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Phylogenetic Analysis of a Frog Virus 3–Like Ranavirus Found at a Site with Recurrent Mortality and Morbidity Events in Southeastern Ontario, Canada: Partial Major Capsid Protein Sequence Alone Is Not Sufficient for Fine-Scale Differentiation

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ABSTRACT: Ranaviruses are emerging pathogens of amphibians. We examined the phylogenetic relationship of ranaviruses from infected *Lithobates sylvaticus* tadpoles 2001–2004 from Oliver Pond, Ontario, Canada. The isolates sequenced are primarily frog virus 3–like, but because of sequence convergence, finer-scale analysis based on the major capsid protein was uninformative.

Ranaviruses are emerging pathogens in amphibian populations on a global scale (Miller et al., 2011). They are large double-stranded DNA viruses of the family *Iridoviridae* (Chinchar, 2002). Ranaviruses have been associated with many mortality and morbidity events in North America since the 1990s (Green et al. 2002; Gray et al., 2009). In southeastern Ontario, Canada, ranavirus-associated morbidity and mortality events began in 1999 at Oliver Pond on the grounds of the James Oliver Ecological Research Station near Peterborough (44°31′N, 78°32′W; Greer et al., 2005). These events primarily affected the wood frog (*Lithobates sylvaticus*) tadpoles, with mortality rates approaching 100% in the first years of the outbreaks (Greer et al., 2005). Infections were continually detected in wood frog tadpoles until 2005, when research at that location ceased (Charbonneau, 2006; Duffus, 2006; Duffus et al., 2008). Greer et al. (2005) reported that the virus present at that site had 98% homology with the major capsid protein (MCP) of frog virus 3 (FV3) and that the MCP sequence did not differ between individuals during their study. However, no further analysis of the phylogenetic relationship of the virus was provided. Duffus et al. (2008) used the same pond in 2005 but failed to provide a sequence analysis of the ranavirus, basing their assumption of an FV3-like virus on the work done by Greer et al. (2005). Here, we examined the phylogenetic affinity of the FV3-like virus present at the pond and discuss the problems with using partial MCP sequences as the only probe to examine ranavirus phylogenetics.

Wood frog tadpoles collected from Oliver Pond between 2001 and 2004 were recovered from the archives at the Berrill Laboratory, Trent University, Peterborough, Ontario, Canada, by A.L.J.D., and liver samples were aseptically removed with sanitized instruments. Instruments were sanitized by immersion in a concentrated (>50%) solution of DECON (Decon Laboratories Limited, East Sussex, UK) for a minimum of 3 min and were rinsed thoroughly to avoid cross-contamination. The hepatic tissue samples (2–3 mm^3) were handled according to Duffus et al. (2008) for DNA extraction. The DNA extract was screened for the presence of the MCP of FV3 following Mao et al. (1997) using primer set MCP 4/5. The PCR products were then run on a 1.5% ethidium bromide–stained agarose gel to determine the presence of the MCP gene by the appearance of an approximately 500 base pair (bp) band.

Positive DNA extracts from each year (2001, $n=2$; 2002, $n=1$; 2003, $n=1$; 2004, $n=2$) were sent to the Natural Resources DNA Profiling and Forensics Center (Ministry of Natural Resources and Trent University, Peterborough, Ontario, Canada) for sequencing in 2005. The sequences were manually verified for scoring errors before analysis. Sequences of the MCP from the following iridoviruses were

TABLE 1. Comparison of the partial major capsid protein sequence of ranaviruses from infected *Lithobates sylvaticus* tadpoles, 2001–2004, from Oliver Pond, Ontario, Canada, to those found via a BLASTn search. All gene sequences with the highest maximum score were included in the Table. The maximum identity provides information on how similar the sequences are and is necessary for comparison. The length of the gene segment used in the BLASTn searches was 670 base pairs.[a]

Sample	Maximum identity (%)	Maximum score	Description	Accession No.
2001 – A	99	942	*Rana grylio* iridovirus, complete genome	JQ654586.1
	99	942	Soft-shelled turtle iridovirus, complete genome	EU627010.1
	99	942	FV3, complete genome	AY548484.1
	99	942	FV3, viral core protein (MCP), complete CDs	U36913.1
2001 - B	97	791	FV3 MCP gene, complete CDs	FJ459783.1
	97	791	Soft-shelled turtle iridovirus MCP gene, complete CDs	DQ335253.1
2002	98	689	FV3 MCP, partial CDs	DQ906049.1
	98	689	FV3 MCP, partial CDs	DQ906048.1
2003	98	857	*Hynobius nebulosus* virus, MCP gene, complete CDs	AB500273.1
	98	857	*Rana catesbeiana* virus, JP MCP gene, complete CDs	AB474588.1
2004 - A	99	955	Soft-shelled turtle iridovirus, complete genome	EU627010.1
	99	955	FV3, complete genome	AY548484.1
	99	955	FV3 viral core protein (MCP) gene, complete CDs	U36913.1
2004 - B	99	922	*Hynobius nebulosus* virus, MCP gene, complete CDs	AB500273.1
	99	922	*Rana catesbeiana* virus, JP MCP gene, complete CDs	AB474588.1

[a] FV3 = frog virus 3; MCP = major capsid protein; CDs = conserved domains; JP = Japan.

obtained from GenBank: frog virus 3 (Accession: AY548484); *Rana grylio* virus (JQ65458); soft-shelled turtle iridovirus (EU627010); and insect iridescent virus type 22 major structural protein gene (M32799.1) as the outgroup.

All MCP sequences were aligned using ClustalW (Larkin et al., 2007; European Bioinformatics Institute, Hinxton, Cambridge, UK) in MEGA 5.0 (Tamura et al., 2011). The ends of the sequences were trimmed to remove the nonoverlapping sections, leaving a 670-bp homologous section for analysis. A phylogenetic tree was constructed using maximum-likelihood methods (Tamura-Nei model), based on the final tree having the highest likelihood of being observed (Nei and Kumar, 2000), with the Jukes-Cantor model of nucleotide substitution, which assumes that substitutions occur at any site with the same likelihood and frequency (Nei and Kumar, 2000). Bootstrap analyses were conducted using 1,000 replicates to ensure adequate statistical power. Sequences were also compared with others found in GenBank through Nucleotide Basic Local Alignment Search Tool (BLASTn) searches.

All sequences held high homology with other ranavirus sequences (97–99%) but not necessarily with the MCP of FV3 (Table 1). In 2001 and 2004, where multiple sequences were available for analysis, the sequences did not hold the same identity with the same comparison sequences from GenBank (Table 1). This demonstrates that, despite previous reports of sequences obtained from

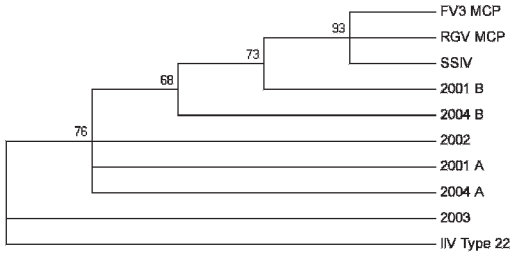

FIGURE 1. Maximum-likelihood phylogenetic analysis of partial major capsid protein sequences of ranaviruses from infected *Lithobates sylvaticus* tadpoles, 2001–2004, from Oliver Pond, near Peterborough, Ontario, Canada. All branches with less than 50% support were removed, and a bootstrap consensus tree was created. Bootstrap values were calculated with 1,000 replicates. FV3 = frog virus 3 (Accession AY548484); RGV = *Rana grylio* virus (JQ65458); SSIV = soft-shelled turtle iridovirus (EU627010); and IIV Type 22 = insect iridescent virus type 22 major structural protein gene (M32799.1; outgroup). The gene segment used to create the phylogeny was 670 base pairs.

different individuals being identical (Greer et al. 2005), there was some sequence diversity in the MCP of the FV3-like virus present in Oliver Pond. The current study likely does not capture the full sequence diversity present because of the small sample size; nor is that diversity captured in phylogenetic analyses of the viral sequences (Fig. 1). The analysis shows several places with poorly defined clades (<75% support) and one additional polytomy that involves three sequences from different years (Fig. 1). The isolate from 2003 does not group with FV3 and falls outside of the clade that holds the rest of the sequences from Oliver Pond. We believe that this is not due to insufficient outgroup divergence but due to the limited nature (i.e., possible homology) of the information contained in the partial sequence of the MCP of the virus being analyzed.

Historically, short sequences of the MCP have been used to both classify ranaviruses and reconstruct their phylogenetic affinities (e.g., Hyatt et al., 2000), likely because of early evidence that the MCP was a good target for examining the relationships between closely related iridoviruses (Tidona et al., 1998). However, as we have shown in this study, the use of only a section of the MCP gene sequence may be misleading, especially when examining closely related viral isolates. Huang et al. (2011), in their study of fish iridoviruses, found that the full length of the MCP gene (~1,300 bp) provides enough information to group different viral isolates into genera of the *Iridoviridae*. They have also shown that interyear variation in this gene can be observed (Huang et al., 2011), which may be very useful for finer-scale phylogenetic analyses. These new methods show that there is still a need for the development of accurate techniques for classifying and studying the phylogenetic relationships between ranaviruses on multiple scales. They also indicated that older studies may need to be interpreted with caution.

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