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Genetic Diversity in Marine Algal Virus Communities as Revealed by Sequence Analysis of DNA Polymerase Genes†

FENG CHEN,* CURTIS A. SUTTLE‡, AND STEVE M. SHORT‡

Department of Marine Science, University of Texas at Austin, Port Aransas, Texas 78373-1267

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Algal-virus-specific PCR primers were used to amplify DNA polymerase gene (*pol*) fragments (683 to 689 bp) from the virus-sized fraction (0.02 to 0.2 μm) concentrated from inshore and offshore water samples collected from the Gulf of Mexico. Algal-virus-like DNA *pol* genes were detected in five samples collected from the surface and deep chlorophyll maximum. PCR products from an offshore station were cloned, and the genetic diversity of 33 fragments was examined by restriction fragment length polymorphism and sequence analysis. The five different genotypes or operational taxonomic units (OTUs) that were identified on the basis of restriction fragment length polymorphism banding patterns were present in different relative abundances (9 to 34%). One clone from each OTU was sequenced, and phylogenetic analysis showed that all of the OTUs fell within the family *Phycodnaviridae*. Four of the OTUs fell within a group of viruses (*Mp*V) which infect the photosynthetic picoplankter *Micromonas pusilla*. The genetic diversity among these genotypes was as large as that previously found for *Mp*V isolates from different oceans. The remaining genotype formed its own clade between viruses which infect *M. pusilla* and *Chrysochromulina brevifilum*. These results imply that marine virus communities contain a diverse assemblage of *Mp*V-like viruses, as well as other unknown members of the *Phycodnaviridae*.

Viruses which infect many ecologically important groups of marine microbes, including heterotrophic bacteria (1), cyanobacteria (26, 32), eukaryotic algae (8, 19, 27), and heterotrophic nanoflagellates (13), have been isolated. This diverse assemblage of viruses coexists with the planktonic communities which are responsible for much of the primary production, nutrient regeneration, and respiration in aquatic environments. Viruses are important for regulating communities of planktonic organisms and may affect the genetic diversity of microbial populations (11, 28, 30).

Most of our knowledge about the genetic diversity of algal viruses comes from studies of viruses which have been isolated and purified in the laboratory. The virus isolates which infect freshwater endosymbiotic *Chlorella*-like algae (31, 34) and the marine photosynthetic flagellate *Micromonas pusilla* (8, 10) are geographically widespread and genetically variable. However, the viruses which have been studied may not be representative of the diversity of algal viruses in natural communities, because the host organism has been selected by culture conditions and only viruses which cause lysis of specific cultivable strains can be examined. Temperate viruses and viruses infecting uncultured algae will be excluded. Defining the diversity and structure of virus populations in nature remains a challenge.

Recently, a PCR-based method was developed for rapidly amplifying and identifying DNA *pol* gene fragments from viruses which infect three genera of distantly related microalgae (*Chlorella* strains NC64A and Pbi, *M. pusilla*, and *Chrysochromulina* spp.), as well as natural virus communities (4, 5). The amplified DNA *pol* segments were sequenced, and it was shown that they provide enough heterogeneity to resolve genetic variation among eight closely related viruses which infect *M. pusilla* (*Mp*V) (6). Phyletic analysis of these sequences indicated that viruses which infect the same algal host are more closely related to each other than they are to viruses which infect other microalgae. Sequence similarities among eight *Mp*V clones ranged from 78 to 99%, whereas CVA-1 and *Cb*V (viruses infecting *Chlorella* strain Pbi and *Chrysochromulina brevifilum*, respectively) were the most different and were only 49% similar (6). Despite the difference in sequences, these phycoviruses are quite closely related and evolved from a common ancestor, even though CVA-1 was isolated from freshwater and *Cb*V was isolated from seawater (6). These studies demonstrated that the DNA *pol* gene is a good marker molecule for examining genetic relatedness and inferring phylogenetic relationships among algal viruses and potentially among other organisms (6).

A further difficulty in studying the genetic diversity of algal viruses in nature is their relatively low abundance. The use of ultrafiltration to concentrate virus-sized particles in seawater has greatly enhanced our ability to obtain large quantities of viruses, relatively free of other contaminating materials (21, 29). In this study we have combined ultrafiltration with PCR to examine the genetic diversity and phylogenetic relationships of natural algal virus communities in the Gulf of Mexico.

MATERIALS AND METHODS

Collection and preparation of viral communities. Natural virus communities were concentrated from the surface or the deep chlorophyll maximum (DCM) at five stations in the Gulf of Mexico (Table 1) between 21 and 28 June 1995. The depth of the DCM was determined by in situ chlorophyll fluorescence, and samples were collected in 30-liter Niskin bottles mounted on a rosette. A submersible pump was used to obtain samples from the surface. The water samples were processed as shown in Fig. 1. Briefly, about 200 liters of water was collected in a 200-liter polyethylene container. A submersible pump was placed in the container and used to pressure filter (<17 kPa) the water through two 142-mm-diameter glass fiber filters (MSF GC50; nominal pore size, 1.2 μm) arranged in

* Corresponding author. Present address: Department of Marine Sciences, University of Georgia, Athens, GA 30602. Phone: (706) 542-5669. Fax: (706) 542-5888. Electronic mail address: fchen2@uga.cc.uga.edu.

† Contribution no. 971 of the Marine Science Institute.

‡ Present address: Department of Earth and Ocean Sciences, Botany and Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4.

TABLE 1. Description of stations and samples from which viral DNA was obtained

Station	Date	Latitude	Longitude	Sample depth (m)	Salinity (‰)	Temp (°C)	Vol		Concn factor	Cartridge(s) used
							Initial (liters)	Final (ml)		
A	21 June	26°14′N	95°11′W	100	36.27	20.75	168	142	1,183	S10 + S1
B10	23 June	25°06′N	93°38′W	Surface	36.31	29.01	200	157	1,273	S10 + S1
B11	23 June	25°06′N	93°38′W	126	36.38	22.14	196	150	1,307	S10 + S1
D	25 June	26°39′N	95°42′W	78.5–86.2	36.35	20.19–21.33	200	770	259	S10 only
E	27 June	26°53′N	96°35′W	73	36.22	21.48	214	517	414	S10 only
G	28 June	27°44′N	96°58′W	Surface	29.98	28.85	200	760	163	S10 only

parallel and a 0.2-μm-pore-size polycarbonate Nuclepore cartridge filter into another 200-liter polyethylene container.

The viruses in the filtrates were concentrated by ultrafiltration through 30,000-molecular-weight-cutoff spiral cartridges (Amicon S10Y30 [S10] with 0.93-m^2 surface area and Amicon S1Y30 [S1] with 0.09-m^2 surface area) (Fig. 1). The virus communities from stations A and B were first concentrated to ca. 1 to 2 liters with the S10 cartridge and in some cases concentrated further to ca. 150 ml with the S1 cartridge (Table 1; Fig. 1). For viruses from stations C, D, and G, only the S10 cartridge was used and the final volume was about 500 to 800 ml. The S10 cartridge was operated with a ProFlux M-12 system (Amicon) at 40 to 50% of pump speed and 50 to 60 kPa of back pressure. Under these conditions it took about 1 h to concentrate 200 liters of prefiltered seawater to a volume of 500 ml. The S1 cartridge was operated with a peristaltic pump as previously described (29). The cartridges were cleaned after use by flushing with at least 12 liters of ultrafiltrate and then stored in 50 mM phosphoric acid. Before each use, the cartridges were flushed with 0.01 N NaOH followed by at least 12 liters of ultrafiltrate.

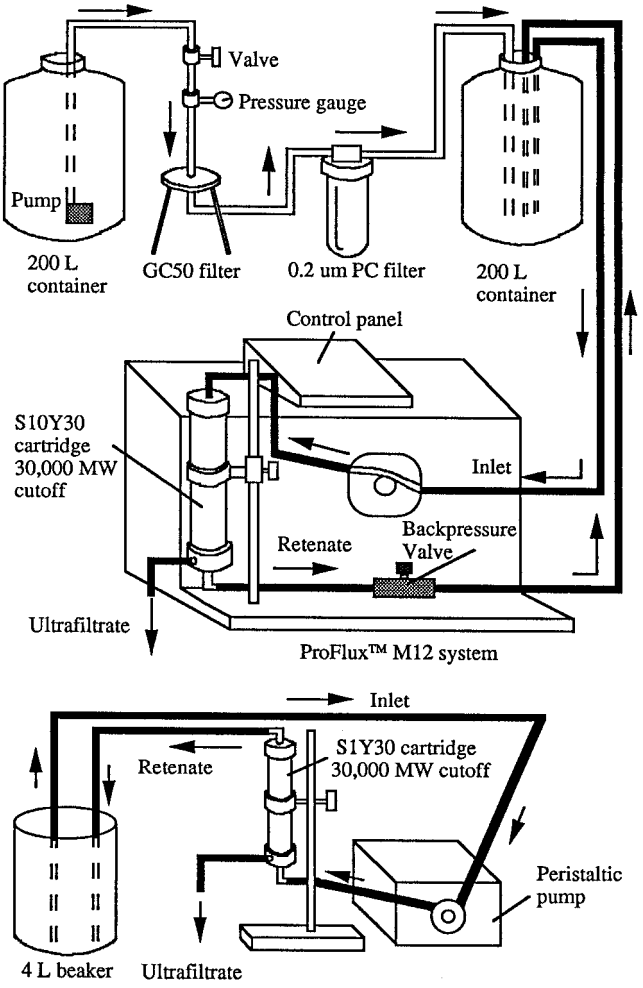

FIG. 1. The ultrafiltration systems used for concentrating natural virus communities from seawater. (Top) Approximately 200 liters of seawater was prefiltered through GC50 glass fiber and 0.2-μm-pore-size polycarbonate (PC) filters, and then the viruses in the filtrate were concentrated into 1 to 2 liters by ultrafiltration with an S10 cartridge mounted on an Amicon ProFlux M12 system. (Bottom) In some cases, the viruses were concentrated into a smaller volume (100 to 200 ml) by a second-step ultrafiltration using an S1 cartridge and a peristaltic pump. MW, molecular weight.

Aliquots from the concentrated virus communities (35 ml from stations A and B and 105 ml from stations D, E, and G) were centrifuged at 25,000 rpm for 90 min in an AH-629 rotor (Sorvall). The pellet was resuspended in 500 μl of distilled water and then divided into two aliquots of 250 μl each. One aliquot was untreated, and the other was chilled in an ice bath for 2 min, heated in boiling water for 2 min, and rechilled in an ice bath for 2 min. The treated and untreated aliquots were stained with DAPI (4′,6-diamidino-2-phenylindole) and examined under an Olympus BX-40 epifluorescence microscope. The treated and untreated aliquots were used for PCR amplification as outlined below.

PCR amplification. The oligonucleotide primers AVS1 and AVS2, which are specific for a group of algal viruses, and a nested primer (POL) which is general for B-family DNA *pol* genes are described elsewhere (4, 5). Each aliquot of treated or untreated sample was subsampled (17.25 μl) and added to 12.75 μl of a PCR mixture containing *Taq* DNA polymerase assay buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$), a 0.16 mM concentration of each deoxyribonucleoside triphosphate, 100 pmol of each primer (AVS1 and AVS2), and 0.625 U of *Taq* DNA polymerase. Mineral oil (20 μl) was added to each reaction tube. Both negative and positive controls were performed to check for DNA contamination and to ensure that amplification was not inhibited by other materials that may have been concentrated along with the viruses. PCR was performed by denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min. The cycle was repeated 30 times with a Minicycler (MJ Research) thermal cycler. Afterwards, 10 μl of the PCR product was purified by electrophoresis on 4% NuSieve GTG agarose (FMC BioProducts, Rockland, Maine) in 0.5× TBE buffer (0.045 M Tris-borate, 1 mM EDTA, pH 8.0) and examined by ethidium bromide staining. For the second-step amplification, the agarose plugs containing the templates were excised from the gels with Pasteur pipettes and then combined with PCR mixtures (4). All of the reaction mixtures and conditions for the second-step PCR were the same as those for the first-step amplification, except that the downstream primer (AVS2) was replaced by the nested primer (POL). The AVS1-POL amplicons were run on 4% NuSieve GTG agarose in 0.5× TBE buffer and visualized as described above.

Cloning and PCR-restriction fragment length polymorphism (RFLP) analysis. The amplified DNA fragments from station B were cloned into the vector pBluescript KS+/− (Stratagene, La Jolla, Calif.) by TA cloning (18). The ligation reaction mixture contained 7 μl of PCR product and 1 μl of T vector, as well as 1 μl of 10× ligase buffer (300 mM Tris-HCl [pH 7.8 at 25°C], 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM ATP) and 3 U of T4 DNA ligase (Promega), in a final volume of 10 μl. The mixture was incubated overnight at 4°C. The ligation reaction was used to transform competent *Escherichia coli* MY1193. Bacterial colonies were screened for α-complementation by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as the substrate (24) on 2× YT (10 g of NaCl, 10 g of yeast extract, 16 g of tryptone in 1 liter of distilled H_2O) agar supplemented with ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml). In order to confirm that transformed cells contained the correct insert, about 25% of each white colony was picked with an Eppendorf pipette tip (volume, 0.2 to 10 μl) and transferred into 24 μl of PCR mixture, which included the AVS1 and AVS2 primers, PCR buffer, and *Taq* DNA polymerase as described above. After 30 cycles of amplification, 5 μl of PCR product from each tube was loaded on an agarose gel (1% SeaKem LE agarose; FMC BioProducts).

The overall procedures for the PCR and RFLP analysis are outlined in Fig. 2. Once a colony was confirmed to contain an insert of the appropriate size, the insert was subjected to RFLP analysis. RFLP was carried out against randomly selected PCR clones to screen for different types of cloned inserts and to avoid sequencing the same clone types (Fig. 2). Each PCR product was digested with two restriction endonucleases, *Hae*III plus *Msp*I (New England Biolabs), which

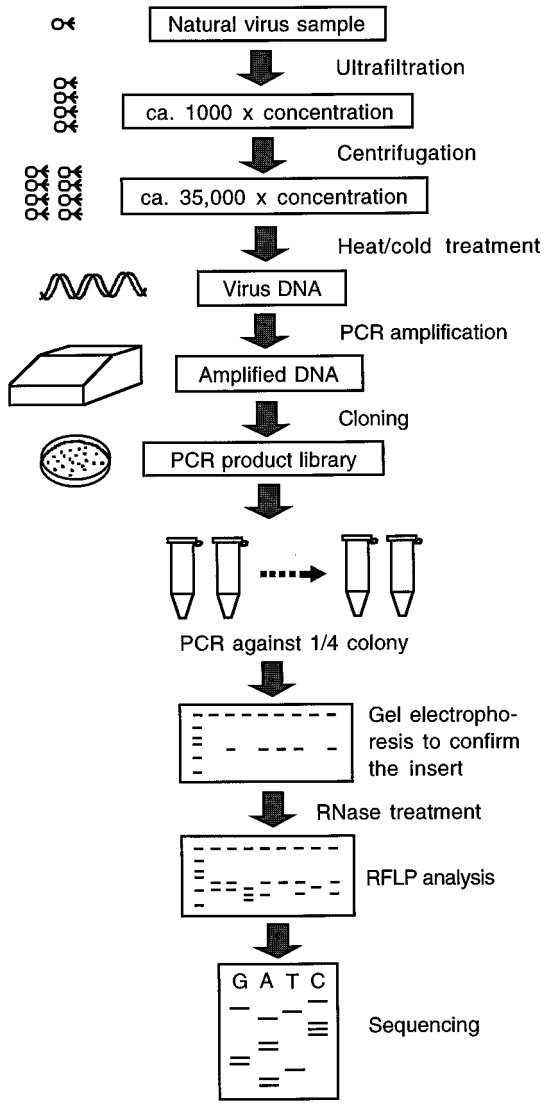

FIG. 2. Flowchart showing the method developed for the PCR and RFLP analysis.

recognize 4-bp restriction sites. A subsample (10 μl) from each PCR mixture was digested with 2 U of each endonuclease in 1× NEB buffer 2 (50 mM NaCl, 10 mM Tri-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol [pH 7.9 at 25°C]) at 37°C for 3 h. Meanwhile, a final RNase concentration of 10 μg/ml was added into the double restriction digestion, resulting in a final reaction volume of 15 μl. The resulting RFLP products were separated on 4% NuSieve agarose gels, stained with ethidium bromide (0.5 μg/ml), and visualized by UV excitation. Amplisize DNA size standard (50 to 2,000 bp; Bio-Rad) was used as a molecular marker.

Sequencing and sequence analysis. Representative clones that could be distinguished by the RFLP analyses were sequenced. The plasmid DNA from selected clones was prepared by a modified alkaline lysis protocol (33). The minipreparation DNA was denatured in 0.2 N NaOH and 0.2 mM EDTA, neutralized, and precipitated with ethanol (3). Manual sequencing was performed by the standard dideoxy chain termination method (25) with a Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp.) and [α-^{35}S]dATP (DuPont). Nucleotide sequences from each RFLP group were aligned with those from 13 known algal viruses (6) with the program CLUSTAL V (14). Evolutionary distances were calculated by the Jukes-Cantor method (15), and a distance tree was constructed with the neighbor-joining algorithm (23) in MEGA version 1.0 (17). The bootstrap method with 100 replicates was employed to estimate the robustness of the tree topologies.

Nucleotide sequence accession numbers. Nucleotide sequences of five representative clones have been submitted to GenBank under the accession numbers U36931 (OTU1), U36932 (OTU2), U36933 (OTU3), U36934 (OTU4), and U36935 (OTU5).

RESULTS

DNA from the virus communities collected at stations A, B, D, and G was amplified with the AVS1 and AVS2 primers (Fig. 3). The yield of PCR products in the heat- and cold-treated samples was higher than that of untreated samples. In the untreated samples from stations B11 and D (Fig. 3, lanes 8 and 11), no PCR products were observed; however, a clear band of about 700 bp was present in the treated samples from these stations (Fig. 3, lanes 9 and 12). The positive control DNA was amplified in all samples; however, no PCR products were obtained from the treated and untreated samples from station E (Fig. 3, lanes 15 and 16). All of the DNA fragments from the first PCR amplification of the treated samples were subjected to a second round of amplification using the nested primers POL and AVS1 (4, 5). All amplifications resulted in a single band of about 500 bp (data not shown), indicating that the original amplifications were from a DNA *pol* gene.

A total of 48 colonies were randomly picked after they tested positive (white color) for α-complementation of β-galactosidase. The 33 white colonies containing an insert of a size similar to that of the original PCR product were identified by adding one-fourth of each colony to a PCR mixture. RNA from bacteria was removed by adding 10 μg of RNase per ml. Restriction digestion with 2 U of each endonuclease (*Hae*III and *Msp*I) at 37°C for 2 h resulted in complete digestion for most of the 33 cloned PCR products and revealed five different restriction patterns, which were grouped into operational taxonomic units (OTUs) (Fig. 4). OTUs 1 through 5 accounted for 34% (11 clones), 27% (9 clones), 18% (6 clones), 9% (3 clones), and 12% (4 clones) of the 33 clones in the library, respectively. At least one clone from each OTU was picked and sequenced to compare the genetic relatedness among the OTUs.

The three clones that were compared within a single OTU (OTU1) shared the highest nucleotide sequence similarities (>98.5%); therefore, only one clone from OTU1 was used for further sequence analysis and phylogenetic reconstruction. Sequencing data revealed that the amplicon sizes of representative clones ranged from 683 to 689 bp and that the GC contents ranged from 44.3 to 48.6% (Table 2). Nucleotide sequence similarities ranged from 50.4 to 87.8% among the OTUs. Phylogenetic analysis showed that the sequences fall within the range of known algal viruses (Fig. 5). OTUs 1, 2, 3, and 4 were widely dispersed within the *M. pusilla* virus group; however, OTU5 formed its own clade between the *Micromonas* and *Chrysochromulina* viruses (Fig. 5).

DISCUSSION

Several interesting results were derived from this study. First, it was shown that algal virus DNA *pol* gene fragments can be amplified from natural virus communities in productive coastal waters and oligotrophic oceanic waters. Second, heating and cooling of concentrated natural virus communities were found to produce DNA that was suitable for PCR amplification. Third, a modified PCR-RFLP method which provides for rapid analysis of cloned PCR products was developed. Fourth, RFLP identified five distinct groups of algal viruses at a single station. Sequence analysis showed that four of the OTUs were widely dispersed within the *Mp*V group, while one OTU formed its own clade between viruses which infect microalgae *M. pusilla* and *C. brevifilum.*

Although the primers employed in this study had previously been used to amplify DNA *pol* gene fragments from natural marine virus communities (4), this is the first time that virus

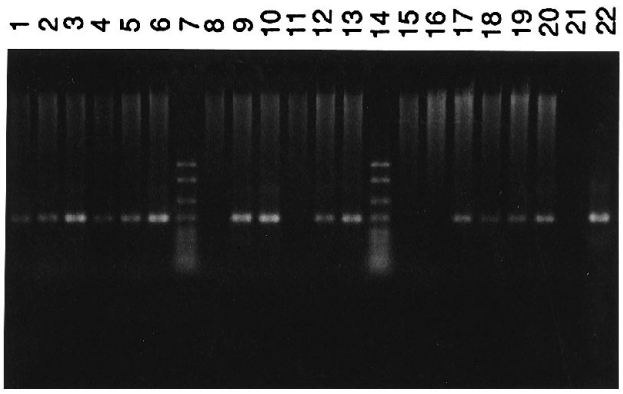

FIG. 3. Analysis of PCR fragments amplified from natural virus communities with the AVS1 and AVS2 primers. An aliquot (10 μl) of PCR product was electrophoresed on 1.5% NuSieve GTG agarose in 1× TBE buffer. Lanes 7 and 14 are molecular size markers corresponding to 2,000, 1,500, 1,000, 700, 500, 400, 300, 200, 100, and 50 bp from top to bottom. Lanes from a single sample have been run beside each other and correspond to untreated samples, heat- and cold-treated samples, and untreated samples plus 0.1 μg of *Mp*V-SP1 DNA, respectively: station A, lanes 1, 2, and 3; station B10, lanes 4, 5, and 6; station B11, 8, 9, and 10; station D, 11, 12, and 13; station E, 15, 16, and 17; station G, 18, 19, and 20. Lanes 21 and 22 are negative (no DNA) and positive (0.1 μg of *Mp*V-SP1 DNA) controls, respectively.

communities from a variety of locations have been screened. A PCR product was obtained in five of six samples that were collected from environments ranging from quite productive to extremely oligotrophic in the Gulf of Mexico. The use of the nested primer (POL) confirmed that the amplified products were from DNA *pol* genes. It is not clear why no PCR product

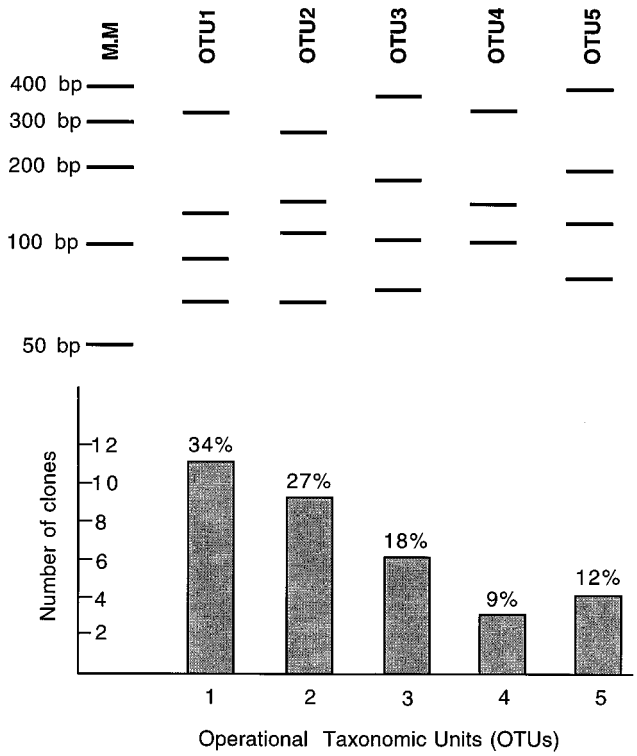

FIG. 4. Schematic representation of the RFLPs for the five OTUs that were identified (top) and the relative abundance of each OTU (bottom). M.M, molecular size marker.

TABLE 2. Amplicon size, GC content, and pairwise nucleotide sequence similarity of the OTUs

OTU	Sequence identity (%) with:				Amplicon size (bp)	GC ratio (%)
	OTU2	OTU3	OTU4	OTU5		
OTU1	75.5	87.8	76.4	50.4	683	44.3
OTU2		77.1	83.6	56.1	683	46.6
OTU3			78.2	54.9	683	47.4
OTU4				55.3	683	47.5
OTU5					689	48.6

was amplified from the sample taken at station E, where the DNA concentration was highest. This sample was taken in a large bloom of *Synechococcus* spp. in which other types of phytoplankton were very rare. Consequently, there were likely very few host cells present.

DAPI-stained particles were not found in the heat- and cold-treated samples, suggesting that the treatment released the viral DNA. Although DNA could be amplified from untreated samples, the relative intensity of the PCR products was greatest in the treated samples. Using the heat-cold treatment to prepare samples for PCR avoids the loss of viral DNA during phenol-chloroform extraction and ethanol precipitation, does not add chemicals which may affect the efficiency of amplification, and is faster.

The genetic diversity of natural communities of algal viruses had not been investigated prior to this study. This is also the first time that DNA *pol* sequences have been used to infer phylogenetic relationships among uncultured organisms in natural environments. Sequence analysis of PCR products amplified from a single station in the central Gulf of Mexico indicated that algal viruses can be genetically diverse within the same water sample. Cottrell and Suttle (10) drew a similar conclusion based on genomic DNA hybridization and found that the genetic diversity between *Mp*V isolates from a single sample can be as large as that between isolates from different oceans. The interactions between algal viruses and their hosts, including virus infection and host defense, lysogeny, and induction, are probably important in maintaining the genetic diversity of both virus and host populations in nature.

Ogunseitan et al. (20) used DNA probes to determine the distribution of bacteriophages which infect *Pseudomonas aeruginosa* in lake water, sediment, soil, and sewage and found that transducing phages and prophages are widely distributed in *Pseudomonas* populations. Therefore, transduction of virus and lysogenic conversion of host cells are potentially important in maintaining the genetic diversity of viruses in natural ecosystems. Brown et al. (2) constructed PCR primers which amplify a 470- to 490-bp segment from six groups of vibriophages isolated from Tampa Bay, Key Largo, Dry Tortugas, Hawaii, and the Gulf of Mexico. The viruses were distinguished from each other on the basis of restriction enzyme digestion and DNA fingerprinting (16). They found that the sequence similarities among these vibriophages ranged from 62 to 74% (2). In our study, the sequence similarities among five different RFLP groups ranged from 50.5 to 87.8% in a single water sample collected from the DCM in offshore water.

Phylogenetic analysis indicated that all of the OTUs belong within the family *Phycodnaviridae*. Four of the five distinct groups that were identified by RFLP analysis, representing 88% of the 33 clones picked from the library (Fig. 4), fell within the *Mp*V group. The fifth group (OTU5) comprised 12% of the clones and did not fall within the three established groups of viruses which infect microalgae (*Mp*V, *Cb*V, and

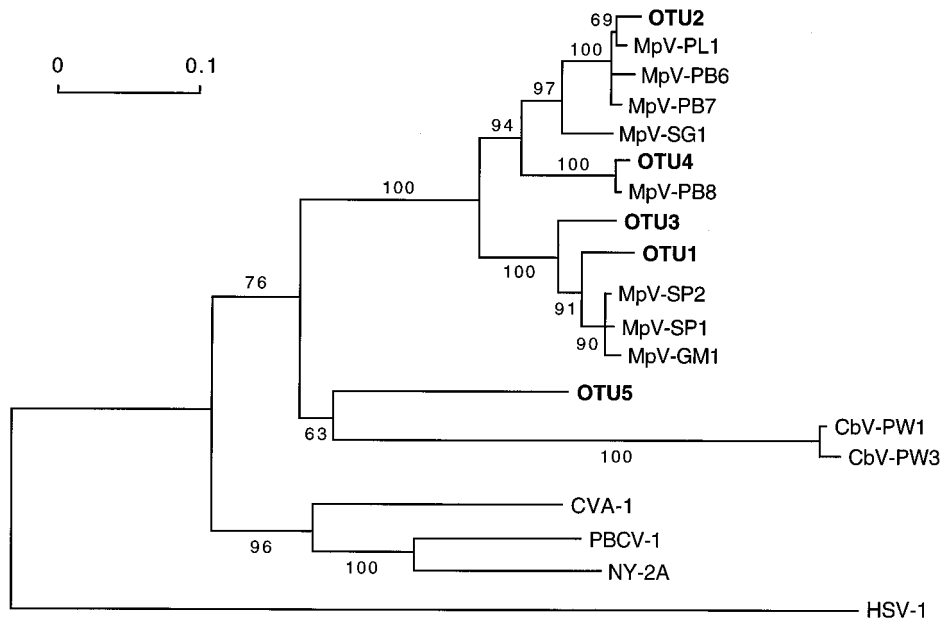

FIG. 5. A neighbor-joining tree showing the phylogenetic relationships among the five OTUs from this study and 13 other algal viruses based on sequence analysis of DNA *pol* gene fragments. Herpes simplex virus type 1 (HSV-1) was used as an out-group. The numbers at the nodes indicate bootstrap values ($n = 100$), and branches with values less than 50 have been collapsed. The scale bar represents 0.1 fixed mutation per nucleotide position.

Chlorella viruses). If the relative proportions of the OTUs are representative of the relative abundances of the different groups in nature, then *Mp*V is the most abundant member of the *Phycodnaviridae* in this sample from the DCM (station B2, 126 m). The abundance of *Mp*V in this sample was estimated to be 5,400 infective viruses per ml, based on a most-probable-number assay (22a). This is equivalent to four *Mp*V viruses ml^{-1} in the natural water, assuming that the viruses were concentrated with 100% efficiency. Cottrell and Suttle (9) reported that the infective *Mp*V concentration ranged from 2.1×10^3 to 1.3×10^5 viruses ml^{-1} in inshore waters of the Texas coast, whereas only 1 of 36 samples had a titer as high as 0.1 ml^{-1} in oligotrophic oceanic water in the Gulf of Mexico. The only place in oligotrophic waters where *Mp*V was detected was at the DCM near the western front of a warm-core eddy (8). Several investigators have observed that *M. pusilla* is particularly abundant at the DCM of coastal and offshore waters (7, 12).

Although viruses within the *Mp*V group appear to be the dominant members of the *Phycodnaviridae* at the DCM at station B, it should be noted that inferring abundance from the relative abundance of PCR products may not be valid. The AVS1 and AVS2 primers were designed on the basis of sequence information for DNA *pol* from three known groups of algal viruses (*Mp*V, *Cb*V, and *Chlorella* viruses) (4). Therefore, the primers may be more efficient at amplifying DNA from these viruses and less efficient at amplifying DNA from unknown viruses. There may be other algal viruses in these samples which were not amplified with this primer pair. As more sequence information from newly isolated algal viruses becomes available, it will be possible to develop more-general primers that have higher specificity for DNA from rare algal viruses. Such applications appear extremely promising for studying the genetics of virus populations in nature (22).

Sequence analysis indicated that DNA *pol* gene segments were not amplified from the DNA of *Cb*V and *Chlorella* viruses at station B, indicating that the viruses may be too rare to be represented among these 33 clones that were examined. However, a new algal virus group (OTU5) which does not fall within the three known groups of algal viruses (*Mp*V, *Cb*V, and *Chlorella* viruses) was found at station B. The RFLP pattern of OTU5 was also quite different from that of other OTUs, indicating that the RFLP screening procedure used provides a practical approach for identifying unknown and uncultivated viruses.

The results from this study provide further evidence that viruses which infect eukaryotic microalgae are a diverse and widely distributed component of planktonic communities. Further research should attempt to elucidate the mechanisms responsible for maintaining this diversity.

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