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Low intraspecific variation of *Frog virus 3* with evidence for novel FV3-like isolates in central and northwestern Canada

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ABSTRACT: *Frog virus 3* (FV3) and FV3-like ranaviruses can infect a variety of cold-blooded aquatic species and present a primary threat to amphibians across the globe. Previous studies of FV3-like viruses have largely investigated higher-level phylogenetic distinctions of these pathogens via portions of the conserved major capsid protein (MCP), and the putative virulence gene vIF-2α. Few studies, however, have investigated the spatial distribution of FV3 variants at the population level — data that can be used to further understand the spatial epidemiology of this disease. In this study, we sequenced the MCP and vIF-2α of 127 FV3-positive amphibians sampled from Canadian water bodies in Ontario, northeastern Alberta, and southern Northwest Territories to explore whether intraspecific genetic variation exists within FV3. There was a lack of variation at the 2 markers across these regions, suggesting that there is a lack of FV3 sequence diversity in Canada, which may hint at a single source of infection that has spread. However, an undocumented variant termed Wood Buffalo ranavirus (WBRV) was detected in samples from 3 sites in Alberta and Northwest Territories that clustered within the FV3-like lineage with 99.3% sequence homology for MCP. For vIF-2α, all sequences were the expected truncated variant except for 6 samples in Ontario. These latter sequences were suggestive of recombination with common midwife toad virus (CMTV). The lack of variation suggests that higher-resolution genome analyses will be required to further explore the spatial spread and intraspecific variation of the disease.

KEY WORDS: *Frog virus 3* · *Ranavirus* · Phylogenetic · Major capsid protein · vIF-2α · Amphibians · Wildlife disease

1. INTRODUCTION

Spatio–temporal phylogeographical analyses of infectious diseases have enhanced our understanding of pathogen dispersion and help to inform management plans to mitigate disease spread (Cullingham et al. 2009, Lemey et al. 2009). For example, phylogeographic studies have been employed to zoonotic and human diseases, such as HIV (Leitner et al. 1996), rabies (Bourhy et al. 2008, Lemey et al. 2009), and influenza (Wallace & Fitch 2008, Lemey et al. 2009), to infer pathogen spread, movement, and maintenance in their host.

Over the past 20 yr, amphibian populations have been declining around the globe (Singh 2002, Stuart et al. 2004, Harp & Petranka 2006). These declines are thought to be linked to a number of anthropogenic factors, including habitat destruction, pollu-

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tion, climate change, agriculture run-off, herbicides, and pesticides (Harp & Petranka 2006, Gray et al. 2007, Schock et al. 2009, Duffus et al. 2015). These stressors, combined with the wildlife trade, have been associated with upsurges in amphibian mortality from disease (Daszak et al. 2001, Gray et al. 2007, Reeve et al. 2013, Brunner et al. 2015). Two broadly dispersed pathogens associated with mass epidemics and significant population declines include the fungus *Batrachochytrium dendrobatidis* (*Bd*), and ranaviruses such as *Frog virus 3* (FV3; Green et al. 2002, Greer et al. 2005, Gray et al. 2009, 2015, James et al. 2015). While *Bd* spread has been relatively well studied (Fisher et al. 2009, Olson et al. 2013), less information exists regarding patterns of ranavirus spread due to their numerous species and strains around the globe.

Ranavirus is a genus of the family *Iridoviridae* that is made up of over 20 systemically infectious species and isolates that target ectothermic vertebrates (Tan et al. 2004, Brunner et al. 2015, Duffus et al. 2015, Forzán et al. 2017). Within this genus, 8 viruses are recognized as distinct species by the International Committee on Taxonomy of Viruses (Chinchar et al. 2017). One of the most prevalent and best characterized species of *Ranavirus* is FV3 (Granoff et al. 1965). Although FV3 is considered a species, other ranaviruses are grouped within the same lineage as FV3 based on factors that include amino acid and nucleotide sequence relatedness, host species, genome size, genetic co-linearity, gene content, and GC content (Tan et al. 2004, Jancovich et al. 2012, 2015b). Examples of FV3-like viruses include, but are not limited to soft-shelled turtle iridovirus (STIV), Rana grylio virus (RGV), and tiger frog virus (TFV), all isolated within various species in China (He et al. 2002, Huang et al. 2009, Lei et al. 2012); Bohle iridovirus (BIV), isolated from *Limnodynastes ornatus* (ornate burrowing frog) in Australia (Marsh et al. 2002); German gecko ranavirus (GGRV), isolated in Germany (Stöhr et al. 2015); and spotted salamander Maine (SSME), isolated in the USA, which has the highest similarity to FV3 with 98.79% sequence identity (Table 1) (Docherty et al. 2003, Morrison et al. 2014). Many other FV3-like ranavirus isolates have been identified, either in regions of the globe other than the region of their initial isolation, or within different species or taxa. FV3 has been identified within the pallid sturgeon *Scaphirhynchus albus* in the USA (T. B. Waltzek unpubl. data), and within the American bullfrog *R. catesbeiana* and the common frog *R. temporaria* in Brazil and the UK, respectively (R. A. Mazzoni et al. unpubl. data, S. J. Price et al. unpubl. data, Teacher et al. 2010). Another FV3-like virus known as *Rana catesbeiana* virus (RCV) has also been isolated in American bullfrogs within Japan, Taiwan, and the USA, likely through international trade (Table 1) (Une et al. 2009, Claytor et al. 2017, C.-Y. Hsieh et al. unpubl. data).

Ranavirus research has primarily focused on phylogenetics of isolates with vast global distances between them, or different taxonomic host origin (e.g. Hyatt et al. 2000, Chinchar 2002, Holopainen et al. 2009, Stöhr et al. 2015); however, there have been fewer investigations pertaining to the spatial and intraspecific variation of a single strain that could inform patterns of disease spread. Recently, phylogenetic comparison of *Ranavirus* strains within the UK were found to cluster into 3 separate, geographically distinct lineages, possibly suggesting 3 separate periods when *Ranavirus* was introduced to the region (Duffus et al. 2017). In most ranavirus studies, the focus has been on the major capsid protein gene (MCP, ORF 90R) due to its conservation and individuality between species, and the viral homolog of eIF-2 (vIF-2α, ORF 26R) based on its suspected role in virulence and its distinct forms between *Ranavirus* lineages (Mao et al. 1997, Hyatt et al. 2000, Chen et al. 2011, Grayfer et al. 2015, Stöhr et al. 2015). Both MCP and vIF-2α genes are common markers used when studying populations of ranaviruses, as they are highly conserved and present variation across species and lineages (Stöhr et al. 2015, Duffus et al. 2017). The MCP is a conserved gene within a species of *Ranavirus* that is often used to identify and categorize *Ranavirus* isolates into their appropriate lineage (Allender et al. 2013, Duffus & Andrews 2013, Kolby et al. 2014, Waltzek et al. 2014). However, the MCP does show evidence of variation within FV3 when sampled across countries and taxonomic classes of hosts (Tan et al. 2004, Holopainen et al. 2009, Mazzoni et al. 2009, Saucedo et al. 2017), making it a reasonable marker to explore intraspecific variability within Canada, especially when marking comparisons of anuran species between northern and southern regions. Furthermore, there are variable sites throughout the entirety of this 1392 bp gene, but many studies only focus on a short 500 bp fragment for ease of amplification (Mao et al. 1997, Greer et al. 2005, Duffus & Andrews 2013), a fragment for which many closely related *Ranavirus* species can be up to 99% identical in sequence (Jancovich et al. 2015b). Thus, a thorough investigation into the intraspecific variability within this marker requires the entire gene (Duffus & Andrews 2013).

Table 1. *Ranavirus* species and isolates used in this study. Species and isolates definition according to Chinchar et al. (2017)

Virus species or lineage	Acronym	Host	Country of isolation	Accession no.	Source
Frog virus 3	FV3	Leopard frog *Rana pipiens*	USA	AY548484	Tan et al. (2004)
Frog virus 3	FV3	Leopard frog *Rana pipiens*	USA	FJ459783	Holopainen et al. (2009)
Frog virus 3	FV3	American bullfrog *Rana catesbeiana*	Brazil	DQ897669	R. A. Mazzoni (unpubl. data)
Spotted salamander Maine virus	SSME	Spotted salamander *Ambystoma maculatum*	USA	KJ175144	Morrison et al. (2014)
Frog virus 3 isolate PSRV-2009	FV3-PSRV	Pallid sturgeon *Scaphirhynchus albus*	USA	KF646249	T. B. Waltzek (unpubl. data)
Frog virus 3 isolate RUK13	FV3-RUK13	Common frog *Rana temporaria*	UK	KJ538546	S. J. Price (unpubl. data)
Frog virus 3 isolate *Op*/2015/ Netherlands/UU3150324001	FV3 *Op*	Strawberry poison dart frog *Oophaga pumilio*	Netherlands	MF360246	Saucedo et al. (2017)
Soft-shelled turtle iridovirus	STIV	Soft-shelled turtle *Trionyx sinensis*	China	EU627010	Huang et al. (2009)
Soft-shelled turtle iridovirus	STIV	Soft-shelled turtle *Trionyx sinensis*	China	DQ335253	Zhao et al. (2007)
Rana grylio virus	RGV	Pig frog *Rana grylio*	China	JQ654586	Lei et al. (2012)
Rana nigromaculata ranavirus	RNR	Dark-spotted frog *Pelophylax nigromaculatus*	China	MF359927	W. Mou (unpubl. data)
Hynobius nebulosus virus	HNV	*Hynobius nebulosus*	Japan	AB500273	M. Murakami (unpubl. data)
Rana catesbeiana virus	RCV	American bullfrog *Rana catesbeiana*	Japan	AB474588	Une et al. (2009)
Rana catesbeiana virus	RCV	American bullfrog *Rana catesbeiana*	Taiwan	FJ207464	C.-Y. Hsieh (unpubl. data)
Rana catesbeiana virus Z2	RCV-Z2	American bullfrog *Rana catesbeiana*	USA	MF187209	Claytor et al. (2017)
Ranavirus KRV-1	KRV	Seoul frog *Rana plancyi chosenica*	South Korea	HM133594	Kim et al. (2011)
Bohle iridovirus	BIV	Ornate burrowing frog *Limnodynastes ornatus*	Australia	AY187046	Marsh et al. (2002)
Tiger frog virus	TFV	Tiger frog *Rana tigrina rugulosa*	China	AF389451	He et al. (2002)
Lacerta monticola ranavirus	LMR	Iberian mountain lizard *Lacerta monticola*	Portugal	KM516757	Stöhr et al. (2015)
Common midwife toad virus	CMTV	Common midwife toad *Mesotriton alpestris cyreni*	Spain	JQ231222	Mavian et al. (2012)
Chinese giant salamander iridovirus	GSIV	Chinese giant salamander *Andrias davidianus*	China	KF512820	Li et al. (2014)
Ambystoma tigrinum virus	ATV	Tiger salamander *Ambystoma tigrinum*	USA	AY150217	Jancovich et al. (2003)

The vIF-2α marker is also specific to different *Ranavirus* lineages. Some lineages do not carry the vIF-2α gene (e.g. grouper iridovirus and Singapore grouper iridovirus), while others have a truncated version of the gene (FV3, STIV, and RGV) lacking three-quarters of the N-terminal (Chen et al. 2011, Grayfer et al. 2015). Complete knockout of this gene results in lower host death rates and increased time until death, suggesting that it plays a role in virulence (Jancovich & Jacobs 2011). There is also evidence that the lack of the N-terminal region in FV3 and STIV causes the viruses to be less virulent than viruses with the complete gene (Majji et al. 2006). The length of the vIF-2α gene in ranaviral isolates is therefore a good indicator of virulence, and it is useful in determining the relationship of new isolates to *Ranavirus* species (Essbauer et al. 2001, Stöhr et al. 2015).

In this study, we investigated the intraspecific genetic variability between isolates of FV3 samples across 2 Canadian regions spanning 2 provinces and 1 territory, sampling over 3 yr to elucidate patterns of genetic diversity present within Canada. The aim was to provide insight into the levels of genetic variation in FV3 isolates and determine if spatial or temporal patterns of variation could be used to assess patterns of disease spread. We began to explore intraspecific variation by expanding to the full MCP gene sequence (1392 bp) for higher resolution in detecting haplotypes at this commonly profiled locus. Along with comparison of haplotypes within Canada, we also compared haplotypes in our study to other FV3 isolates from previous studies. We chose to sample at both a fine and a coarse geographical scale, with samples taken from Ontario, Alberta, and the Northwest Territories. Further, given the importance of vIF-2α in pathogenicity (Jancovich & Jacobs 2011), we also screened for size variants and sequenced this gene to estimate virulence, as well to determine the relationship between our haplotypes to pre-existing ranaviruses (Essbauer et al. 2001, Stöhr et al. 2015).

2. MATERIALS AND METHODS

2.1. Sample collection

Sixteen field sites were visited across central Ontario in June and August 2016, with only 4 sites having FV3-positive specimens (Table 2, Fig. 1). Tadpoles were euthanized and collected in 95% ethanol. Nitrile gloves were changed between handling individuals, and field equipment (nets, waders, boots) was disinfected with 10% bleach for 15 min between sites to prevent cross-contamination. All samples were kept on ice until transported to Trent University, where they were refrigerated at 4°C until extraction.

Seven field sites were sampled in Alberta and Northwest Territories from April to July 2015, 2016, and 2017 to assess *Ranavirus* presence. Tissue samples were tested for FV3 using PCR from each field site across all 3 yr (Table 2, Fig. 1). Adult toe and juvenile tail clips were taken from specimens with sterile scalpel blades. All samples were kept in ethanol and stored at −20°C until transportation and extraction. Samples were transported to and processed at Laurentian University.

2.2. Extraction

Tails of tadpoles (n = 215) were clipped and toe clips (n = 94) were put into tubes with 200 µl lysis buffer. All clippings (n = 309) had 20 mg ml^{-1} proteinase K added and were incubated at 56°C for 2 h, vortexing every 30 min. All samples were then extracted with a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocols.

2.3. PCR amplification and sequencing

Primers were newly designed to amplify the MCP of FV3 and SSME (see Table S1 in the Supplement at www.int-res.com/articles/suppl/d134p001_supp.pdf), excluding all other *Ranavirus* isolates and species. Samples were run on conventional PCR targeting the MCP, which was performed in 15 µl volume reactions consisting of 1× PCR buffer (Promega), 0.2 mmol l^{-1} dNTPs, 1.5 mM $MgCl_2$, 0.15 mg ml^{-1} bovine serum albumin, 0.3 µmol l^{-1} forward (5′-TCC ACA GTC ACC GTG TAT CTT-3′) and reverse (5′-TGC AGC AAA CGG ACA CTT-3′) primers, 0.2 U Taq polymerase (Promega), and 4 µl of template DNA. PCR conditions consisted of 5 min at 95°C, then 38 cycles of 30 s at 95°C, 30 s at 58°C, and 2 min at 72°C, followed by 2 min at 72°C. Samples were amplified at the vIF-2α region with newly designed primers (Table S1) and PCR was performed in 15 µl volume reactions as detailed above except with 0.3 µmol l^{-1} forward (5′-AAC AAA TGC AAT GAC TGT AAA TG-3′) and reverse (5′-ACA CAA AGG GGC ACA GTC-3′) primers, and 3 µl template DNA. PCR conditions consisted of 5 min at 95°C, then 35 cycles of

Table 2. Site locations (see Fig. 1) and life stages of all collected samples, with breakdown of all samples positive for *Ranavirus*. FV3: *Frog virus 3*; WBRV: Wood Buffalo ranavirus

Site	Date	Coordinates		Life stage			FV3 prevalence		Haplotype
		Lat. (°N)	Long. (°W)	Tadpole	Meta	Adult	+/total	%	FV3/WBRV/ Recom (of total +)
Ontario									
KEN	Jun 2016	44.57991	78.42634	6	0	0	6/6	100	0/0/6
MOA	Aug 2016	44.27409	77.34493	7	0	0	1/7	14	1/0/0
STL	Aug 2016	44.45036	78.64189	7	0	0	2/7	29	2/0/0
TIM	Aug 2016	46.53696	80.94803	0	0	1	1/1	100	1/0/0
Alberta									
Toadlet pond	2015	59.445	112.362	0	4	15	13/25	52	0/13/0
Toadlet pond	2016	59.445	112.362	0	0	2	2/2	100	2/0/0
Toadlet pond	2017	59.445	112.362	25	0	2	8/27	30	8/0/0
Wolf Creek	2015	59.927	111.747	16	0	10	0/26	0	0/0/0
Northwest Territories									
Antoinette's pond	2015	60.108	112.263	4	31	10	6/45	13	6/0/0
DnP Wetlands	2015	60.034	112.911	3	0	18	0/21	0	0/0/0
Preble pond	2017	60.033	113.189	5	0	0	0/5	0	0/0/0
KM 190	2015	60.048	113.137	30	7	10	21/47	45	0/21/0
KM 190	2017	60.048	113.137	12	0	18	12/30	40	2/10/0
KM 196	2017	60.028	113.027	58	0	8	55/66	83%	54/1/0

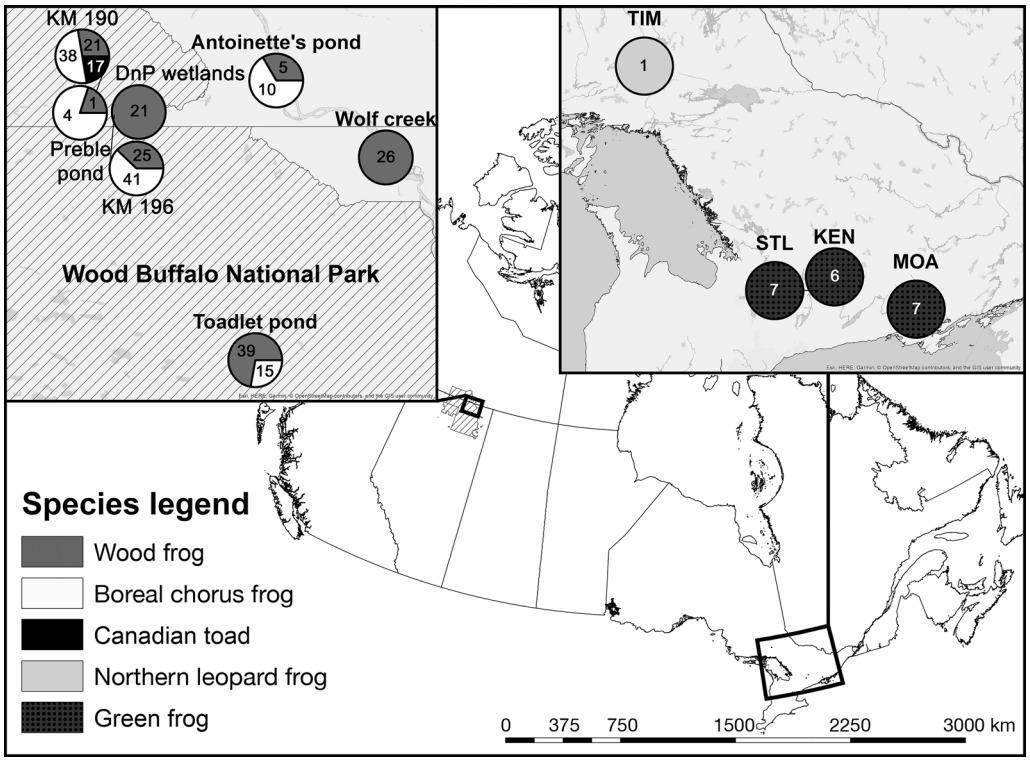

Fig. 1. Sites across Ontario, Alberta, and Northwest Territories, Canada. Numbers represent the total number of anuran species collected at each site between 2015 and 2017

30 s at 95°C, 30 s at 53°C, and 90 s at 72°C, followed by 2 min at 72°C. Three positive controls of an FV3-like isolate cultured in epithelioma papulosum cyprini cells were included at concentrations of 200, 20, and 2 pfu μl^{-1} as well as a negative control. Amplified products were separated using gel electrophoresis on a 1.5% agarose gel, and visualized using ethidium bromide under ultraviolet light.

Amplified products were purified using ExoSAP (New England Biolabs) then sequenced using a Big Dye® Terminator version 3.1 cycle sequencing kit (Life Technologies) and run on an ABI 3730 sequencer.

2.4. BLAST and phylogenetic analysis

Completed sequences were aligned using ClustalW (Larkin et al. 2007) and manually verified for calling errors using MEGA7 (Kumar et al. 2016) using reference FV3 genes (MCP and vIF-2α) from GenBank accession no. AY548484. MCP sequences were trimmed to 1392 bp of the entire coding region, while vIF-2α sequences were trimmed down to the appropriate coding region. Sequences were analyzed through Nucleotide Basic Local Alignment Search Tool (BLASTn) to determine sequence similarity. vIF-2α sequences were then trimmed to the reference sequence of best fit, and all sequences were then translated to determine the estimated protein sequences of unknown haplotypes.

Samples with <100% sequence homology to FV3 and SSME (GenBank accession nos. AY548484 and KJ175144, respectively) were aligned in MEGA7 (Kumar et al. 2016) to the MCP and vIF-2α of other FV3-like *Ranavirus* isolates to determine variable nucleotide positions. Sequences compared to unknown samples (both MCP and vIF-2α) included FV3-like ranaviruses for MCP, along with common midwife toad virus (CMTV)-like ranaviruses for vIF-2α, and *Ambystoma tigrinum virus* (ATV, AY150217) (Jancovich et al. 2003) as an outgroup (see Table 1 for sequence data from GenBank). Nucleotide substitution model optimization was performed through MEGA7 for MCP and vIF-2α to determine the best-fitting model for phylogenetic tree construction. For the MCP, the HKY+G model (Hasegawa et al. 1985) was the best-fitting model, and for vIF-2α, the HKY model (Hasegawa et al. 1985) was determined as the best fit. Phylogenetic trees were constructed using maximum-likelihood methods in MEGA7 (Kumar et al. 2016), with 1000 bootstrap replicates.

3. RESULTS

3.1. Intraspecific variation between FV3-like ranaviruses at the MCP

A total of 127 out of 309 tissue samples were positive for *Ranavirus* and were fully sequenced at the MCP gene. Ninety-four of the 127 samples were fully sequenced at the vIF-2α gene due to low viral copy DNA. A total of 5 anuran species were infected with *Ranavirus* (Table S2): green frog *Rana clamitans* (n = 9), northern leopard frog *R. pipiens* (n = 1), wood frog *Lithobates sylvaticus* (n = 53), boreal chorus frog *Pseudacris maculata* (n = 61), and Canadian toad *Anaxyrus hemiophrys* (n = 3). The majority of sequences (82 samples, within all species except Canadian toad) had 100% similarity to the MCP of 4 FV3-like isolates (Table 3): FV3 isolated in *R. pipiens* from the USA (Holopainen et al. 2009), SSME isolated in *Ambystoma maculatum* from the USA (Docherty et al. 2003, Morrison et al. 2014), FV3-RUK13 isolated in *R. temporaria* in the UK, and PSRV-2009 isolated in pallid sturgeon *Scaphirhynchus albus*. However, compared to the reference FV3 genome submitted to GenBank in 2004 (AY548484; Tan et al. 2004), there was one nucleotide difference at position 648 between all sequences (99.9% similarity; Table 3). This genome was originally isolated in 1962, when FV3 was first described, likely explaining the 1 bp difference from all other FV3-like sequences (Claytor et al. 2017).

3.2. Wood Buffalo ranavirus isolate

At the MCP, a previously undocumented haplotype, WBRV (referenced as Wood Buffalo ranavirus, as all 3 sites presenting this variant were in Wood Buffalo National Park; 45 samples total) had high homology with FV3, with 99.3% sequence similarity and 9 nucleotide changes (Table 3). Of the 9 nucleotide changes, 3 were non-synonymous mutations (S235A, A238E, and T290A). The vIF-2α region for the WBRV haplotype was truncated (231 bp), and there was a 99.6% homology to FV3 (AY548484) with one non-synonymous substitution (R29P; Table 4).

3.3. Possible FV3/CMTV recombinant

Interestingly, the vIF-2α region of samples from one site, KEN (Ontario), had a band approximately 1200 bp in length (Fig. S1), which when trimmed to the coding region presenting a 99.8% sequence similarity to the eIF-2 protein of the Chinese giant salamander iridovirus (GSIV; Li et al. 2014) — also referred to as Andrias davidianus ranavirus (ADRV) — an isolate of the *Common midwife toad virus* lineage (CMTV-like viruses; Table 3, Table S2). Overall, there were 3 haplotypes found using these 2 genes (Fig. 2).

3.4. Phylogeny of novel isolates

A maximum likelihood phylogeny of the MCP placed the new WBRV isolate outside the FV3 clade (Fig. 3), yet with relatively low bootstrap support. However, the phylogenetic tree of the vIF-2α (Fig. 4) clustered the WBRV isolate within with FV3 clade with high bootstrap support, providing a more reliable tree than the phylogenetic tree based on the MCP. Concatenation of the 2 genes demonstrated higher bootstrap values, strengthening the phylogeny of the FV3 and WBRV samples (Fig. S2). Meanwhile, the samples from KEN with the CMTV-like vIF-2α gene clustered with SSME and FV3 when comparing the MCP, yet clustered with CMTV and GSIV when comparing the vIF-2α gene (Fig. 4).

4. DISCUSSION

In this study, we sequenced the MCP and the putative viral homologue of eIF-2α (vIF-2α) to investigate the intraspecific genetic variability of FV3 across 3 Canadian regions over 3 yr. Out of the 127 tissue samples, there were a total of 3 FV3-like haplotypes: the FV3 isolate; a possible recombinant isolate that had the MCP of a FV3-like virus, but with a non-truncated vIF-2α gene of the CMTV lineage; and a novel isolate with nucleotide changes at both the MCP and vIF-2α, denoted as WBRV.

4.1. FV3 diversity in Canada

The FV3 isolate sequenced in this study showed no genetic variation across Ontario, Alberta, and Northwest Territories, and was present in 4 of the 5 sampled anuran species (except Canadian toad) all at different life stages. Our results showed that *Ranavirus* in Canada was either FV3 or a distant FV3-like isolate, with a lack of intermediate haplotypes between the two. Likewise, this FV3 isolate clustered with FV3 isolates found in frogs, salamanders, and fish in

Table 3. Polymorphic sites of the entire major capsid protein (MCP) of 15 FV3-like ranaviruses with ≥98.5% sequence similarity to the reference FV3 (AY548484). Includes 3 samples from the present study (**bold**): Wood Buffalo ranavirus (WBRV), an unknown haplotype discovered in Northwest Territories and Alberta, Canada; FV3/CMTV, a potentially recombinant isolate between FV3 and CMTV; and FV3-CAN (Canadian isolate), which had no genetic variation across northern and southern provinces and territories. Abbreviations for isolate names were based on given names of sequences from GenBank. Several sequences share similar names but were sampled from different species or location. See Table 1 for full virus names. N/A: not applicable

Given name (isolate)	Accession no.	Country of origin	Variable nucleotide position – MCP																								
			11	12	18	20	39	42	90	171	186	249	261	268	279	292	297	306	327	414	432	443	460	579	612	648	660
FV3	**AY548484**	**USA**	**T**	**A**	**T**	**C**	**C**	**C**	**G**	**G**	**C**	**G**	**C**	**G**	**T**	**G**	**C**	**C**	**C**	**C**	**G**	**C**	**A**	**C**	**C**	**C**	**C**
FV3-CAN	**N/A**	**Canada**																								**G**	
FV3/CMTV	**N/A**	**Canada**																								**G**	
FV3-RUK13	KJ538546	UK																								G	
SSME	KJ175144	USA																								G	
FV3-PSRV	KF646249	USA																								G	
FV3	FJ459783	USA																								G	
FV3	DQ897669	Brazil					A									A	A									G	
FV3 *Op*	MF360246	Netherlands/ Nicaragua[a]																			A					G	
STIV	EU627010	China																				T				G	
RGV	JQ654586	China																				T				G	
STIV	DQ335253	China																				T	G			G	
RNR	MF359927	China	A	T																		T				G	
HNV	AB500273	Japan			A						T								A		T	T				G	
RCV	AB474588	Japan																				T				G	
RCV	FJ207464	Taiwan												A								T				G	
KRV	HM133594	South Korea																				T				G	
WBRV	**MH332773**	**Canada**																							**T**	**G**	
BIV	AY187046	Australia				T		T	A									A			A				T	G	T
TFV	AF389451	China								T		A	T		C		A			T				A		G	
			681	703	711	713	783	816	833	867	868	889	893	913	989	1017	1023	1101	1174	1183	1200	1212	1278	1285	1324	1368	1380
FV3	**AY548484**	**USA**	**G**	**T**	**T**	**C**	**A**	**T**	**C**	**G**	**A**	**C**	**T**	**T**	**T**	**T**	**A**	**G**	**C**	**C**	**C**	**T**	**T**	**A**	**A**	**C**	**C**
FV3-CAN	**N/A**	**Canada**																									
FV3/CMTV	**N/A**	**Canada**																									
FV3-RUK13	KJ538546	UK																									
SSME	KJ175144	USA																									
FV3-PSRV	KF646249	USA																									
FV3	FJ459783	USA																									
FV3	DQ897669	Brazil																									
FV3 *Op*	MF360246	Netherlands/ Nicaragua[a]	A																	T			C	G			
STIV	EU627010	China			C					A																	
RGV	JQ654586	China			C					A																	
STIV	DQ335253	China			C					A			C														
RNR	MF359927	China			C					A								A									
HNV	AB500273	Japan			C				T		G											C					
RCV	AB474588	Japan			C				T		G															T	
RCV	FJ207464	Taiwan			C				T		G														G		
KRV	HM133594	South Korea			C				T		G														G		
WBRV	**MH332773**	**Canada**		**G**	**C**	**A**	**G**	**C**			**G**			**C**		**G**											
BIV	AY187046	Australia			C						G	A				G	G					C	C	G			T
TFV	AF389451	China			C		G		T		G				C	G	G		A		A	C	C	G			

[a]Reported in The Netherlands but imported from Nicaragua

Table 4. Polymorphic sites of vIF-2α gene of 15 ranavirus isolates. Table includes 3 samples from the present study (**bold**): Wood Buffalo ranavirus (WBRV), an unknown haplotype discovered in Northwest Territories and Alberta, Canada; FV3-like isolate discovered in Ontario with CMTV-like vIF-2α gene (FV3/CMTV), a potentially recombinant isolate between FV3 and CMTV; and FV3-CAN (Canadian isolate), which had no genetic variation across northern and southern provinces and territories. Sequences were compared to the truncated vIF-2α of FV3 (231 bp); therefore, the complete extent of genetic variation is not seen within non-truncated samples. —: gene sequences had fewer nucleotides than referenced FV3 sequence. See Table 1 for full virus names. N/A: not applicable

Given name (isolate)	Accession no.	Country of origin	Length (bp)	Variable nucleotide position – vIF-2α																				
				1	2	3	6	7	8	9	10	11	12	13	14	15	18	19	21	23	24	25	26	27
FV3	**AY548484**	**USA**	**231**	**A**	**T**	**G**	**T**	**A**	**G**	**A**	**C**	**T**	**T**	**G**	**G**	**A**	**G**	**T**	**T**	**A**	**A**	**A**	**C**	**A**
SSME	KJ175144	USA	231																					
FV3-CAN	**N/A**	**Canada**	**231**																					
LMR	KM516757	Portugal	211	—	—	—																		
RCV-Z2	MF187209	USA	231																					
WBRV	**MH332772**	**Canada**	**231**																					
STIV	DQ335253	China	200	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
RGV	JQ654586	China	200	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
BIV	AY187046	Australia	198	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
TFV	AF389451	China	200	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
CMTV	JQ231222	Spain	780	G	C	C	C	T	T	T	G	G	G	C	A	C	C	C	G	G		G	G	T
FV3 *Op*	MF360246	Netherlands/ Nicaragua[a]	780	G	C	C	C	T	T	T	G	G	G	C	A	C	C	C	G	G		G	G	T
FV3/CMTV	**MH332774**	**Canada**	**725**	**G**	**C**	**C**	**C**	**T**	**T**	**T**	**G**	**G**	**G**	**C**	**A**	**C**	**C**	**C**	**G**	**G**		**G**	**G**	**T**
GSIV	KF512820	China	780	G	C	C	C	T	T	T	G	G	G	C	A	C	C	C	G	G		G	G	T
ATV	AY150217	USA	780	G	C	C	C	T	T	T	G	G	G	C	A	C	C	C	G	G	G	G	G	T
				28	31	44	45	49	62	74	90	100	146	150	153	158	159	160	161	166	167	172	184	192
Giv**FV3**	**AY548484**	**USA**	**231**	**C**	**G**	**G**	**T**	**A**	**G**	**G**	**C**	**A**	**C**	**G**	**C**	**G**	**G**	**C**	**T**	**A**	**C**	**C**	**C**	**T**
SSME	KJ175144	USA	231																					
FV3-CAN	**N/A**	**Canada**	**231**																					
LMR	KM516757	Portugal	211																					
RCV-Z2	MF187209	USA	231																					
WBRV	**MH332772**	**Canada**	**231**										**G**											
STIV	DQ335253	China	200	—	A		C																	
RGV	JQ654586	China	200	—	A		C																	
BIV	AY187046	Australia	198	—	—	A	C					G												C
TFV	AF389451	China	200	—	A	A	C			A		G		A						G		A		C
CMTV	JQ231222	Spain	780	G	A	A	C					G			A	A	C	T	G	G			T	C
FV3 *Op*	MF360246	Netherlands/ Nicaragua[a]	780	G	A	A	C	C				G												
FV3/CMTV	**MH332774**	**Canada**	**725**	**G**	**A**	**A**	**C**	**C**				**G**			**A**					**G**	**T**		**—**	**—**
GSIV	KF512820	China	780	G	A	A	C	C				G			A					G				C
ATV	AY150217	USA	780	G	A	A	C		A		T	G												

[a]Reported in The Netherlands but imported from Nicaragua

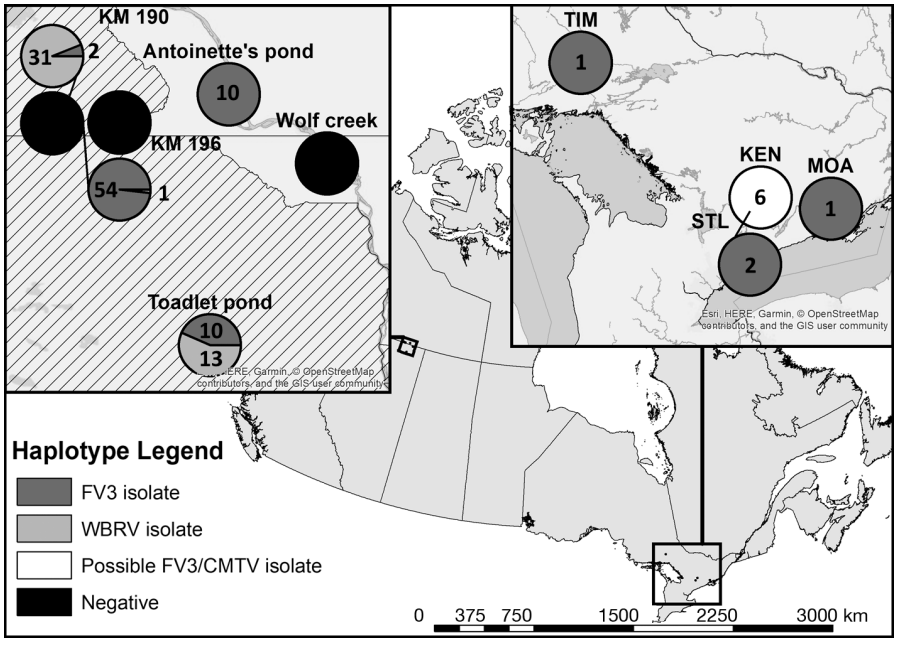

Fig. 2. Haplotype frequencies across Ontario, Alberta, and Northwest Territories, Canada. Colors represent the different haplotypes in the major capsid protein (MCP) and vIF-2α genes, and numbers in the pie charts represent the total number of samples infected with a given isolate over 3 yr. WBRV: Wood Buffalo ranavirus. Other virus names as in Table 1

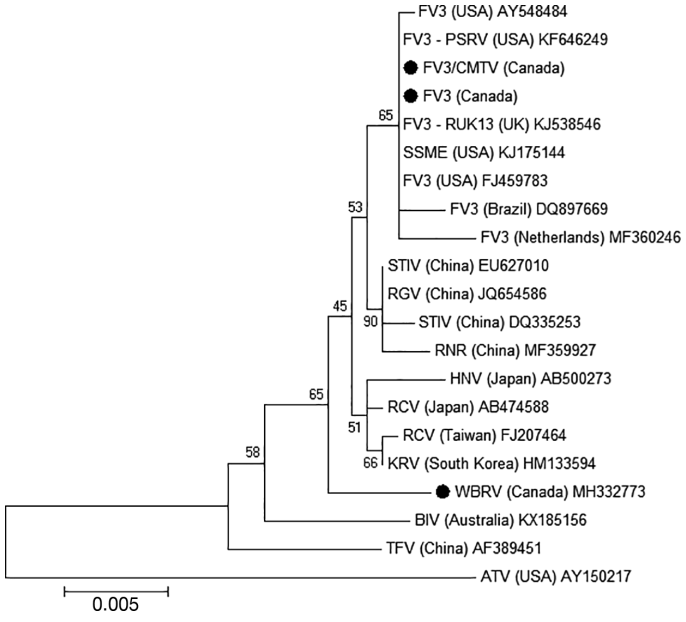

Fig. 3. Maximum likelihood tree of the major capsid protein (MCP) gene of 20 FV3-like ranaviruses, with ATV as an outgroup. The 3 samples found within this study are shown with black circles. Sequences were named based on given names from GenBank sequences. WBRV: Wood Buffalo ranavirus. See Table 1 for other full virus names. See Table 3 for nucleotide polymorphisms

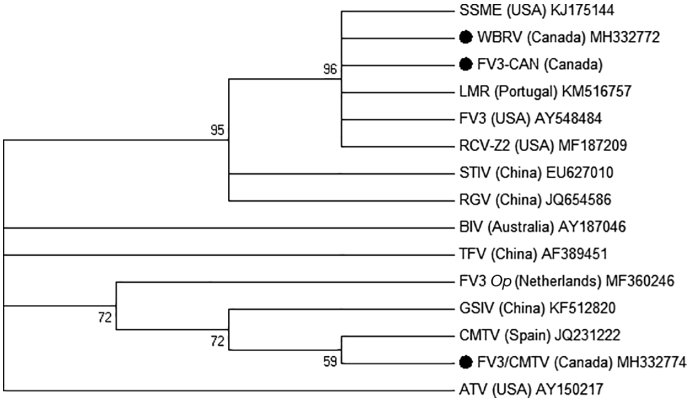

Fig. 4. Maximum likelihood consensus tree of the vIF-2α gene including 11 FV3-like *Ranavirus* isolates, 2 CMTV-like isolates, and ATV as an outgroup. Sequences with a black circle indicate samples sequenced during the present study. WBRV: Wood Buffalo ranavirus; FV3-CAN: FV3 isolate found within Canada; FV3/CMTV: FV3-like isolate discovered in Ontario with CMTV-like vIF-2α gene. See Table 1 for full virus names. See Table 4 for nucleotide polymorphisms

the USA, as well as in frogs in the UK. This lack of variation across multiple countries and taxonomic classes suggests that the virus may have moved rapidly over space and time, or that these markers are too conserved for this specific type of analysis.

Despite the lack of genetic variation across Canada and other countries at these 2 markers, insertions, deletions, and structural rearrangements are important sources of genetic variability in the FV3 genome (Morrison et al. 2014). The SSME isolate of FV3 has 100% sequence homology with FV3 (Holopainen et

al. 2009) at the MCP and vIF-2α; however, there are multiple deletions, insertions, and rearrangements throughout the genome (Morrison et al. 2014). While we sought to explore the intraspecific variation at the MCP and vIF-2α to gain a higher level of phylogenetic distinctions and virulence patterns among the FV3-like virus within Canada, the lack of diversity and intermediate variants at these 2 markers suggest that other genes or whole genome analyses will be required. It is possible that our samples were recent point infections that had not had time to evolve, or that these 2 genes are under strong selective pressure. For instance, the vIF-2α gene, due to its role in virulence, may be under purifying selection, preventing deleterious mutations as already evidenced in ATV (Ridenhour & Storfer 2008). The MCP may also be under selective forces, but there is currently no literature that supports this claim.

The lack of genetic diversity across provinces and territories contrasts with what would be expected of viruses, as they generally have highly mutagenic natures. However, this lack of variation could likely be due to the nature of double-stranded DNA viruses, such as *Ranavirus* (Duffy et al. 2008, Jancovich et al. 2015b, Price 2015). For instance, herpesvirus, a notable double-stranded DNA virus, has an estimated universal substitution rate of 10^{-9} substitutions per site per year in human strains, whereas by comparison, rapidly evolving RNA viruses have substitution rates around 10^{-2} to 10^{-5} substitutions per site per year (Drake & Hwang 2005, Shackelton et al. 2006, Duffy et al. 2008, Price 2015). While these mutation rates are specific to human strains, herpesviruses in sea turtles have an estimated substitution rate that is 2 to 5 times slower than that of human herpesviruses (Herbst et al. 2004). *Poxviridae* are another family of nucleocytoplasmic large double-stranded DNA viruses like *Iridoviridae*, and genomes within this family have estimated substitution rates of around 0.5–7 × 10^{-6} substitutions per site per year, making them faster than herpesviruses, yet slower than RNA viruses (Babkin & Shchelkunov 2006, Hughes et al. 2010, Babkin & Babkina 2011). Currently, the mutation rate in iridoviruses is unknown, preventing a better understanding of the lack of variability witnessed in our study (Ridenhour & Storfer 2008).

4.2. Novel FV3-like variant

Aside from the FV3 haplotype, the new WBRV isolate clustered within the FV3-like lineage of ranaviruses in the MCP phylogenetic tree, although it was not more closely related to other FV3-like isolates found in North America (FV3 and SSME). Based on the low bootstrap values of clades for the MCP, we cannot definitively establish the relationship between these different clusters based on this gene alone. The phylogenetic tree based on the vIF-2α formed a cluster between FV3, SSME, and WBRV, with a higher bootstrap support, suggesting that the inclusion of other genes, or even complete genomes, should provide a better picture of the relationship of WBRV and other FV3-like isolates.

While the difference in virulence between WBRV and FV3 is unknown, it is likely that the isolate with higher infectability may outcompete the other virus. The vIF-2α gene showed little variability between the FV3 strain and WBRV, with the same length as the truncated version of the gene and only one synonymous nucleotide difference between them. This suggests that virulence levels may be similar between both isolates. However, there are likely other genes that play roles in virulence, as shown in the SSME isolate, which is associated with lower virulence and mortality rate in frogs when compared to wild type FV3, both of which have truncated vIF-2α genes with 100% sequence homology (Morrison et al. 2014). Therefore, a complete phylogenomic analysis would be required to understand the extent of genetic variation between FV3, SSME, and the novel isolate WBRV.

4.3. Potential FV3/CMTV recombinant

Samples from KEN (Ontario) produced a MCP sequence that was identical to FV3 and SSME; however, their vIF-2α sequences had a 99.9% homology to a CMTV-like *Ranavirus*. While it is possible there may be co-infection of 2 *Ranavirus* strains at this site, the primers for vIF-2α were designed to bind to any *Ranavirus*, and there were no signs of double-banding of the vIF-2α on the gel during electrophoresis (Fig. S2). Although evidence of competition between *Ranavirus* strains has yet to be observed, general parasite competition often selects the most virulent strains, leaving others for extinction (Antia et al. 1994, Brunner et al. 2015). There is evidence for recombination between FV3 and CMTV, as seen in The Netherlands, where FV3 in a strawberry poison dart frog *Oophaga pumilio* imported from Nicaragua had a non-truncated eIF-2 protein, and a 99.7% homology to the samples from KEN (Saucedo et al. 2017). Similarly, in a ranaculture in the USA, an isolate from 2006 had recombinant sequences from a CMTV-like

Ranavirus, with the major parent being an FV3-like *Ranavirus* (Claytor et al. 2017). This recombinant virus was also found to have increased virulence compared to its FV3 counterpart, likely due to the CMTV-like genes acquired through recombination (Claytor et al. 2017). This study reported a recombinant in a facility within the USA; yet, the present study is the first to document a CMTV-like virus in North America within the wild. CMTV-like viruses are assumed not to be native to North America, as all documented cases are from regions in Europe and Asia (Balseiro et al. 2009, Geng et al. 2011, Chen et al. 2013, Price et al. 2017). It is possible that CMTV-like viruses are also present in North America and may have been for some time; however, due to a lack of surveillance in an FV3-predominant region, their presence may have gone undetected. The lack of the N-terminal region in FV3-like ranaviruses causes the viruses to be less virulent than viruses with the complete gene (Majji et al. 2006), suggesting that this recombination may be under positive selection towards higher virulence (Abrams et al. 2013).

4.4. Future steps

Previous studies have focused on the presence and absence of *Ranavirus* within amphibian populations (Greer et al. 2005, Miller et al. 2007, 2015, Forzán & Wood 2013). However, with the expanding knowledge of *Ranavirus* genetics, studies should focus at the population level by comparing virus isolates from various time periods or sites within a region in order to improve our understanding of *Ranavirus* movement and evolutionary history. Our study focused on 2 regions across Canada; future sampling should either encompass the entire country or focus on finer sampling within a province or territory to further explore the expanse and number of haplotypes. The virus may also have high vagility across the landscape, causing the lack of observed genetic variation, so above all, the evolution rate of ranaviruses requires further study, and more variable markers are necessary to understand spatio–temporal movement of FV3.

The MCP is an excellent marker to identify pathogens as isolates, categorize them into lineages, and detect genetic variation across continents and taxonomic classes (Chinchar 2002, Holopainen et al. 2009, Duffus & Andrews 2013, Jancovich et al. 2015b), and the length of the vIF-2α gene is often assessed to estimate virulence of new isolates (Essbauer et al. 2001, Majji et al. 2006, Stöhr et al. 2015). However, these markers are not variable enough to demonstrate intraspecific variation across Canada, and to further understand the relationships between FV3 samples across Canada, WBRV, and the possible recombinants, additional analyses should be performed, such as genomic co-linearity, gene content, and amino acid and nucleotide sequence identity (Tan et al. 2004, Majji et al. 2006, Jancovich et al. 2012, Cheng et al. 2014, Stöhr et al. 2015, Claytor et al. 2017). Furthermore, comparisons of complete viral genomes of apparently identical samples across the landscape are necessary to determine variable regions that could be used for evolutionary history and epidemiology of ranaviruses (Duffus et al. 2017).

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