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Phylogenetic Analysis of a Chlorovirus Isolated from New York State

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ABSTRACT

Chloroviruses are large icosahedral viruses that contain about 50 structural proteins, and typically infect certain green eukaryotic algae. Recently, chlorovirus sequences were isolated from throat swabs of healthy adults and were associated with cognitive impairment. Here, we analyzed a pond water sample from New York for the presence of chlorovirus. Cytopathic effects were observed in *Chlorella heliozoae*, strain SAG 3.83. A portion of the chlorovirus polymerase gene was amplified by the polymerase chain reaction using degenerate primers. The PCR product was bidirectionally sequenced. The sequence of the polymerase gene was compared to known chloroviruses and shown to be 98% identical to ATCV-1. The sequence of the environmental isolate was phylogenetically compared to related chloroviruses, and genetic relatedness to known viruses is presented.

1 INTRODUCTION

Chloroviruses are plaque-forming DNA viruses that are part of the family *Phycodnaviridae*. Their genomes are quite large for viruses, but only roughly 40% of the proteins encoded have known functions, including many that are novel for a virus (Van Etten & Dunnigan, 2012). Chloroviruses typically infect certain eukaryotic green algae (Argakova et al., 2006), and are found in freshwater sources in relatively high titers (up to thousands of plaque forming units) around the world (Yolken et al., 2014). While chloroviruses are ubiquitous in the northeastern United States, Canada, and other parts of the world, we have not been able to isolate any from Florida. The reason for this is unclear, and still remains to be resolved. Chlorovirus hosts typically live within the protozoan *Paramecium bursaria*, the coelenterate *Hydra viridis* or the heliozoon *Acanthocystis turfacea*. They can therefore be considered zoochlorellae since they can symbiotically live within these protozoans or invertebrates. Zoochlorellae are resistant to chloroviruses in their symbiotic states (Wilson et al., 2009). However, there are three zoochlorellae that can grow independently of their host, and therefore allow for a detailed study of the virus life cycle and replication mechanisms. These hosts are *Chlorella* NC64A, *Chlorella* SAG 3.83 and *Chlorella Pbi* (Van Etten & Dunnigan, 2012). Chloroviruses infect their hosts by fusing with the host membrane and enzymatically degrading the cell wall. The virus then releases its genome into the host cytoplasm, and replication takes place. Capsids assemble in the cytoplasm and are released by lysis.

A recent study unexpectedly found DNA of *Acanthocystis turfacea* chlorella virus 1 (ATCV-1) in human oropharyngeal samples. Studies performed on mice revealed that ATCV-1 could potentially disrupt pathways that are involved in learning, memory formation and immune response to viral exposure (Yolken et al., 2014). The authors then performed a series of tests on 92 individuals living in the Baltimore, Maryland metropolis area. The tests documented the individuals' performances on visual motor speed, memory function, and general knowledge. In individuals who were found to have ATCV-1 DNA present in their throat swabs, memory and visual motor speed functions were significantly decreased. These results were independent of demographic variables such as age, race, sex, education level, socioeconomic status, place of birth and cigarette smoking. However, no differences were noted between individuals who did or did not have ATCV-1 present in their throats for the general knowledge test. Therefore, this study suggests that chloroviruses may affect cognitive functions in humans by decreasing memory capacity and spatial awareness.

In the experiment described here, we examined a pond water sample from Blydenburg County Park in Smithtown, New York for chloroviruses. We were able to observe cytopathic effects in *Chlorella heliozoae*, strain SAG 3.83, which indicated the presence of chlorovirus (designated MG1). We then analyzed the section of the virus's genome that encoded DNA polymerase B (polB) because this specific polymerase is highly conserved throughout many known viruses. By using molecular and computational techniques, we compared it to other known polymerase sequences of different chloroviruses, and created a cladogram to display the results.

2 METHODS

Isolation of virus particles

Water samples were collected from a freshwater lake in Blydenburg County Park in Smithtown, New York. The samples were filtered through a $0.45\,\mu\mathrm{m}$ filter, and filtrate added cultures of *Chlorella sp* (ATCC 30562), *Chlorella heliozoe* (SAG 3.83) and *Chlorella sp* (NC64A). All chlorella species were provided by Dr. James Van Etten (University of Nebraska, Lincoln) and maintained in Modified Bold's Basal Medium (MBMM). Cultures were incubated at room temperature under fluorescent light and monitored for clearing as evidence of viral replication. A TCID50 assay was performed using Chlorella heliozoae strain SAG 3.83 to confirm the presence of chlorovirus. After the virus was isolated, its DNA was extracted.

DNA Amplification

Chloroviruses from the pond water sample were isolated and amplified using polymerase chain reaction (PCR). Two degenerate primer pairs were used to amplify the DNA polB region of the genome. The primer designs shown in Table 1 were analyzed and selected based off of the following qualities: 1) primer lengths varied between 15–30 bases 2) the G/C content composed 40–60% of the primer 3) formation of internal secondary structures was avoided 4) continuous Gs or Cs at the $3'$ end was avoided 5) similar melting temperatures.

The PCR kit from Promega Corporation (Madison, WI) was used to perform the reaction. A final volume of $50\,\mu\mathrm{L}$ was required to complete the reaction, so a $25\,\mu\mathrm{L}$ of GoTaq Colorless Mater Mix

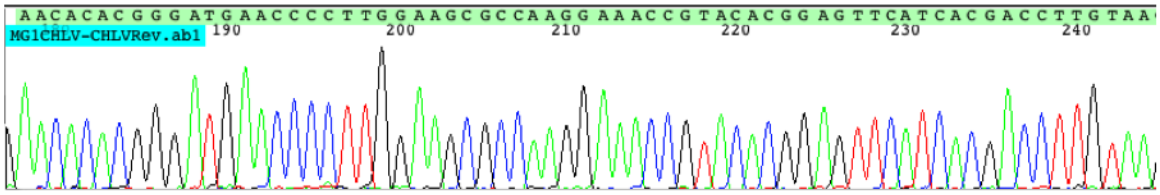

Fig. 1: Example electropherogram of DNA sequencing analysis for the reverse sequence of MG1CHLV

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PBCV-1      AGGGTGTG-----------------------GTGCCCGCTCTATTGGACGATCTCGCGAAG
MG1         TTGTTCTGGTCCTTACCTTCGGGCAGCTTCATCTTCACCATGATGGAATCTGTATCACCA
ATCV-1      TTGTTCTGGTCCTTACCTTCGGGCAGCTTCATCTTCACCATGATGGAATCTGTATCACCA
              * * **                        *   * *  *  ****   * *  *

PBCV-1      TTCAGGAA-----ACTAGCGAAGAAGCATATG-----------GCAGAAGCTAAACGAAA
MG1         TACACTACTACAGACCCCGGGAGCAGCTCCAGAGCACGCCGAGCTGCAACATCAATCATG
ATCV-1      TACACTACTACAGACCCCGGGAGCAGCTCCAGAGCACGTCGAGCTGCAACATCAATCATG
            * **  *       **    * ** ***    *                  **  * **  *

PBCV-1      TGGTGATGATTTCAAAGAAGCGTTGTACGATGCTCAACAACGG-----------------
MG1         TTGCGACCCGTCGCAGTCACCGATGCTGCAATCGGAACACACGGGATGAACCCCTTGGAA
ATCV-1      TTGCGACCCGTCGCAGTCACCGATGCTGCAATCGGAACACATGGGATGAACCCCTTGGAT
            * * **    *   *   * ** **    *  *  ****   *

PBCV-1      ------------TCGTTCAAAGTTGTGATGAACAGTG---TGTACGGTTTCCTGGGGGCT
MG1         GCGCCAAGGAAACCGTACACGGAGTTCATCACGACCTTGTAAGAACGTTGACTGGCATCG
ATCV-1      GCGCCAAGGAAACCGTACACAGAGTTCATCACGACCTTGTAAGAACGTTGGCTGGCATCG
                        *** **  *   * ** *  *          *  ***  ****   *

PBCV-1      TCGAAGG-------------GATTCATTCCTTGTGTTCCGATCGCGGCGTCTGTGACTGC
MG1         TACAACGCCTCCTTGAAGTCATCTCCCTCCTTGTGCGCCTGTGCCATCAGCTTCTTTGCG
ATCV-1      TACAACGCCTCCTTGAAGTCATCTCCCTCCTTGTGCGCCTGTGCCATCAGCTTCTTCGCG
            *  ** *                 **  ********  **  *  *  *  **

PBCV-1      AACGGGCAGAAAGATGATTGAGCATACTGCAAAACGCGCGGTGGAACTGCTACCTGGGTC
MG1         TTCTTCCTGAACTTTGC------CAGGTCGTCGAGAAGAGCTGGGACAACACCCTGGGAC
ATCV-1      TTCTTCCTGAACTTTGC------CAGGTCGTCGAGAAGAGCTGGGACAACACCCTGGGAC
              *   * ***   **          *    *   * * *** **  *  ****** *

PBCV-1      TGAGGTTATTTACGGAGATACAGATAGCGTGATGGTCAAGATGAAACTTCCTGATGATAA
MG1         TTCTGCGAATACCTAAACTTGCCAATGCCT----------------------GTCTGGA-
ATCV-1      TTCTGCGAATACCTAAATTTGCCAATGCCT----------------------GTCTGGA-
            *   * * *  *  *  *    *  ** *                       *    *
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Fig. 2: Sequence alignment for MG1 and known chloroviruses ATCV-1 and PBCV-1. MG1 displays 98% sequence identity with ATCV-1. The asterisks denote perfect conservation. The dashes indicate there are base pairs that are missing and the sequence is not conserved in that region.

(2X) was used. 1 µL of forward primer (10 µM) and 1 µL of reverse primer (10 µM) were added to the Master Mix. The volume of the DNA-free nuclease water and the DNA template were adjusted according to the concentration of the DNA isolated from the virus sample. The reaction was then carried out under the following cycle parameters: initial incubation at 95 °C for 2 min; 30 cycles of denaturation for 1 min; annealing at 58 °C for 1 min; extension for 1 min at 72 °C, and a final extension of 5 min at 72 °C. The finished products were stored indefinitely at 4 °C for further use.

Upon completion of the reaction, 5 µL of the final products and 2 µL bromophenol blue were loaded into a 1% agarose gel, and were electrophoresed at 80 V for 60 min. A 2-log ladder standard (New England Biolabs) that positioned at a 300 bp DNA fragment also ran alongside the products. TBE was used as the electrophoresis buffer. The gel was stained with ethidium bromide, and was visualized using a UV light. Bands of interest were excised and the DNA was extracted using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) according to the manufacturers protocol. The extractions were stored for additional analysis at 20 °C.

Sequencing

The DNA was sequenced by GENEWIZ, Inc. (South Plainfield, NJ) by bidirectional sequencing.

Alignment

The product was then run through NCBIs BLAST search tool to find other viruses of genetic relatedness. DNA polymerase sequences of closely and distantly related viruses were collected from NCBI and aligned using Clustal Omega.

Tree Construction

The final result was generated with the trimmed sequences from Gblocks. Clustal Phylogeny was used to create a cladogram with the neighbor-joining method with distance correction.

3 RESULTS

Analysis of the DNA sequences from MG1

Electropherograms were created using CodonCode Aligner, and were analyzed and compared for each strand of polymerase B DNA sequence (example shown in Figure 1). Each colored peak represents a single base pair. Peaks that are clearly defined and separated are strong indicators of a clean PCR product, and can confirm the accuracy of the sequenced DNA. We were able to sequence approximately 500 nucleotides each from three samples: MG1CHLV forward and reverse, MG2CHLV forward and reverse,

Table 1. Primer designs for amplification of the DNA polB region of MG1

Primers	Primer Length	G/C content	Melting Temp (°C)
FOR: 5′—CACAACATGTCTCCGGAGAC—3′	20	55%	55.4
REV: 5′—GCHGCAATYGGRACACATGG—3′	20	56.7%	55.2–61.8

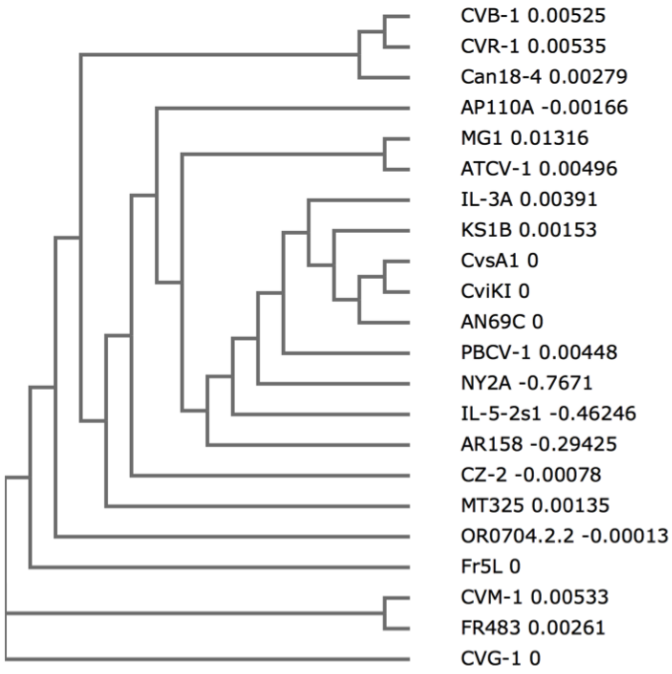

Fig. 3: Cladogram generated from polymerase sequences of known viruses from Family *Phycodnaviridae*, and known viruses from other families that are closely related to *Phycodnaviridae*. This cladogram hypothesizes the evolutionary history of known viruses to predict where MG1 would fit. Numbers indicate branch lengths

and MG1JW forward and reverse. There were very few differences in terms of nucleotides between the three samples. However, we found that MG1 was the cleanest and most accurate of the three samples, so it was used in the multiple sequence alignment.

Multiple sequence alignment

The sequence of MG1 DNA polymerase was aligned with the known DNA polymerase sequences of ATCV-1 and PBCV-1 (*Paramecium bursaria* chlorovirus-1), two chloroviruses that are also from family *Phycodnaviridae*. Results indicated that MG1 was 98% identical to ATCV-1. Results from BLAST also indicated that PBCV-1 was 80% related to ATCV-1. It was included to help diversify the input nucleotides and create a more accurate alignment.

4 DISCUSSION

After receiving the sequenced results from GENEWIZ, we analyzed the electropherogram and determined the PCR product was clean and accurate. As noted in the Results section, the multiple sequence alignment revealed that MG1s polymerase B sequence is 98% related to ATCV-1. This was also confirmed by the cladogram generated by Clustal Phylogeny (Figure 3), which showed that MG1 and ATCV-1 were part of the same clade, further proving that they are closely related. Therefore, we determined that MG1 is highly similar to ATCV-1, but additional sequencing will be necessary to determine whether MG1 is a distinct chlorovirus or a strain of ATCV-1 since only one region of the genome was targeted for sequencing. The study published in PNAS determined that ATCV-1 isolated from human throat swabs were associated with cognitive deficiencies. Therefore, the presence of MG1 and ATCV-1 in recreational waters may be of concern to local residents.

As mentioned in the Introduction, chloroviruses have a cosmopolitan presence with titers ranging between 1-100 plaque forming units (PFU)/mL. Sometimes titers can reach up to a hundred thousand PFUs (Van Etten & Dunnigan, 2012). Despite this fact, we decided to use a chlorovirus isolated from New York because we were unable to successfully isolate specimens from Florida.

When ATCV-1 was first discovered in 2005 by researchers in Germany, they were able to identify it as a novel virus due to structural differences as well as genetic differences when compared to PBCV-1 (Bubeck & Pfitzner, 2005). The scientists amplified the DNA polB region of ATCV-1 using the following degenerate primers under the conditions given by Chen & Suttle (1995):

FOR: [5′—GA(A/G)GGIGCIACIGTI(T/C)TIGA(T/C)GC—3′]
REV: [5′—(G/C)(A/T)(A/G)TCIGT(A/G)TCICC(A/G)TA—3′]

They found that many of the proteins encoded in ATCV-1 and ATCV-2 were homologous to PBCV-1, although the degree of variability ranged between 0% to 88%. Furthermore, the positions of the proteins on the genomes of ATCV-1 and PBCV-1 were different. When the researchers analyzed the evolutionary relationship of ATCV-1 to chloroviruses and other large DS DNA viruses, they found that the main capsid proteins from members in family *Phycodnaviridae* were closely related to capsid proteins from members of family *Iridoviridae*, a virus that commonly infects insects and other vertebrates. They also found that a phylogenetic tree based on sequences from DNA polymerases of various algal viruses suggested that Pbi and NC64A chloroviruses were in separate, but close branches. Additional research also suggested that *Phycodnaviridae* was closely related to *Herpesviridae*. Our tree suggests similar information in regards to PBCV-1 and ATCV-1. Since our tree is strictly limited to sequences from mainly family Phycodnaviridae, the more distantly related DS DNA families such as *Herpesviridae* and *Mimiviridae* are not shown. Moreover, the branch lengths do not have any significant meaning in terms of phylogeny because the tree is unscaled and cannot predict exactly how far these viruses have diverged from the common ancestor.

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