias davidianus*	2010	China[M]	Jiang et al. (2011)	KF033124	106,719
Common midwife toad virus (CMTV-NL)	Edible frog	*Pelophylax esculentus*	2013	Netherlands[W]	van Beurden et al. (2014)	KP056312	107,772
Tortoise ranavirus 1 (ToRV1)	Egyptian tortoise	*Testudo kleinmann*	1996	Germany[M]	Stöhr et al. (2015)	KP266743	103,876
German gecko ranavirus (GGRV)	Leaf-tailed gecko	*Uroplatus fimbriatus*	2001	Germany[M]	Stöhr et al. (2015)	KP266742	103,681
Frog virus 3 (FV3)	Leopard frog	*Lithobates pipiens*	1962	USA[W]	Tan et al. (2004)	NC_005946	105,903
Rana grylio virus (RGV)	Pig frog	*Lithobates grylio*	1995	China[M]	Lei et al. (2012)	JQ654586	105,791
Epizootic haematopoietic necrosis virus (EHNV)	Redfin perch	*Perca fluviatilis*	1984	Australia[W]	Jancovich et al. (2010)	NC_028461	127,011
European sheatfish virus (ESV)	Sheatfish	*Silurus glanis*	1989	Germany[M]	Mavian et al. (2012b)	NC_017940	127,732
Soft-shelled turtle iridovirus (STIV)	Soft-shelled turtle	*Trionyx sinesensis*	1997	China[M]	Huang et al. (2009)	EU627010	105,890
Ambystoma tigrinum virus (ATV)	Sonoran tiger salamander	*Ambystoma tigrinum stebbinsi*	1995	USA[W]	Jancovich et al. (2003)	NC_005832	106,332
Spotted salamander Maine (SSME)	Spotted salamander	*Ambystoma maculatum*	1998	USA[W]	Morrison et al. (2014)	KJ175144	105,070
Tiger frog virus (TFV)	Tiger frog	*Hoplobatrachus tigerinus*	1999	China[M]	He et al. (2002)	AF389451	105,057
Bohle iridovirus (BIV)	Ornate burrowing frog	*Limnodynastes ornatus*	1992	Australia[W]	Hick et al. (2016)	KX185156	103,531
Pike-perch iridovirus (PPIV)	Pike-perch	*Sander lucioperca*	1995	Finland[M]	Holopainen et al. (2016)	KX574341	108,041
Short-finned eel ranavirus (SERV)	Short-finned eel	*Anguilla austalis*	1999	Italy[M*]	Subramaniam et al. (2016)	KX353311	126,965
Ranavirus maximus (Rmax)	Turbot	*Scophthalmus maximus*	1999	Denmark[M]	Ariel et al. (2016)	KX574343	115,510
Cod iridovirus (CoIV)	Atlantic cod	*Gadus morhua*	1979	Denmark[W]	Ariel et al. (2016)	KX574342	114,865
Testudo hermanni ranavirus (CH8/96)	Hermann's tortoise	*Testudo hermanni*	1996	Germany[M]	Stöhr et al. (2015)	KP266741	105,811

Isolates derived from wild[W] or managed[M] populations.
[*] SERV was isolated from short-finned eel imported from New Zealand.

and the functions were predicted based on BLASTP searches against the GenBank non-redundant protein sequence database provided by the National Center for Biotechnology Information.

2.4. Multiple genome-wide alignments and phylogenomic analyses

The genomes of RCV-Z, RCV-Z2, and 19 ranavirus genomes obtained from GenBank were first reordered to ensure the 5′ end of each genome began with the arbitrarily chosen FV3 ORF1R as previously described for the German Gecko ranavirus (GenBank acc. KP266742) (Table 1). The 21 reordered genomes (Dataset21, D21) were aligned using Mauve 2.4 (Darling et al., 2004) to visualize genomic inversions and obtain the locally collinear blocks (LCB) alignments. The D21 LCB alignments were then concatenated in Geneious v.7 (Kearse et al., 2012). The concatenated D21 and partitioned D21 alignments were then used in Maximum Likelihood (ML) and Bayesian phylogenetic analyses. ML analyses were performed in IQ-TREE with the Bayesian information criterion used to determine the best model fit for each partition and 1000 non-parametric bootstrap analyses to test the robustness of the hypothesis (Nguyen et al., 2015). The same models were also implemented in the Bayesian analyses performed in MrBayes v.3.2 (Ronquist et al., 2012) with default priors for topology (uniform) and branch lengths (Exp and 10). The Markov chain was run for a maximum of 1.1 million generations. Four independent analyses were conducted, each with 1 cold and 3 heated chains with the default heating parameter (temperature = 0.2). Every 1000 generations were sampled and the first 25% of the Markov chain Monte Carlo samples discarded as burn-in.

2.5. Recombination analyses

For the recombination analyses, only the 15 FV3- and CMTV-like ranaviruses (Dataset15, D15) were included: 1) because our study focused on bullfrog FV3- and CMTV-like ranaviruses, 2) to reduce possible noise due to the high genetic diversity within D21 (i.e., due to the divergent marine fish ranaviruses), 3) initial recombination analyses revealed little to no recombination when divergent marine fish ranaviruses (e.g. SERV and CoIV-like ranaviruses) were analyzed alongside the reptile and amphibian isolates (i.e., FV3- and CMTV-like ranaviruses), and 4) to reduce the computational time of analyses. The D15 was aligned into LCBs and concatenated as described above prior to performing genome-wide recombination analyses. Putative recombination breakpoints were visualized in the Recombination Detection Program (RDP) BETA4.67 (Martin et al., 2010) with default settings except the linear DNA option was selected given iridoviruses possess linear double-stranded DNA genomes. The following algorithms within RDP4 were employed: RDP (Martin et al., 2000), GENECONV (Padidam et al., 1999), BootScan (Martin et al., 2005), MaxChi (Smith et al., 1992), Chimaera (Posada and Crandall, 2001), SiScan (Gibbs et al., 2000), and 3Seq (Boni et al., 2007). Putative recombination events were considered significant when $P \leq 0.01$ was observed for the same event using six or more algorithms. ML and Bayesian phylogenetic analyses were performed as described above on the following D15 alignments: concatenated, concatenated with recombinant sites removed as implemented within RDP4, and concatenated recombinant sites (i.e., only regions of recombination considered).

2.6. US22 protein family analyses

In double-stranded DNA viruses, US22 proteins have been shown to counter diverse antiviral responses by interacting with specific host proteins (Zhang et al., 2011). Thus, we scrutinized the 22 US22 amino acid sequences present in the 15 FV3- and CMTV-like ranaviruses to determine whether they possess the predicted conserved secondary structures previously defined for this protein family (Zhang et al., 2011). To accomplish this, the US22 amino acid sequences were aligned using the MAFFT server (http://mafft.cbrc.jp/alignment/server/) with default parameters. The aligned amino acid sequences were then uploaded in the ClustalW/Jalview server (http://www.pedb.org/scripts/clustalw.php) to generate the amino acid consensus sequence. The amino acid consensus was then used to predict conserved protein domains using the HMMER server (http://www.ebi.ac.uk/Tools/hmmer/) with default parameters and secondary structure using the JPred 4 server (http://www.compbio.dundee.ac.uk/jpred/) with default parameters.

We also sought to determine how many times US22 orthologous genes have been acquired and maintained over the evolutionary history of these ranaviruses. To accomplish this the ranaviral US22 amino acid sequences were then used in BLASTP searches against the NCBI non-redundant protein database to acquire 34 cellular (i.e. vertebrates including fish, amphibians, and reptiles) US22 amino acid sequences. The 53 ranaviral and cellular US22 amino acid sequences were then aligned in MAFFT and ML and Bayesian phylogenetic analyses performed as described above. In addition to phylogenetic analyses, the positional conservation of the US22 genes within the ranaviral genomes was considered in defining the orthologous gene clusters.

3. Results

3.1. Assembly, genome annotation, and BLASTP analyses

De novo assembly of 4,388,046 paired end reads for RCV-Z generated the full viral genome in a single contiguous sequence of 106,890 bps encoding 98 putative ORFs ranging from 49 to 1294 amino acids and a G+C content of 55.5% (Supplemental Table 1). A total of 2,419,906 paired-end reads (55.15%) mapped back to the genome sequence at an average coverage of 5883 reads per nucleotide. The BLASTP searches revealed nearly all ORFs (95/98) displayed highest identity (typically 91% or greater) to CMTV-like ranaviruses (e.g., CMTV, ADRV, CGSIV, CH8/96, RCV-Z).

De novo assembly of 4,330,026 paired end reads for RCV-Z2 generated the full viral genome in a single contiguous sequence of 104,968 bps encoding a 101 putative ORFs ranging from 43 to 1293 amino acids and a G+C content of 56.08% (Supplemental Table 2). A total of 2,903,694 paired-end reads (67.06%) mapped back to the genome consensus sequence at an average coverage of 7494 reads per nucleotide. The BLASTP searches revealed the majority of ORFs (77/101) with highest identity to FV3-like ranaviruses (e.g., FV3, SSME, RGV, STIV, GGRV). The top scoring matches for the other 26 ORFs were CMTV-like ranaviruses (e.g., ADRV, CGSIV, CH8/96). Comparative genomic analyses revealed RCV-Z2 is missing two ORFs orthologous to FV3 ORFs 5 and 6 (RefSeq acc. NC_005946) that encode a US22 protein and a hypothetical protein, respectively.

3.2. Multiple genome-wide alignments and phylogenomic analyses

The analysis in Mauve 2.4 returned the genomic arrangement of 21 ranaviruses (D21) in seven locally collinear blocks (LCBs) including: large red block (orthologous to FV3 ORFs 1–10; RefSeq NC_005946), small red block (FV3 ORFs 11–12), green block (FV3 ORFs 13–22), blue block (FV3 ORFs 23–51), purple block (FV3 ORFs 52–77), olive green block (FV3 ORFs 78–95), and light brown block (FV3 ORFs 96–98) (Fig. 1). Genomic inversions resulted in the seven LCBs being arranged in five different genomic arrangements (GA) including GA1: EHNV-like ranaviruses (e.g., SERV, ESV, EHNV, ATV), GA2: CoIV-like ranaviruses (e.g., CoIV and Rmax), GA3: CMTV-like ranaviruses (e.g., ADRV, CH8/96, CMTV, PPIV, RCV-Z, ToRV1), GA4: FV3-like ranaviruses (e.g., BIV, FV3, GGRV, RGV, SSME, STIV, TFV), and GA5: recombinant ranavirus (RCV-Z2).

The best-fit model for the D21 concatenated LCB alignment was GTR+G4. The best fit models for the D21 partitioned by LCB alignment were: TVM+G4 for the large red block, K3P+G4 for the small red block,

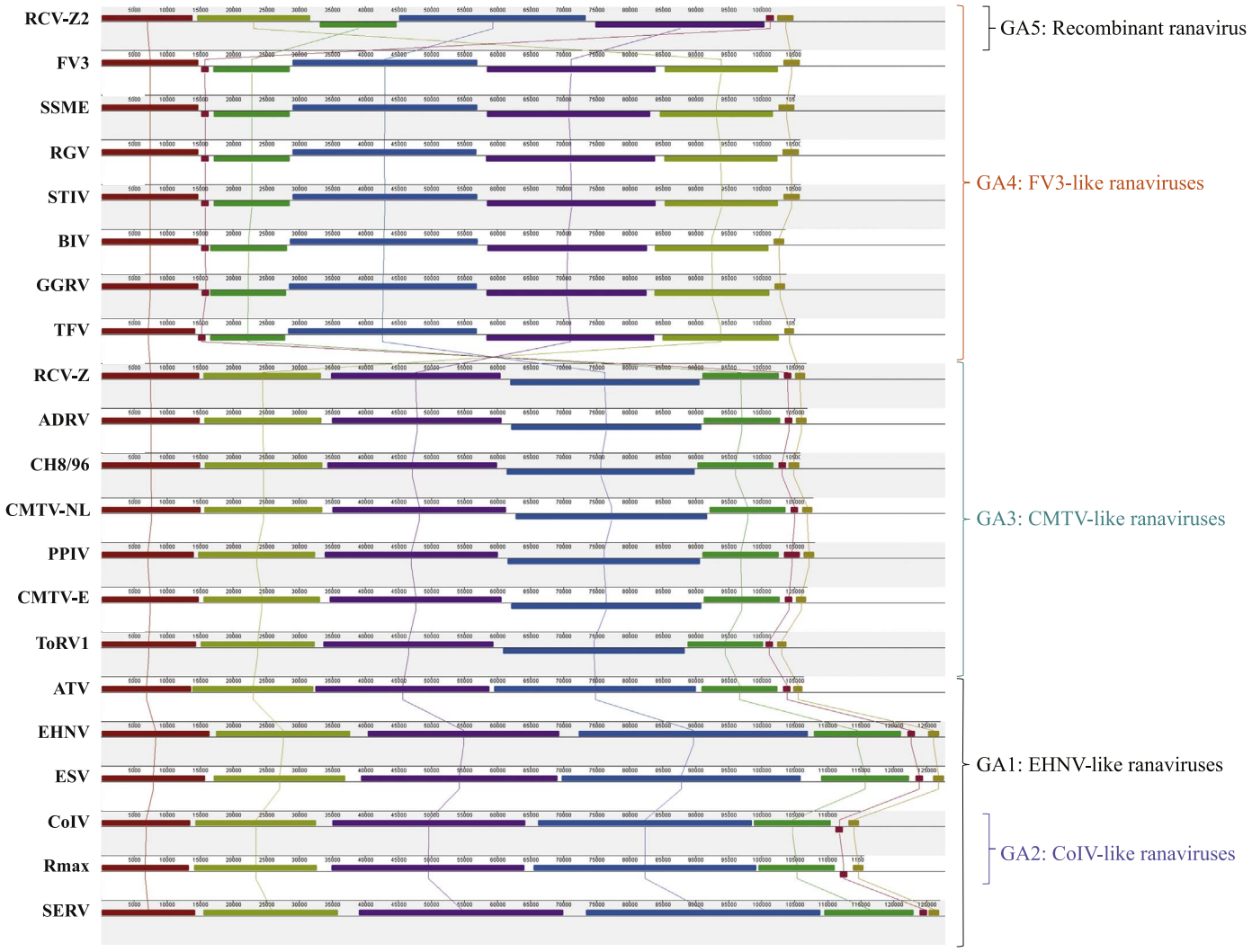

Fig. 1. Whole-genome alignment of 21 ranaviruses displaying 7 locally collinear blocks displayed as colored blocks arranged into 5 genomic arrangements (GA) including: GA1: EHNV-like ranaviruses, GA2: CoIV-like ranaviruses, GA3: CMTV-like ranaviruses, GA4: FV3-like ranaviruses, and GA5: recombinant ranavirus RCV-Z2. Regions inverted relative to the short-finned eel ranavirus (SERV) are set below those that match in the forward orientation. Lines collate aligned blocks between genomes. Refer to Table 1 for virus abbreviations.

GTR+G4 for the green block, GTR+G4 for the blue block, TVM+G4 for the purple block, TVMe+G4 for the olive green block, and K3Pu+G4 for the light brown block. The D21 ML and Bayesian phylogenetic analyses generated identical well-resolved trees with supported FV3-, CMTV-, and CoIV-like clades irrespective of whether the data were analyzed with or without partitions (Fig. 2A). The RCV-Z ranavirus was supported within the CMTV-like ranaviruses group as the sister species to ADRV (Figs. 2A, 3B, 3C). The RCV-Z2 ranavirus isolated eight years later at the same ranaculture facility was supported within the FV3-like ranaviruses clade as the sister group to the clade of North American FV3 ranaviruses (i.e., FV3 and SSME; Figs. 3B, 3C).

Mapping the five putative inversion events (A-E) onto the resolved ranavirus phylogeny resulted in five genomic arrangements (GA1-5; Figs. 2A, 2B). The EHNV-like ranaviruses represent the ancestral state (GA1). A minor inversion (A) of the small red block (orthologous to FV3 ORFs 11–12) resulted in GA2. A medium-sized inversion (B) of the blue block (FV3 ORFs 23–51) resulted in GA3. A large inversion (C) involving the olive green (FV3 ORFs 78–95), purple block (FV3 ORFs 52–77), blue block, green block (FV3 ORFs 13–22), and small red block resulted in GA4. Finally, a large inversion (D) involving the olive green block, purple block, blue block, green block, and small red block followed by a second large inversion (E) involving the purple block, blue block, and green block resulted in GA5.

3.3. Recombination analysis

Analysis of D15 revealed 15 significant recombination events with seven observed within RCV-Z2 alone (approximately 20% of its genome) and none in RCV-Z (Fig. 3A; Supplemental Table 3). The minor parent (i.e., apparent contributor of the smaller recombinant fragment) of all seven recombinant events within the RCV-Z2 (FV3-like ranavirus) genome was RCV-Z (CMTV-like ranavirus). The hypothetical major parents (i.e., apparent contributor of the rest of the sequence) of all seven recombination events were FV3-like ranaviruses with North American isolates (FV3, SSME) chosen as the top candidates in 6/7 events (Supplemental Table 3). Fig. 4 lists the 30 RCV-Z2 ORFs completely or partially (italics) predicted to fall within recombination break points including: *putative replicating factor* (ORF1), *putative myristylated membrane protein* (ORF2), *putative NTPase* (ORF8), putative DNA repair protein RAD2 (ORF11), *immediate early protein ICP-46* (ORF15), *transcription elongation factor SII* (ORF25), Ribonuclease III (ORF26), putative ATPase-dependent protease (ORF27), USS family protein (ORF79), *putative interleukin-1 beta convertase precursor* (ORF85), ribonucleoside diphosphate reductase beta subunit (ORF86), *putative NTPase/helicase-like protein* (ORF92), putative LITAF/PIG7 possible membrane associated motif in LPS-induced tumor necrosis factor alpha factor (ORF94), putative myeloid

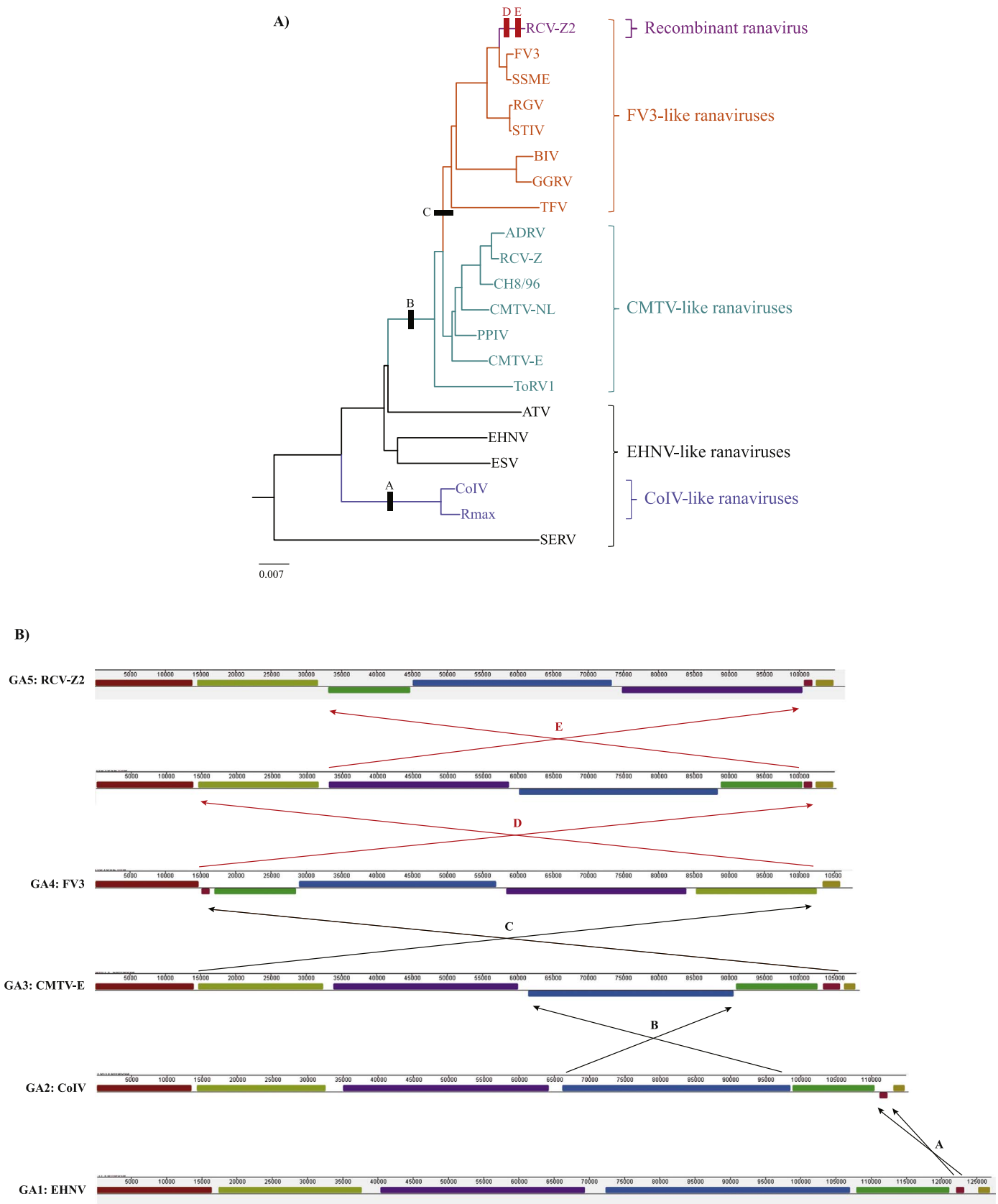

Fig. 2. (A) Maximum Likelihood (ML) phylogram depicting the relationship of RCV-Z and RCV-Z2 to 19 ranaviruses based on their aligned genomes (concatenation of 7 locally collinear blocks). All nodes were supported by bootstrap values > 75% from the ML analysis and by posterior probabilities values > 0.9 from the Bayesian analysis. The branch lengths represent the number of inferred substitutions as indicated by the scale. (B) Five putative genomic inversion events depicted as crossing arrows (A-E) that gave rise to 5 genomic arrangements (GA1-5). The black arrows indicate the inversion events (A-C) resulting in GA2, CoIV-like ranaviruses; GA3, CMTV-like ranaviruses; and GA4, FV3-like ranaviruses; respectively. The red arrows indicates two potential inversions (D, E) resulting in GA5, recombinant ranavirus RCV-Z2. Inversion invents (A-E) are mapped as tick marks onto the resolved tree in their most parsimonious configuration. Refer to Table 1 for virus abbreviations.

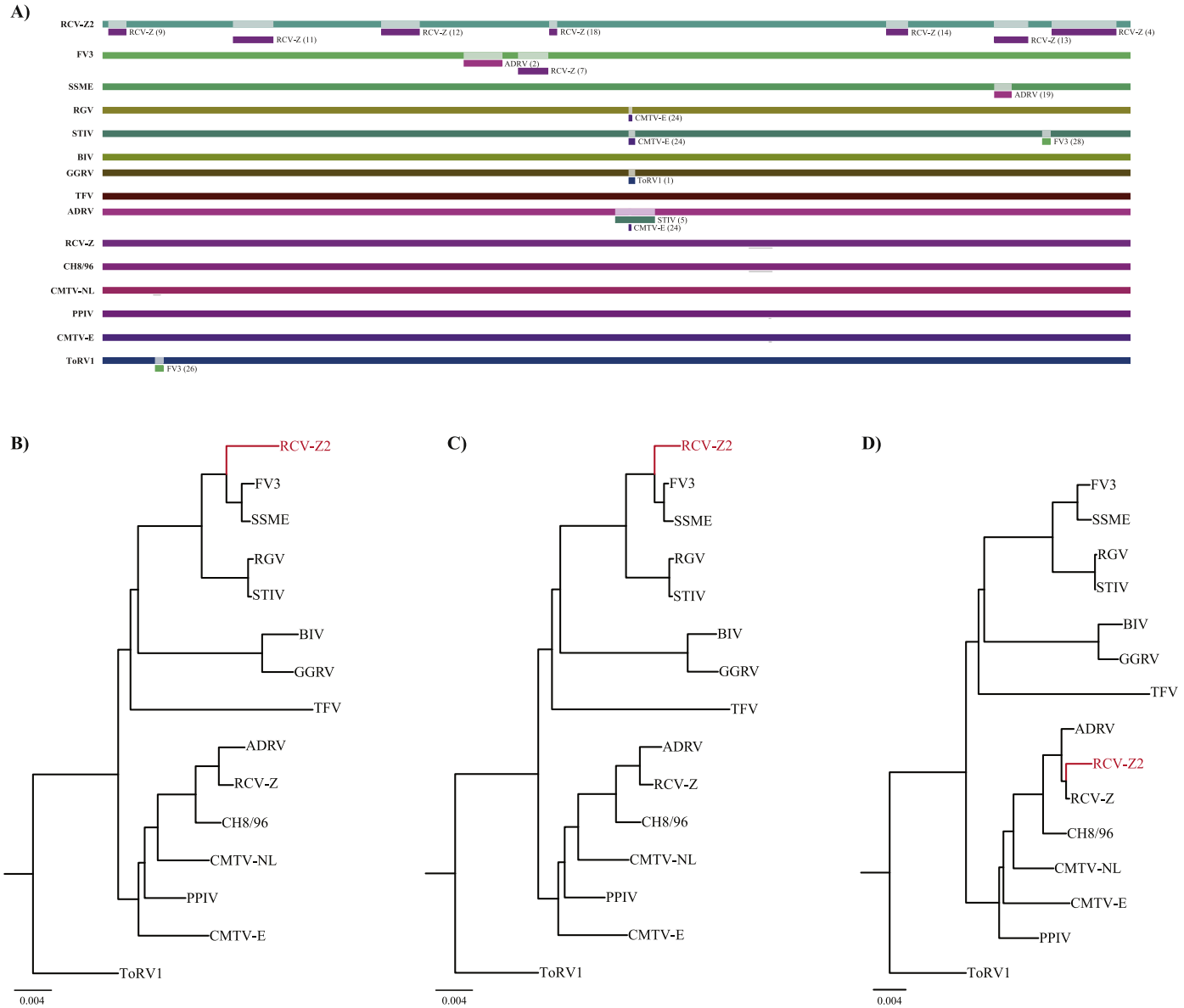

Fig. 3. (A) Schematic representation of 15 significant recombination events across the genomes of 15 FV3- and CMTV-like ranaviruses. The recombination events are represented by small numbered blocks set below the non-recombinant regions of each genome. The donor (minor parent) for each of the recombinant events is provided to the right of each small colored block. Refer to Supplemental Table 3 for the details of the 15 recombination events. (B) Maximum Likelihood phylograms depicting the relationship of RCV-Z and RCV-Z2 (labeled in red) to 13 other FV3- and CMTV-like ranaviruses based on their aligned genomes (concatenation of 5 locally collinear blocks), (C) aligned genomes with recombinant regions removed, and (D) recombinant regions only. All nodes were supported by bootstrap values > 75%. The branch lengths represent the number of inferred substitutions as indicated by the scale. Refer to Table 1 for virus abbreviations.

cell leukemia protein (ORF100), and 16 ORFs of unknown function (9, 10, 12, 13, 14, 28, 41, 78, 80, 87, 93, 95, 96, 97, 98, 99).

Partitioned ML and Bayesian phylogenetic analyses were not conducted on the D15 alignments because partitioned versus concatenated D21 alignments generated identical trees with similar high node support values (reported above). The best-fit model for the D15 concatenated LCB alignments was TVM+I. The D15 ML and Bayesian analyses with and without recombinant sites generated well-resolved trees identical to the D21 analyses including the position of RCV-Z within the CMTV-like ranaviruses group and the recombinant RCV-Z2 within the major parental FV3-like ranaviruses clade (Figs. 2A, 3B, 3C). The D15 ML and Bayesian analyses based on the concatenated recombinant site alignment produced nearly identical trees; however, the recombinant RCV-Z2 was now supported within the CMTV-like ranaviruses group as the sister species to its predicted minor parent, RCV-Z (Fig. 3D).

3.4. US22 protein family analyses

The amino acid alignments of the ranaviral US22 proteins resulted in the prediction of four α-helices and six β-sheets within the US22 domain (Fig. 5) as previously described (Zhang et al., 2011). The best-fit model for the ML and Bayesian phylogenetic analyses was JTT+I +G4. The ML and Bayesian phylogenetic analyses of the ranaviral and cellular US22 amino acid sequences generated nearly identical well-resolved trees with four orthologous ranaviral US22 gene clusters observed (Fig. 6). These analyses support four independent ranaviral capture events from amphibian donors and the gain of a US22 gene in RCV-Z2 through recombination with a CMTV-like ranavirus donor (i.e., RCV-Z) as described below (Figs. 6 and 7).

The first US22 orthologous gene cluster was present in all FV3- and CMTV-like ranaviruses except PPIV and RCV-Z2 (Figs. 5 and 6). The position of the first US22 orthologous gene cluster was conserved

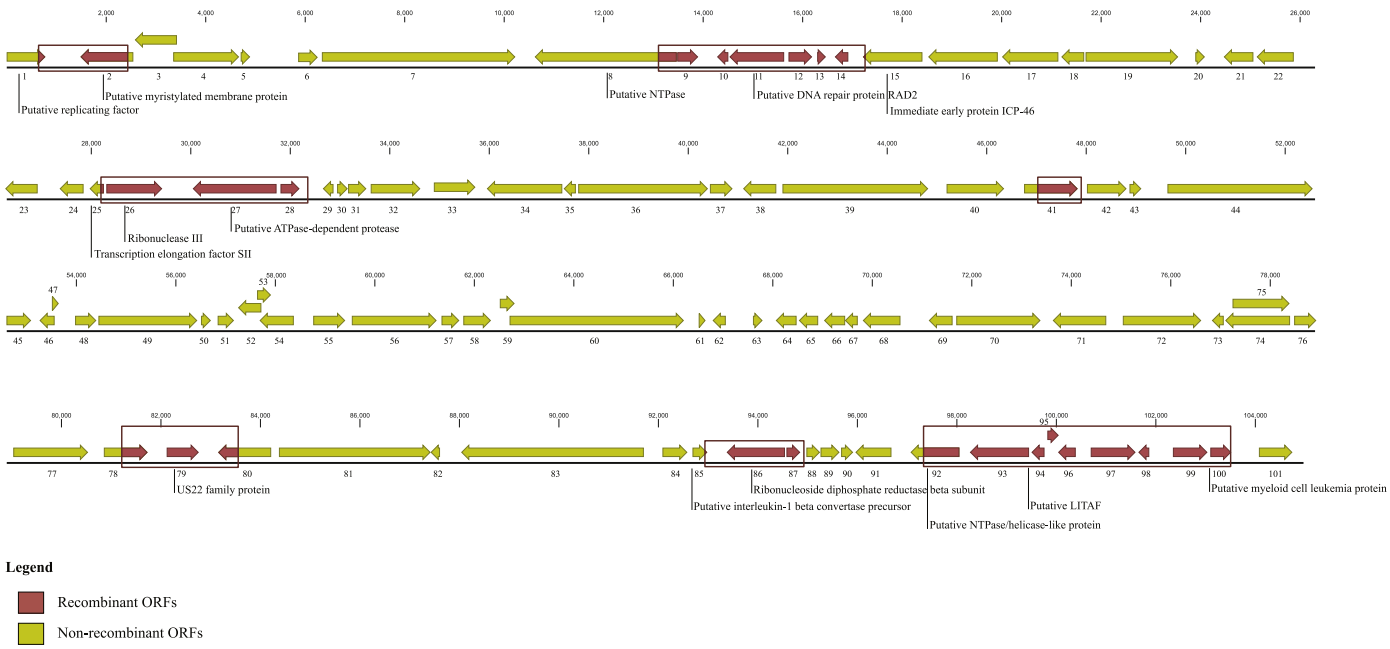

Fig. 4. Schematic representation of the RCV-Z2 genome annotation. The predicted open reading frames (ORFs) are indicated by yellow arrows. The 7 recombinant regions and associated 30 ORFs are indicated with red arrows and surrounded by red boxes. The functions (if known) of the recombinant ORFs are listed below the respective red arrows.

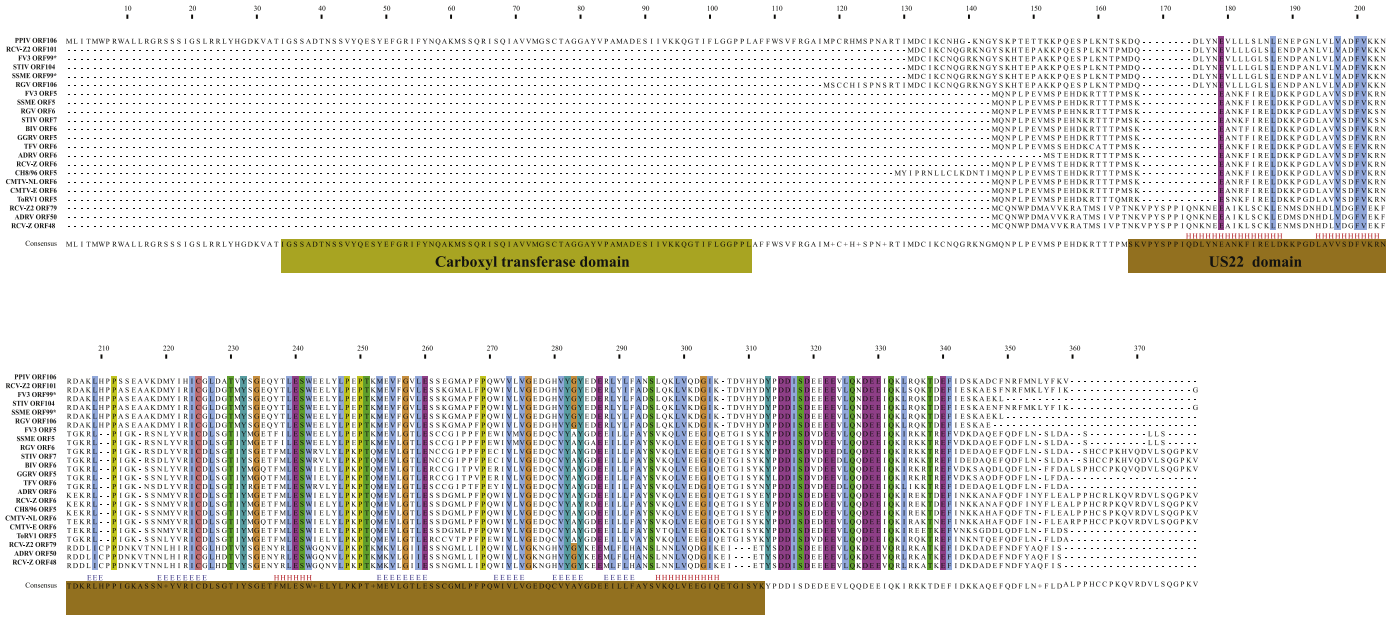

Fig. 5. Multiple amino acid sequence alignment of the ranaviral US22 proteins found in FV3- and CMTV-like ranaviruses. Predicted secondary structures are shown above the alignment consensus with runs of red Hs representing an α-helix and runs of blue Es representing a β-sheet. Conserved protein domains denoted by light brown (carboxyl transferase domain) or dark brown (US22 domain) boxes set below the consensus sequence. Refer to Table 1 for virus abbreviations.

among the FV3- and CMTV-like ranaviruses at the beginning of their genomes (i.e., ORFs 5–7 in these ranaviruses; Fig. 6) between the same two hypothetical proteins (orthologous to FV3 ORFs 4 and 6; RefSeq acc. NC_005946). The presence of this US22 gene cluster in nearly all FV3- and CMTV-like ranaviruses suggests the gene was acquired before they split from their most recent common ancestor (Figs. 6 and 7).

A second orthologous US22 gene cluster was observed within a subset of the FV3-like viruses including RGV (ORF106), STIV (ORF104), RCV-Z2 (ORF101), and unannotated in FV3 (RefSeq acc. NC_005946, positions 105,280-105,903 joined to positions 1–43) and SSME (RefSeq acc. KJ175144, positions 104,447-105,070 joined to positions 1–43). The position of this second US22 orthologous gene cluster was conserved among these FV3-like ranaviruses at the end of their genomes (i.e., ORFs 101–106; Fig. 6) after the putative myeloid cell leukemia protein (orthologous to ORF97 in FV3). Like the other horizontal gene transfer events, phylogenetic analyses suggested this US22 gene was acquired from an amphibian host after the aforementioned FV3-like ranaviruses split from their most recent common ancestor (i.e., BIV, GGRV; Fig. 7).

The CMTV-like ranavirus PPIV appears to have acquired a third cellular US22 gene near the end of its genome (PPIV ORF106) between two hypothetical proteins (PPIV ORFs 105 and 107). PPIV ORF105 is orthologous to ADRV ORF99, while PPIV ORF107 is present in ADRV but was not annotated (ADRV GenBank acc. KF033124). The PPIV ORF106 was the only US22 gene that encoded a carboxyl transferase domain upstream of the US22 domain (Fig. 5). The fact that PPIV is the

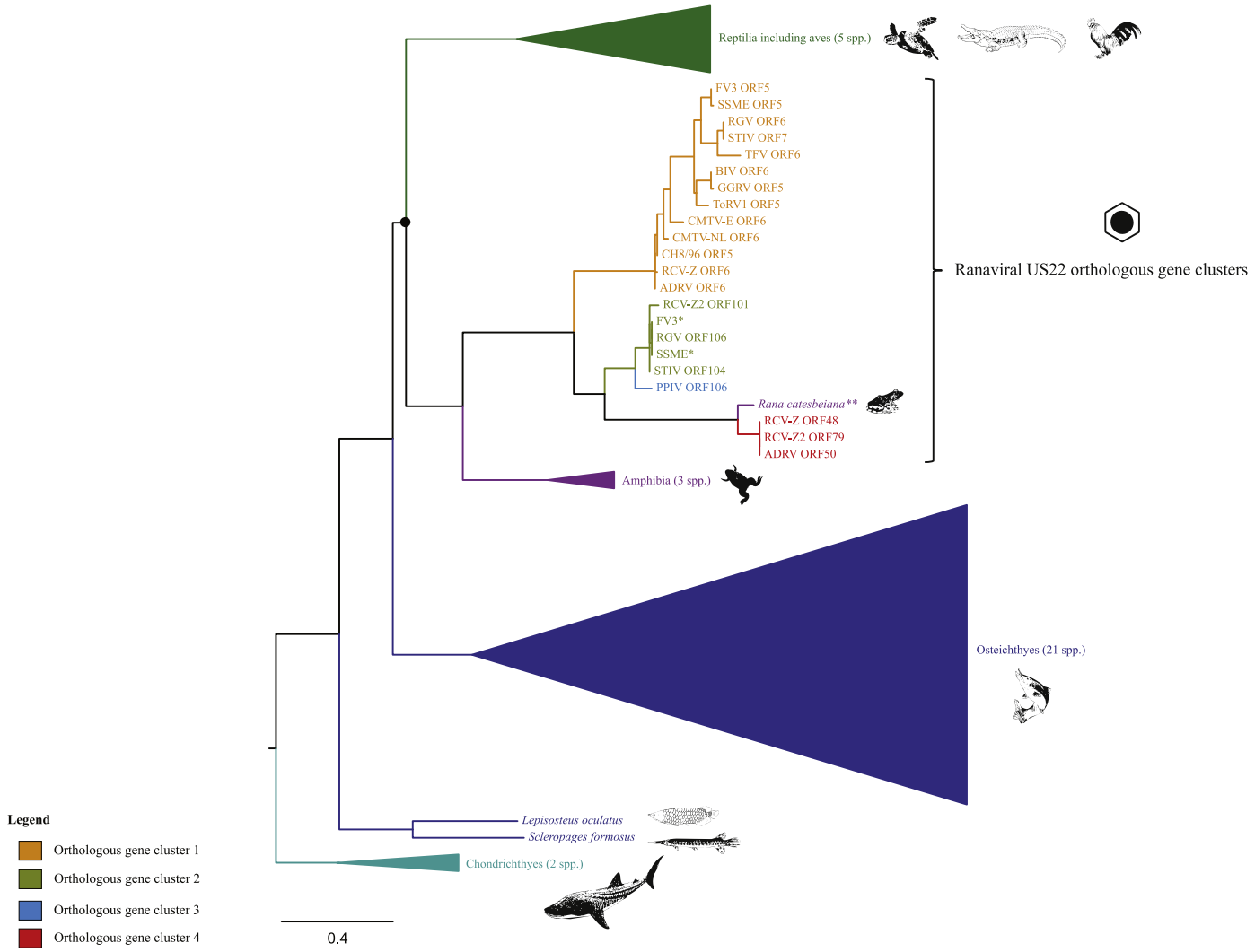

Fig. 6. Maximum Likelihood phylogram of cellular and ranaviral US22 proteins. All nodes were supported by bootstrap values > 75%. The branch lengths represent the number of inferred substitutions as indicated by the scale. * These US22 genes were not originally annotated in the genomes of FV3 and SSME (see Section 3 for details). ** Cellular US22 gene from bullfrog (*Rana catesbeiana*). Refer to Table 1 for virus abbreviations.

only CMTV-like ranaviruses to possess this US22 gene, its unique genomic position within the PPIV genome, and given it carries an upstream carboxyl transferase domain supports its independent acquisition from the other US22 orthologous gene clusters (Figs. 5–7).

A fourth US22 orthologous gene cluster was observed within the CMTV-like ranaviruses ADRV (ORF50; GenBank acc. KF033124) and RCV-Z (ORF48) as well as the recombinant ranavirus RCV-Z2 (ORF79). The position of the fourth US22 orthologous gene cluster was conserved among these CMTV-like ranaviruses in the middle of their genomes between two hypothetical proteins (orthologous to ADRV ORFs 49 and 52). The RCV-Z and RCV-Z2 US22 orthologs were genetically identical and nearly identical to ADRV (99%, 605/606) and exhibited high nucleotide identity (92%, 443/480) and coverage (75%, 480/639) to the cellular US22 gene (i.e., bullfrog; GenBank acc. BT081530). The ML phylogenetic analysis conclusively placed the bullfrog US22 gene as the sister group to the fourth ranaviral US22 orthologous gene cluster (i.e., ADRV ORF50, RCV-Z ORF48, RCV-Z2 ORF79; Fig. 6). This suggests that RCV-Z likely acquired this cellular US22 ortholog from its bullfrog host before vertically passing the gene along to ADRV, its closest relative, and donating the gene through recombination to RCV-Z2 (Figs. 6 and 7).

4. Discussion

In this study, we employed a comprehensive genome-wide alignment approach to gain a better understanding of phylogenomic patterns (i.e., genomic inversion and recombination events) of 21 ranaviruses including two bullfrog isolates obtained from epizootics that occurred at the same U.S. ranaculture facility (Majji et al., 2006; Miller et al., 2007). The phylogenomic hypotheses presented here (Figs. 2A, 3A, 3B) are largely congruent with both the results of our initial partial genomic analyses (data not shown) as well as those of other studies based on the concatenation of differing numbers of conserved genes (Eaton et al., 2010, 2013; Abrams et al., 2013; Jancovich et al., 2015; Stöhr et al., 2015; Epstein and Storfer, 2016). This congruence is noteworthy because the time spent generating genome-wide alignments using Mauve 2.4 (Darling et al., 2004) was significantly less than the manual construction of partial genome datasets based on the concatenation of conserved genes. Although recombination did not appear to influence tree topology when analyzed at a genomic scale (compare Figs. 3B to 3C), analysis of the recombinant regions alone resulted in an important topological difference in the position of RCV-Z2 that switched from the FV3-like ranaviruses

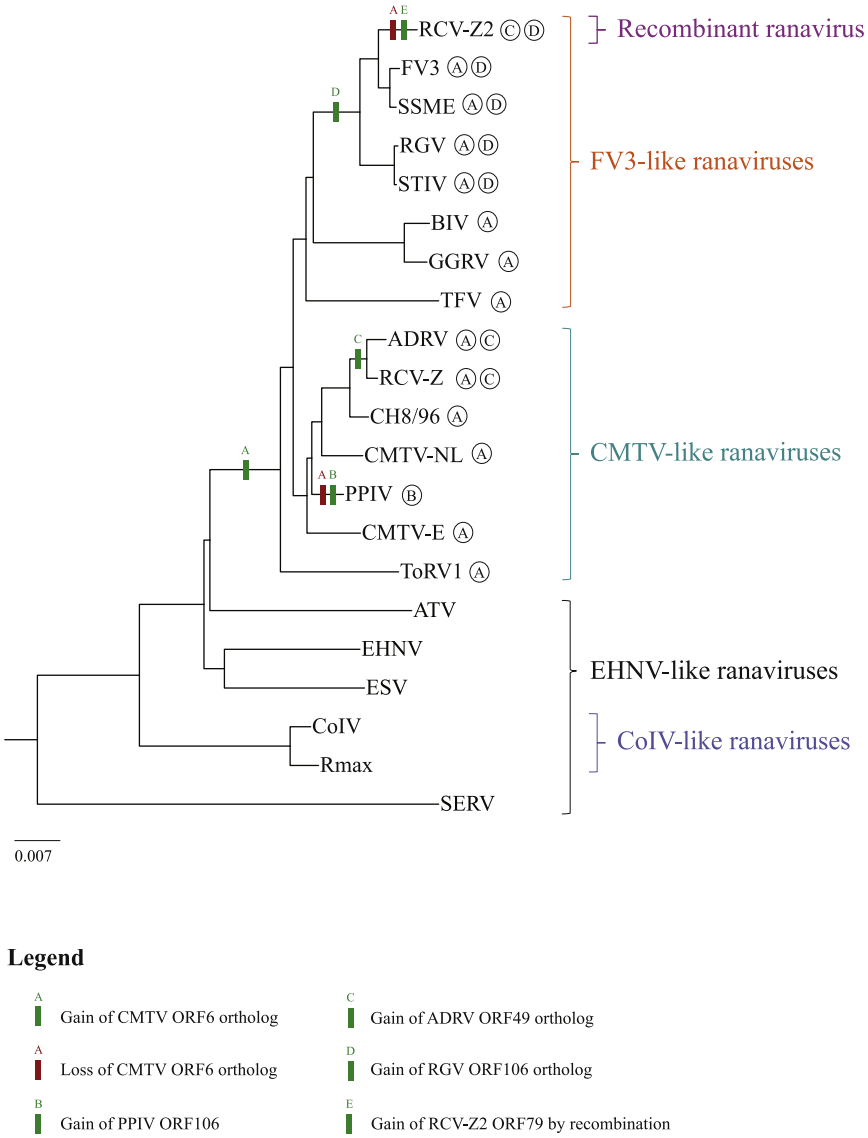

Fig. 7. Depiction of five US22 protein acquisition events (A-E) observed among the 21 ranaviruses analyzed in this study. Maximum Likelihood phylogram of 21 ranaviruses taken from Fig. 2. Acquisition events are mapped as green tick marks onto the tree in their most parsimonious configuration. Red tick marks denote US22 gene losses. Refer to Table 1 for virus abbreviations.

clade (predicted non-recombinant major parent) to the CMTV-like ranaviruses group (predicted minor parent donating the recombinant sequences) (Fig. 3D). These data suggest ranavirus phylogenetic studies relying on partial genomic datasets should consider the potential influence of recombination as has been noted previously for other DNA and RNA viruses (Martin et al., 2011; Weber et al., 2015).

Our study provides insight into ranaviral genomic architectural changes over their evolutionary history by mapping the most parsimonious position of five inversion events onto the branches of the resolved phylogeny (Figs. 1, 2A, 2B). The ancestral genomic arrangement is represented by the EHNV-like ranaviruses (GA1) with the short-finned eel ranavirus representing the most basal branch (Subramaniam et al., 2016). The CoIV-like ranaviruses clade display GA2 that likely arose following a minor inversion that occurred after their spilt from an EHNV-like ranavirus ancestor (Ariel et al., 2016). The CMTV-like ranaviruses, including the bullfrog isolate RCV-Z, display GA3 that arose from a medium-sized inversion that likely occurred after their split from their most recent common ancestor (MRCA) with the EHNV-like ranaviruses (Mavian et al., 2012a; Chen et al., 2013; Stöhr et al., 2015). The FV3-like ranaviruses display GA4 that arose from a large inversion that likely occurred following their split from their MRCA with the CMTV-like ranaviruses (Mavian et al., 2012a; Chen et al., 2013; Stöhr et al., 2015). Phylogenomic analysis of the other bullfrog RCV-Z2 isolate revealed a novel fifth genomic arrangement (GA5) that is hypothesized to have occurred through two additional inversion events (Fig. 2B). Although it is unknown whether undersampled members of related iridovirus genera (e.g., *Lymphocystivirus* and *Megalocytivirus*) possess comparable diversity in genomic architecture, other nucleo-cytoplasmic large DNA virus families such as poxviruses possess conserved core regions devoid of large genomic inversions (exceptions observed in avipoxviruses) and instead display variable inverted terminal repeat regions (Gubser et al., 2005; Jancovich et al., 2015). The significance of ranavirus genomic inversions remains unknown; however, a genomic inversion in *Ambystoma tigrinum virus* ORF52R (Putative 3-beta-hydroxy-delta-5-C27 steroid oxidoreductase-like protein) resulted in truncation of the gene (Jancovich et al., 2003). The mechanism generating ranavirus genomic inversions also remains unknown; however, high rates of *in vitro* recombination have been suggested (Chinchar and Granoff, 1986; Jancovich et al., 2003, 2015). Future work is needed to elucidate the significance and mechanism(s) leading to ranavirus genomic diversity and any relationship to their ecology, host range evolution, and pathogenesis (Jancovich et al., 2015).

Phylogenomic analysis of the 1998 bullfrog isolate RCV-Z support it as a member of the CMTV-like ranavirus group. CMTV-like ranaviruses were first described from a mass mortality event in 2007 among Spanish common midwife toads *Alytes obstetricans* (Balseiro et al., 2009) and subsequently described in European amphibians (Kik et al., 2011; Price et al., 2014; Van Beurden et al., 2014), reptiles (Stöhr et al., 2015), and fish (Holopainen et al., 2016), as well as Chinese giant salamanders in China (Dong et al., 2011; Geng et al., 2011; Jiang et al., 2011; Chen et al., 2013; Wang et al., 2014). Thus, the North American ranaculture CMTV-like ranavirus RCV-Z expands the known host and geographic range of these emerging ranaviruses. Our data corroborate the findings of Majji and colleagues (2006) who reported that the RCV-Z isolate differed from wild-type FV3 by its increased virulence, restriction fragment profiles, and possession of a full-length copy of the viral ortholog of eukaryotic translational initiation factor 2 alpha (eIF-2α). The latter is a putative viral immune evasion protein that acts to block the antiviral activity of interferon-induced protein kinase R and enhances virus replication (Rothenburg et al., 2011).

Recombination is recognized as a driving force in the generation of chimeric pathogenic viruses (Strayer et al., 1983; Weaver et al., 1997; Esposito et al., 2006, Gonzalez-Candelas, 2011, Vuilleumier et al., 2015) and viral adaptation to new hosts (Hoelzer et al., 2008; Bhatt et al., 2013; Lefeuvre and Moriones, 2015; Ding et al., 2017). We documented recombination between FV3- and CMTV-like ranaviruses resulting in the generation of the pathogenic chimeric ranavirus RCV-Z2. Genomic scanning and phylogenomic analyses support the minor parent (donor) to be the CMTV-like ranavirus isolate RCV-Z that appears to have contributed all seven recombinant sequences to the hypothetical FV3-like ranavirus major parent predicted to be a North American FV3 isolate (Fig. 3, Supplemental Table 3). The isolation of the RCV-Z bullfrog isolate in 1998 (Majji et al., 2006) followed by the isolation of the recombinant RCV-Z2 ranavirus at the same ranaculture facility eight years later (Miller et al., 2007) suggests the chimeric ranavirus could have evolved onsite. The recombinant ranavirus RCV-Z2 has previously been shown to be lethal in a ranaculture facility and exhibited greater virulence when compared to wild-type FV3 in experimental challenges involving various amphibian species (Hoverman et al., 2010, 2011). It is possible that one or more of the CMTV-like ranavirus genes acquired through recombination are responsible for the reported increased virulence of the recombinant FV3-like ranavirus RCV-Z2. In addition, the RCV-Z2 isolate: 1) has been shown to be highly pathogenic to amphibians species of conservation concern (Sutton et al., 2014; Earl et al., 2016), 2) can be transmitted to fish (Brenes et al., 2014), and 3) has the capability of causing local populations of common and rare amphibian species to go extinct (Earl and Gray, 2014; Earl et al., 2016). Hence, the presence of RCV-Z2 in North America represents a significant conservation threat to biodiversity.

Previous reports of recombination in double-stranded DNA (dsDNA) viruses include bacteriophages, herpesviruses, and poxviruses (Hershey and Rotman, 1948; Fenner and Comben, 1958; Perez-Losada et al., 2015). *In vitro* homologous recombination has been used to generate extensive ranavirus knock out mutants to replace specific ranavirus genes with various selectable markers (Jancovich et al., 2003; Chen et al., 2011, Andino Fde et al., 2015; Aron et al., 2016). Moreover, Chinchar and Granoff (1986) demonstrated that co-infection with two different *temperature-sensitive* (*ts*) mutants resulted in the generation of wild-type virus via recombination. In that study, which was designed to map the positions of various *ts* mutants, recombination took place at a high rate and was directly proportional to the distance between *ts* mutants. Recent studies have confirmed recombination among naturally occurring ranavirus isolates based on analyses of individual genes (Abrams et al., 2013; Epstein and Storfer, 2016). Epstein and Storfer (2016) detected recombination among the genomes of 16 ATV strains; however, the significance of the recombination could not be established. Using a subset of the ranaviruses analyzed in our study, Abrams and colleagues (2013) performed selection analyses to determine genes potentially involved with ranavirus host range evolution. Twelve genes were found to be under positive selection and 25/71 genes displayed significant recombination signatures including 2/12 of the same genes under positive selection, the ATPase-dependent protease (orthologous to FV3 ORF79; RefSeq acc. NC_005946) and the immediate early protein ICP-46 (FV3 ORF91). The ATPase-dependent protease was the only gene to exhibit positive selection along the branch that separates the fish EHNV-like ranaviruses and the amphibian FV3-/CMTV-like ranaviruses suggesting it may have been fundamental to the initial switch from fish to amphibians and possibly subsequent jumps (Abrams et al., 2013). Although selection analyses were beyond the scope of our study, we also detected evidence of recombination within the ATPase-dependent protease and immediate early protein ICP-46 genes in the RCV-Z2 genome (Fig. 3A, Supplemental Table 3). The acquisition of these recombined genes could increase viral replication in bullfrogs (Xia et al., 2009).

Like the members of many dsDNA viral families, ranaviruses have adapted to their respective hosts through the acquisition of cellular genes via recombination (Tidona and Darai, 2000). The US22 protein family belongs to the SUKH superfamily which includes a diverse group of proteins found in prokaryotes, eukaryotes, and dsDNA viral families that infect vertebrates including adenoviruses, alloherpesviruses, herpesviruses, iridoviruses, and poxviruses (Zhang et al., 2011). In dsDNA viruses, US22 proteins have been shown to counter diverse antiviral responses by interacting with specific host proteins (Zhang et al., 2011). In ADRV, the acquisition of two US22 genes (ORFs 6 and 49; GenBank acc. KC865735) has been argued as potential driver of virulence and host specificity (Chen et al., 2013). Inspection of the genomes of the 21 ranaviruses included in this study revealed every amphibian FV3- and CMTV-like ranavirus possess one or two US22 genes displaying the conserved SUKH protein family domains (Zhang et al., 2011) (Figs. 5–7). Phylogenetic analyses of ranaviral and cellular (e.g. fish, amphibians, and reptiles including birds) US22 genes suggest four independent ranaviral capture events from amphibian donors and the gain of a US22 gene in RCV-Z2 by recombination with a CMTV-like donor (i.e., RCV-Z; Figs. 6 and 7). A similar analysis based on a subset of the ranaviral and cellular US22 genes included in our analysis generated a nearly identical tree (Price, 2014) to our comprehensive tree (Fig. 6). Price (2014) found three of the four ranaviral US22 orthologous gene clusters we reported here (Fig. 6), missing only the US22 gene acquisition in PPIV as its genomic sequence was not determined until 2016 (Holopainen et al., 2016). The repeated acquisitions of US22 genes by amphibian-infecting FV3- and CMTV-like ranaviruses suggest that these genes may play an important role in the pathogenesis and host range evolution of these emerging pathogens (Chen et al., 2013; Price, 2014).

Although the functions of many of the predicted recombined RCV-Z2 genes are unknown, the presence of vCARD, the ribonucleoside diphosphate reductase beta subunit, LITAF, and an RNase III-like molecule among the CMTV-like genes found within RCV-Z2 is instructive as all have been linked to either immune evasion or enhanced virus replication among ranaviruses or other vertebrate viruses (Langelier et al., 2002; Lembo et al., 2004; Hussain et al., 2010; Eaton et al., 2013; Andino Fde et al., 2015; Aron et al., 2016; Allen et al., 2017). This result suggests that recombination events, in which immune evasion or viral efficiency genes have been acquired from a more pathogenic donor, provide a mechanism for increasing the virulence of an otherwise avirulent or lower virulence virus. Continued research is needed to elucidate the role inversions, recombination, and selection play in the evolution of virulence and host range evolution among ranaviruses.

In our study, we compared the phylogenomic patterns of two bullfrog ranavirus isolates to 19 different ranaviruses isolated from poikilothermic vertebrate hosts around the world (Supplemental

Fig. 1). Amphibians are traded internationally as pets or as part of zoological collections, for food or fishing bait, educational or scientific research, and as biological control agents (Schlaepfer et al., 2005; Andreone et al., 2006). Amphibians in the live animal trade can carry exotic pests and parasites (Franke and Telecky, 2001) and spread diseases (Daszak et al., 1999). Kolby et al. (2014) reported that 57% of amphibians in the Hong Kong trade were infected with ranaviruses. The housing of amphibians before and during importation typically involves grouping diverse species together at high densities, an arrangement that allows opportunities for amplification and transmission of virulent pathogens among species (Ewald, 1994; Pavlin et al., 2009). In the western United States, tiger salamanders have been infected with virulent strains of *Ambystoma tigrinum virus* through the bait trade (Jancovich et al., 2005; Picco and Collins, 2008; Epstein and Storfer, 2016). It is believed that the trade in American bullfrogs may have contributed to the spread of the amphibian fungus *Batrachochytrium dendrobatidis* (Daszak et al., 1999) and ranaviruses into new areas (Schloegel et al., 2009). It is noteworthy that ranavirus outbreaks have occurred in cultivated bullfrogs in North America (Majji et al., 2006; Miller et al., 2007) and South America (Mazzoni et al., 2009), as well as in invasive bullfrogs in Japan (Une et al., 2009) and Belgium (Sharifian-Fard et al., 2011). The bullfrog isolate RCV-Z, isolated from diseased American bullfrogs in 1998, was found to be the closest relative to ADRV. Given that, perhaps RCV-Z spread to China via the unregulated international bullfrog trade (see below) and led to outbreaks in the Chinese giant salamander beginning in 2010 (Dong et al., 2011; Geng et al., 2011; Jiang et al., 2011; Chen et al., 2013; Wang et al., 2014).

The annual United States trade in amphibians is estimated at millions of animals, body parts, and products (Schlaepfer et al., 2005). The American bullfrog is both the most commonly cultivated amphibian for human consumption and is the most frequently traded species globally (Altherr et al., 2011). From 2008–2013, > 8,000,000 North American bullfrogs were imported into, and > 17,000 were exported from, the United States (data from C.M. Romagosa compilation of USFWS Form 3–177). Schloegel et al. (2009) reported that 8.5% of live bullfrogs imported into three U.S. ports from 2000 to 2005 were infected with ranavirus. When exported, bullfrogs are usually live, but some international trade calls for frozen frog legs (Teixeira et al., 2001). The major countries that import frog legs are France, the United States, Belgium, and Luxembourg (United Nations Statistics Division, 2008). Thus, the distribution of the CMTV-like ranaviruses in Europe, China, and the United States may be linked to the international trade of bullfrogs.

The American bullfrog may have played an important role in the global dissemination of virulent ranaviruses given they are: 1) globally cultivated under intensive conditions, 2) susceptible to multiple ranaviruses (i.e., FV3- and CMTV-like ranaviruses) and thus may serve as mixing vessels for the evolution of pathogenic chimeric ranaviruses (i.e., FV3 x CMTV recombinants), and 3) regularly traded internationally. Ranaculture facilities may pose a significant risk to wild fish, amphibians, and reptiles if infected animals or water are released into the environment. Continued efforts are needed to properly evaluate the risk ranaculture facilities may pose to wildlife through pathogen pollution and the role international trade has played in the global dissemination of ranaviruses (Picco and Collins, 2008). In addition, regulatory authorities should consider creation or enforcement of regulations that require pathogen-free trade of amphibians following the World Organization for Animal Health (OIE) protocols (Schloegel et al., 2010) and the decontamination of wastewater at ranaculture facilities to reduce the likelihood of spillover of ranaviruses to wild populations.

Conflicts of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.07.028.

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