Vol. 63: 61–67, 2011
doi: 10.3354/ame01478

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online February 23

NOTE

Novel phycodnavirus genes amplified from Canadian freshwater environments

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ABSTRACT: Extant and newly designed primers for the polymerase chain reaction (PCR) were used to amplify phycodnavirus DNA polymerase (*polB*) gene fragments from numerous samples collected at different times of the year from 3 freshwater environments in Ontario, Canada. Overall, a total of 143 cloned PCR fragments were sequenced and 106 putative phycodnavirus *polB* gene fragments were identified. Although most of these 106 gene fragments were very closely related (i.e. >97% identical) to *polB* sequences from chloroviruses, or environmental sequences related to prasinoviruses, 16 represented 2 new types of phycodnavirus *polB* genes. More specifically, *polB* fragments that formed a new clade of chloroviruses were amplified from Lake Ontario using newly designed *Chlorovirus*-specific PCR primers, and a *polB* sequence most closely related to genes from the prymnesioviruses PgV-03T and CbV-PW1 was amplified from a pond sample from Mississauga, Ontario, using the degenerate algal virus-specific PCR primers AVS1 and AVS2. Thus, the results of the present study provide evidence for a new type of *Chlorovirus*, and the first observation of *polB* sequences from freshwater phycodnaviruses that are presumed to infect algae other than chlorophytes.

KEY WORDS: Phycodnaviruses · Algal viruses · *Chlorella* viruses · Prymnesioviruses · Freshwater · DNA polymerase · *polB*

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INTRODUCTION

Phycodnaviruses are a remarkable group of viruses because they are closely related to the largest known viruses, the *Mimiviridae* (Monier et al. 2008), they have large, diverse genomes (Dunigan et al. 2006, Iyer et al. 2006), they encode surprising biochemical pathways (e.g. Markine-Goriaynoff et al. 2004, Wilson et al. 2005, Vardi et al. 2009), and they infect one of the most ecologically important groups of organisms on Earth, eukaryotic algae. To study the genetic richness of these viruses in the environment, researchers have designed polymerase chain reaction (PCR) primers to target phycodnavirus B-family DNA polymerase genes (*polB*) (Chen & Suttle 1995), and the major capsid protein (Larsen et al. 2008). Although neither primer set is truly universal for the *Phycodnaviridae*, each has the ability to amplify its targets from several phycodnavirus taxa (Larsen et al. 2008). For example, the algal virus-specific *polB* primers AVS1 and AVS2 have been used to amplify genes from different types of chloroviruses, prasinoviruses and prymnesioviruses (Chen & Suttle 1995, Brussaard et al. 2004, Bellec et al. 2009). However, with the exception of 1 operational taxonomic unit (OTU, or a DNA sequence ≤97% identical to any other) from the Gulf of Mexico (Chen et al. 1996), and 2 from Kane'ohe Bay, O'ahu, Hawai'i, USA (Culley et al. 2009), *polB* fragments amplified from natural environments using the AVS primers have all been most closely related to cultivated marine prasinoviruses (Short & Short 2008, Clasen & Suttle 2009, Culley et al. 2009).

Considering that the only known freshwater phycodnaviruses are chloroviruses, the fact that all phycodnavirus *polB* sequences amplified from freshwater environments have been most closely related to *Prasinovirus* genes is somewhat puzzling (Short & Short

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2008, Clasen & Suttle 2009). To resolve this issue and to determine whether other types of phycodnavirus could be PCR-amplified from freshwater environments, 2 complementary research strategies were employed. First, new group- and taxon-specific PCR primers were designed to amplify *polB* fragments from chloroviruses, and second, samples collected from different locations during different seasons were surveyed.

MATERIALS AND METHODS

At various times of the year during the ice-free period between 2007 and 2009, surface water samples of 1 l were collected from several freshwater habitats in Ontario, Canada, including Lake Ontario (43° 32.614′ N, 79° 34.995′ W), a storm water pond on the University of Toronto Mississauga (UTM) campus in Mississauga (43° 32.759′ N, 79° 39.574′ W), and Crawford Lake (43° 28.140′ N, 79° 56.954′ W). Following collection, all samples were immediately transported back to the laboratory for processing as described in Short & Short (2008) except that all samples collected after 2007 were filtered through 47 mm diameter 0.45 µm pore size Durapore® PVDF membrane filters of (Millipore) only, and samples collected in 2009 were centrifuged at 118 000 × *g* for 3.5 h in an SW32Ti rotor (Beckman Coulter). Samples collected in 2007 were pre-filtered through 47 mm diameter GC50 glass-fibre filters (Advantec MFS), and samples collected before 2009 were centrifuged using an SW40Ti rotor (Beckman). The pre-filtration step was abandoned because it was not necessary for the small volumes (<200 ml) that were processed, and the centrifugation procedure was changed to increase sample throughput. Neither of these changes would negatively affect the virus preparations because it has been shown that filtration through glass-fibre filters can actually cause loss of virus particles (Wommack et al. 2010), and the calculated time to pellet phycodnaviruses with the SW32Ti rotor is ca. 3.3 h if a very conservative sedimentation coefficient of 65S is assumed for phycodnaviruses; sedimentation coefficients range from 42 to >1000 S for all known viruses (Lawrence & Steward 2010). Following centrifugation, the supernatant was decanted, and pelleted material was resuspended and subjected to a freeze/thaw treatment as described in Short & Short (2008).

PCR primer sets specifically targeting the chloroviruses ATCV-1, CVM-1 and PBCV-1, as well as a degenerate primer set targeting all three (CHLVd) (Table 1) were designed from aligned *Chlorovirus polB* genes (Fig. 1). The efficacy of these newly designed primers was determined by amplifying gene fragments from filtered (47 mm diameter, 0.45 µm pore size Durapore® PVDF), freeze/thaw-treated cellular lysates from the viruses PBCV-1, CVM-1 and ATCV-1 grown on *Chlorella* NC64a, *Chlorella* Pbi and *Chlorella* SAG 3.83, respectively. The *Chlorovirus*-specific primers and the algal virus-specific primers AVS1 and AVS2 (Chen & Suttle 1995) were used to PCR-amplify gene fragments from a variety of freeze/thaw-treated environmental samples. All PCR reactions, and the cloning and sequencing methods, were conducted as described in Short & Short (2008), except that the annealing temperatures for PCR with the ATCVs, CVMs, PBCVs and CHLVd primer sets were 48, 50, 44 and 52°C, respectively, and a C1000™ thermal cycler (Bio-Rad Laboratories) was used. Each 50 µl PCR reaction was electrophoresed in 1.5% LE agarose (Promega) gels in 1X TAE (40 mM Tris, 20 mM acetic acid, 1.0 mM EDTA, pH 7.6), and gels were stained in a 0.5 µg ml^{-1} ethidium bromide solution and visualized on a Molecular Imager® ChemiDoc™ XRS system (Bio-Rad Laboratories). Amplified fragments of the appropriate size (i.e. as noted in Table 1) were excised from the gels, extracted using a QIAquick Gel Extraction kit (Qiagen), and cloned using pGEM®-T Vector System II (Promega) as described in Short & Short (2008). Plasmid inserts were sequenced at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children, Toronto, Ontario, Canada. Only full-length

Table 1. Newly designed *Chlorovirus*-specific DNA polymerase (*polB*) PCR primers

Primer name	Virus targeted/expected amplicon size (bp)	Forward primer[a,b]	Reverse primer[a,b]
ATCVs	ATCV-1/610	AAG AAA GGT GCC TAC TTT GAA C	AGG TCG TTC GGA GCT TTG TAC T
CVMs	CVM-1/645	AAG AAG GGA GCA TAC TTC ACG C	CAA AAT GTA AGG GTA ATA GAT CTT C
PBCVs	PBCV-1/600	CTT ATC GCA GCT CTC GAT TTT G	GTT CGG TGC TCG GAA ATC CTT C
CHLVd	PBCV-1, ATCV-1, CVM-1/560–575	CCW ATC GCA GCW CTM GAT TTT G	ATC TCV CCB GCV ARC CAC TT

[a]Shown in the 5′ to 3′ orientation
[b]Codes for mixed bases are: B = C, G, T; M = A, C; R = A, G; V = A, C, G; W = A, T

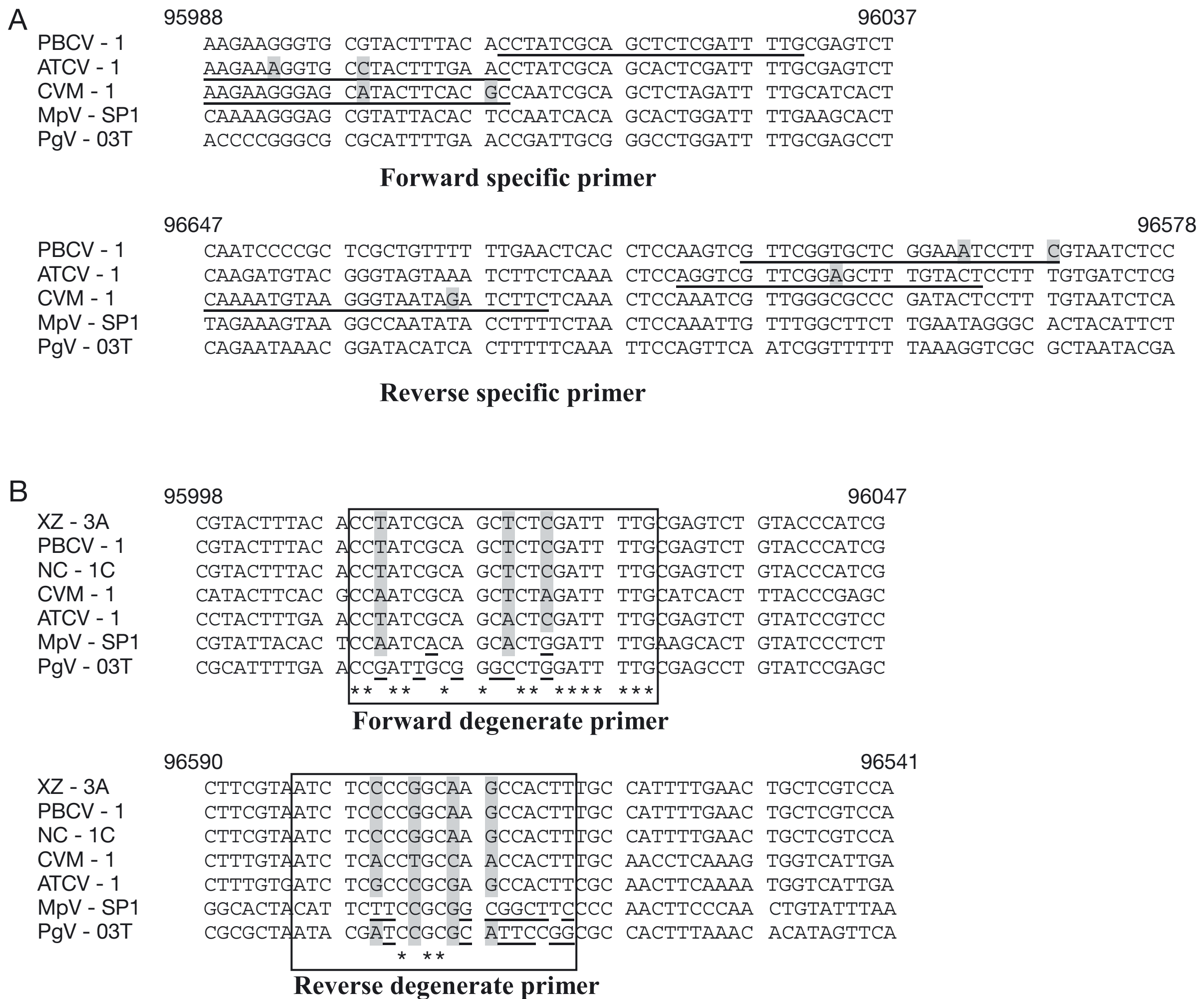

Fig. 1. Partial sequence alignments of *Chlorovirus* and other phycodnavirus *polB* genes used for PCR primer design. Gene fragments, shown in the 5′ to 3′ orientation, were aligned using ClustalW with Mega 4.0 default parameters. Numbers above the alignments represent nucleotide positions relative to the PBCV-1 genome; sequences for the reverse primers are shown as reverse complements. (A) Alignment of *Chloroviruses* (PBCV-1, ATCV-1, CVM-1), a *Prasinovirus* (*Micromonas pusilla* virus MpV-SP1) and a *Prymnesiovirus* (*Phaeocystis globosa* virus PgV-03T) was used for the design of *Chlorovirus* gene-specific PCR primers. Forward and reverse oligonucleotides are underlined; shaded nucleotides are unique to the primer. (B) Alignment of 5 chloroviruses (XZ-3A, PBCV-1, NC-1C, CVM-1, ATCV-1), and other phycodnaviruses (MpV-SP1, PgV-03T), was used to design degenerate *Chlorovirus*-specific PCR primers. Boxes indicate the region from which forward and reverse degenerate primers were designed. Asterisks below the boxes indicate conserved nucleotide positions; shaded nucleotides indicate degenerate positions; underlined nucleotides indicate positions that do not match the primer sequence

sequences without ambiguous base calls were used for further analysis.

Sequences encoding the conserved DNA polymerase amino acid motif 'YGDTDS' were compared to cultivated nucleo-cytoplasmic large DNA viruses as described in Short & Short (2008). Briefly, using default parameters in Phylogeny.fr (Dereeper et al. 2008), inferred amino acid sequences were automatically aligned and curated using MUSCLE (Edgar 2004) and Gblocks (Castresana 2000), respectively. The resulting alignment of 94 confidently aligned amino acid positions was used to recreate phylogenies via Bayesian inference (BI) and maximum likelihood (ML). BI was conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) with 2 runs, 4 chains, 10^6 generations, sampling every 100 generations, a burnin value of 0.25, and mixed models of amino acid substitution. The ML phylogeny was reconstructed using PhyML (Guindon & Gascuel 2003) using the default parameters in Phylogeny.fr. Tree viewing and drawing were conducted using Mega 4.0 (Tamura et al. 2007) and Adobe Illustrator CS (Adobe Systems), respectively.

RESULTS AND DISCUSSION

Because previous reports have already demonstrated that the primers AVS1 and AVS2 can be used to amplify phycodnavirus genes from freshwater environments (Short & Short 2008, Clasen & Suttle 2009), only 2 samples were amplified with these primers. These samples were collected from Lake Ontario, Canada, on July 16, 2008, and the UTM pond on September 18, 2009, and were amplified, cloned, and sequenced as components of other ongoing studies in our laboratory. Interestingly, 2 *polB* fragments that were not putative *Prasinovirus* genes were amplified using AVS1 and AVS2; prior to this study all freshwater sequences amplified with these primers have been closely related to marine *Prasinovirus polB* genes. The gene fragment LO16jul08.23 was nearly identical (98% identical over 689 nucleotides) to *polB* of the *Chlorovirus* FR483, and was obtained from 36 cloned fragments (generated by PCR) of a July 16, 2008, Lake Ontario sample (Fig. 2); the other 35 sequences from that clone library were all putative *Prasinovirus* gene

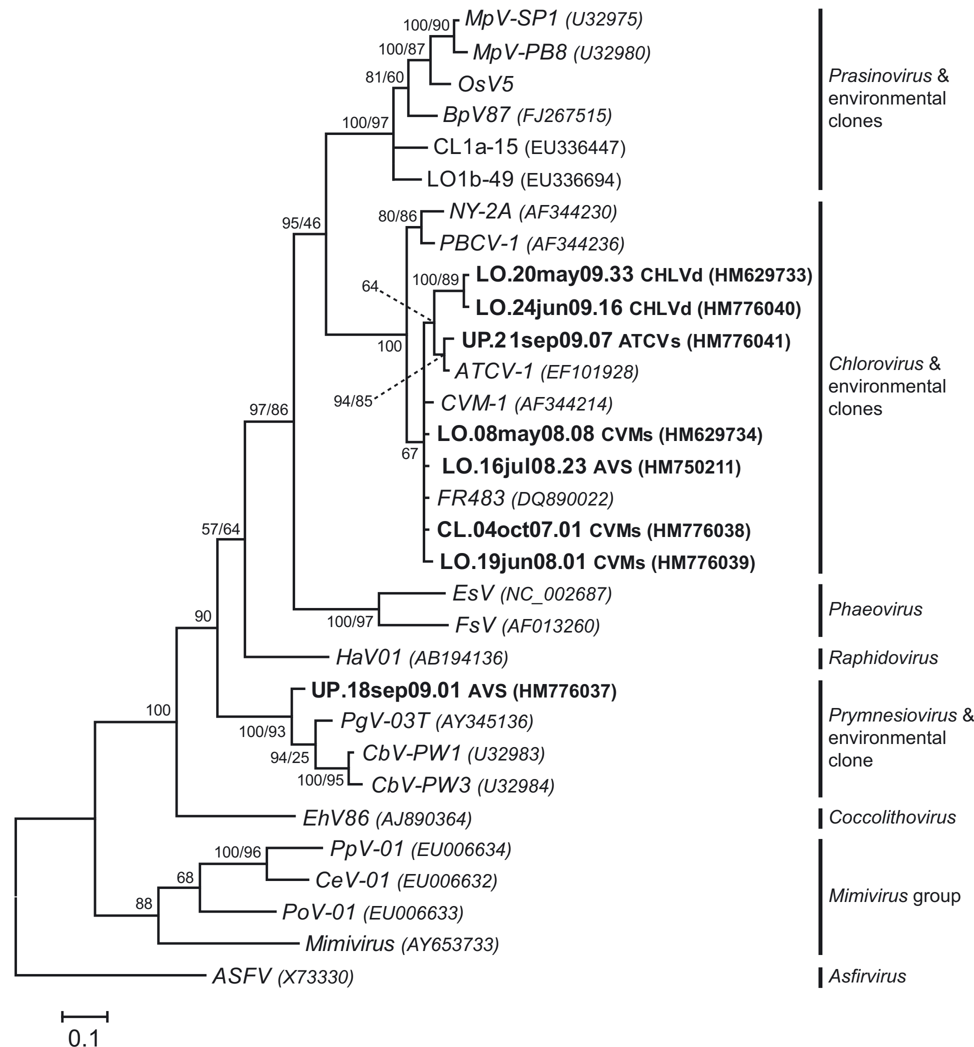

Fig. 2. Phylogenetic tree of phycodnaviruses and other nucleo-cytoplasmic large DNA viruses based on Bayesian inference (BI) of inferred amino acid sequences from partial DNA polymerase genes. Where the maximum likelihood (ML) tree topology matched the BI topology, numbers at branch nodes indicate the posterior probability followed by the aLRT (approximate likelihood ratio test) branch support from SH-like (Shimodaira-Hasegawa-like) values. Where the topology varied between BI and ML trees, only posterior probability values are shown. Sequences obtained in this study are in bold; sequences from cultivated viruses and other environmental clones are in italics and normal type, respectively. Names of the sequences obtained in this study include their origin (Lake Ontario: LO; Crawford Lake: CL; or the University of Toronto Mississauga pond: UP), date of sample collection (as day/month/year), an arbitrary clone number, and the PCR primer set used to obtain the sequence. GenBank accession numbers are listed in parentheses beside each sequence, and clusters containing sequences from cultivated viruses are indicated to the right of the tree and are labelled with the virus genera. Scale bar indicates the proportion of changes per site

fragments (Short et al. in press). Additionally, the gene fragment UP.18sep09.01 was amplified from a UTM pond sample collected on September 18, 2009, and was most closely related to *polB* genes from prymnesioviruses that infect *Phaeocystis globosa* and *Chrysochromulina brevifilum* (Fig. 2). Furthermore, this putative *Prymnesiovirus polB* fragment was the only sequence recovered from the September 18, 2009, UTM pond sample; all 13 cloned fragments that were sequenced were identical.

When testing the newly designed *Chlorovirus* gene-specific PCR primer sets ATCVs, CVMs and PBCVs on virus lysates, each primer set amplified DNA fragments from their target viruses only. For example, the CVMs primers amplified gene fragments from cellular lysates of CVM-1, but not from lysates of PBCV-1 or ATCV-1. In contrast, the *Chlorovirus*-specific degenerate primers were able to amplify DNA fragments of the expected size from all 3 of the *Chlorovirus* lysates tested. Because no other phycodnavirus isolates were available at the time of this study, it was not possible to determine whether the *Chlorovirus*-specific degenerate primers could amplify *polB* genes from other phycodnaviruses. However, as these degenerate primers have 11 mismatches when compared to the *polB* sequences of the virus MpV-SPI (Fig. 1), and, with respect to their *polB* genes, prasinoviruses such as MpV-SP1 are the closest relatives of chloroviruses (Fig. 2), it seems unlikely that they could amplify any genes other than *Chlorovirus polB*. Moreover, the fact that all identifiable sequences obtained with the degenerate primers were putative *Chlorovirus polB* genes suggests that these newly designed degenerate primers were specific for chloroviruses. PCR of environmental samples using the *Chlorovirus*-specific primers CVMs and ATCVs produced amplicons of the expected size only, whereas the primers PBCVs and degenerate *Chlorovirus*-specific primers (CHLVd) produced the expected amplicons as well as fragments that did not correspond to the expected size (Table 2).

PCR reactions with all *Chlorovirus*-specific primers except PBCVs produced amplified *polB* gene fragments that clustered among known phycodnaviruses (Fig. 2, Table 2). Overall, only 57 *polB* sequences were obtained from 94 cloned fragments that were originally amplified with the *Chlorovirus* gene-specific and degenerate PCR primers (Table 2). Without exception, sequences from the other 37 cloned fragments were unidentifiable (i.e. the sequencing reactions failed, or BLAST comparisons to the GenBank nucleotide collection revealed no significant homologies). PCR with the ATCVs primers resulted in the successful amplification of a *polB* gene fragment from only a single sample collected from the UTM pond on September 21, 2009. In contrast, reactions with the CVMs primers led to the amplification of *polB* gene fragments from many samples collected at various times of the year from all 3 freshwater locations (Table 2). Although reactions with the PBCVs primers produced amplicons of the expected size for several samples, no *polB* sequences were recovered from any cloned PBCVs amplicons (Table 2). Because sequences related to PBCV-1 were not recovered with any phycodnavirus primers used in this study, it seems very unlikely that the PBCVs amplicons were bona fide *Chlorovirus* genes. In fact, it is plausible that viruses related to PBCV-1 were not present or were below the level of detection in the samples tested, or that PBCVs primers are not useful for obtaining environmental *Chlorovirus* sequences. Although the disappointing results with the PBCVs primers highlight challenges associated with PCR primer design and amplification of environmental samples, the recovery of *Chlorovirus polB* fragments via PCR with the other *Chlorovirus* gene-specific primers demonstrates the utility of virus-specific primers for environmental surveys. Furthermore, sequences obtained with the CVMs primers have already been useful for developing quantitative molecular methods for assessing virus dynamics *in situ* (Short et al. in press).

For many samples, reactions with the primers CHLVd produced amplicons of the expected size. However, these primers also produced nonspecific amplicons from almost every sample, suggesting that the reaction conditions could not be made stringent enough to prevent spurious amplification (Table 2); increasing the stringency of PCR with CHLVd always resulted in failed amplification. Nonetheless, of the 33 CHLVd cloned fragments that were sequenced, 24 were identified as putative *Chlorovirus polB* fragments; 9 could not be identified because they shared no significant homology to any sequences in GenBank. Of these 24 *polB* fragments, 21 were >97 % identical to each other and to *polB* sequences from viruses infecting *Chlorella* Pbi (e.g. FR483 and CVM-1). The other 3 *polB* fragments amplified with CHLVd were considered novel *Chlorovirus* gene fragments and were recovered from the May 20, 2009, and June 24, 2009, Lake Ontario samples. Two of these fragments (LO.20may09.33 and LO.24jun09.16) are shown in Fig. 2 to highlight the fact that they were obtained from more than 1 sample, but all 3 were >97 % identical to each other. These particular *polB* fragments were considered novel because they clustered among known chloroviruses but formed their own distinct clade (Fig. 2). Also, nucleotide BLAST comparisons of the novel sequences to the GenBank nucleotide collection (nr/nt database) revealed that the top hits for LO.20may09.33 and LO.24jun09.16 were DNA polymerase genes of PBCV-1 and NY-2C, respectively, each with only 74 % sequence identity over the entire

Table 2. PCR and sequencing results for samples targeted with *Chlorovirus*-specific primers. UTM: University of Toronto Mississauga. –: no amplification; +: correct size product; ns: non-specific amplification

Sample date	Sample location	Amplification results (no. of *polB* sequences/no. of plasmids sequenced)			
		CVMs	ATCVs	PBCVs	CHLVd
27 Sep 07	Lake Ontario	–	–	+ ns (0/4)	+ ns
4 Oct 07	Crawford Lake	+ (4/5)	–	+ ns	+ ns
10 Oct 07	Lake Ontario	+ (9/10)	–	+ ns	+ ns
8 May 08	Lake Ontario	+ (8/10)	–	ns	+ ns (5/5)
5 Jun 08	Lake Ontario	+ (2/2)	–	ns	+ ns
19 Jun 08	Lake Ontario	+ (4/5)	–	ns	ns
14 Aug 08	Lake Ontario	–	–	ns	ns
9 Oct 08	Lake Ontario	–	–	ns	ns
22 Jan 09	Lake Ontario	–	–	ns	+ ns
4 May 09	UTM pond	+ (2/2)	–	ns	+ ns
20 May 09	Lake Ontario	+	–	ns	+ ns (4/5)
2 Jun 09	UTM pond	+ (3/3)	–	+ ns (0/2)	+ ns (5/13)
9 Jun 09	Lake Ontario	+	–	ns	+ ns
9 Jun 09	UTM pond	+	–	ns	+ ns (5/5)
24 Jun 09	UTM pond	+	–	ns	ns
24 Jun 09	Lake Ontario	+	–	+ ns (0/5)	+ ns (5/5)
23 Jul 09	Lake Ontario	+	–	ns	–
23 Jul 09	UTM pond	–	–	+ ns (0/5)	ns
21 Sep 09	Lake Ontario	–	–	+ ns	+ ns
21 Sep 09	UTM pond	–	+ (1/8)	ns	+

query length (578 nucleotides). Although the BLAST results seem to suggest that the novel sequences were more closely related to PBCV-1 than ATCV-1, the phylogeny based on 83 confidently aligned amino acids placed the novel *Chlorovirus* fragments in a group nearest to ATCV-1 (Fig. 2). However, because the sequences used for the BLAST searches and the phylogenetic analysis are different (nucleotides versus informative amino acid positions), these results are not contradictory and highlight the fact that LO.20may09.33 and LO.24jun09.16 represent a unique, novel group of Lake Ontario chloroviruses.

The results of the present study reinforce and extend previous studies demonstrating that Canadian freshwaters are home to diverse phycodnaviruses (Short & Short 2008, Clasen & Suttle 2009) by reporting sequences that fall outside previously observed clusters of environmental phycodnavirus *polB* sequences (e.g. Chen et al. 1996, Short & Suttle 2002, Short & Short 2008, Clasen & Suttle 2009, Culley et al. 2009), and by demonstrating the existence of *polB* fragments from unknown types of phycodnaviruses. Amplification with degenerate *Chlorovirus*-specific primers led to the recovery of Lake Ontario sequences that represent a new type of *Chlorovirus.* The notion that the CHLVd amplicons LO.20may09.33 and LO.24jun09.16 represent unknown chloroviruses is supported by the reasoning of Clasen & Suttle (2009) because the genetic distances between these amplicons and the *polB* genes of cultivated chloroviruses are greater than the distances between different types of cultivated chloroviruses (e.g. ATCV-1 and CVM-1), or different viruses within the genera *Prasinovirus* (Bellec et al. 2009), or *Prymnesiovirus* (Brussaard et al. 2004) (Fig. 2). Similar reasoning can be applied to the UP.18sep09.01 AVS fragment to support its identification as a novel *Prymnesiovirus* gene.

Although the hosts for the viruses that encode the novel *Chlorovirus*-like *polB* gene fragments are unknown, numerous free-living *Chlorella* spp. have been observed in past phycological surveys of Lake Ontario (Munawar & Munawar 1996), and gene fragments closely related to *Chlorella* spp. *psbA* have recently been amplified from Lake Ontario (e.g. GenBank accession numbers HM629672, HM629554 and HM629626). Thus, the coincidence of potential hosts and host sequences in the same environment permits speculation that the viruses encoding the novel *Chlorovirus*-like *polB* sequences infect free-living *Chlorella*-like algae, and supports their putative identification as chloroviruses. Similarly, a gene fragment (GenBank accession number HQ112284) most closely related to *psbA* from prymnesiophyte algae such as *Emiliania huxleyi, Chrysochromulina polylepis* and *Prymnesium parvum* was amplified from the same UTM pond sample from which the putative *Prymnesiovirus* fragment UP.18sep09.01 originated. Again, this coincidence supports speculation that the UP.18sep09.01 fragment originated from a novel type of *Prymnesiovirus*. The discovery of a *polB* sequence closely related to *Prymnesiovirus* genes provides the first evidence that the host range of freshwater phy-

codnaviruses extends beyond chlorophyte algae, and the discovery of new *Chlorovirus* sequences demonstrates that new types of chloroviruses await isolation.

Acknowledgements. We thank C. Short and N. Musrap for their assistance with sample collection and processing. *Chlorovirus* isolates and *Chlorella* cell cultures were generously provided by J. L. Van Etten. This work was supported by the Canadian Foundation for Innovation Leaders Opportunity Fund and NSERC Discovery grants awarded to S.M.S

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Editorial responsibility: Paul del Giorgio, Montreal, Canada

Submitted: August 9, 2010; Accepted: November 24, 2010
Proofs received from author(s): February 7, 2011