J. Mar. Biol. Ass. U.K. (2006), **86**, 485–490
Printed in the United Kingdom

Phylogenetic analysis of PgV-102P, a new virus from the English Channel that infects *Phaeocystis globosa*

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A new virus that infects the harmful algal bloom-forming microalga *Phaeocystis globosa* was isolated from surface water in the English Channel off the coast of Plymouth, UK, in May 2001. Phylogenetic analysis of the DNA polymerase gene revealed the virus isolate, designated PgV-102P, belongs to the family Phycodnaviridae, a group of large double-stranded DNA viruses known to infect algae. Basic characterization of PgV-102P revealed it was a lytic virus with a relatively slow culture lysis period of 10-days. The genome size (176 kbp) and capsid diameter (98 nm) of PgV-102P fall at the bottom end of the range expected for phycodnaviruses. Interestingly, PgV-102P did not cluster with other *P. globosa* viruses; instead, it was more closely related to other prymnesioviruses that infect the marine prymnesiophyte *Chrysochromulina brevifilum*. We discuss the effectiveness of DNA polymerase as a diagnostic marker. Although it is ideal for determining what family or even genus an algal virus belongs to, it is clear that the DNA polymerase gene does not have sufficient resolution when looking for relationships within algal virus genera.

INTRODUCTION

Viruses are ubiquitous and abundant components of the marine environment with concentrations reaching up to 100 million ml^{-1} (Bergh et al., 1989). It is now recognized that viruses play important roles in oceanic biogeochemical cycling (Fuhrman, 1999) and are thought to act as catalysts by accelerating transformation of organic matter from particulate to a more bio-available dissolved form (Suttle, 2005). A wide range of different viruses are known to infect phytoplankton (Brussaard, 2004). Indeed, infection of bloom-forming phytoplankton is key in succession dynamics, allowing succession of different communities of phytoplankton following the lysis event (Wilson et al., 2002).

Viruses are known to infect globally-important oceanic phytoplankton such as *Phaeocystis* spp. (Jacobsen et al., 1996; Brussaard et al., 2004; Baudoux & Brussaard, 2005) which form dense and often nuisance blooms in polar waters and coastal shelf seas of northern Europe (Lancelot et al., 1987). *Phaeocystis* spp. have important roles in CO_2 and sulphur cycling which ultimately have major implications in global climate regulation (Liss et al., 1994) and infection by viruses can exacerbate this process (Malin et al., 1998). The role of viruses in regulating *P. globosa* blooms has received a great deal of attention recently following the publication of a large European Union-funded study focusing on bloom dynamics and biological control of *Phaeocystis* (Veldhuis & Wassmann, 2005). *Phaeocystis globosa* forms dense monospecific blooms as a mixture of (mainly) colonial and unicellular cells that collapse suddenly in a virus-induced crash (Brussaard et al., 2005a). Consequently, this leads to a rapid shift in bacterial community composition which takes advantage of the organic nutrients released (Brussaard et al., 2005b). Intense blooms of *P. globosa* can lead to anoxia and an impressive formation of foam on beaches during their decline (Lancelot et al., 1987), hence their label as harmful algal blooms (HABs). Inevitably, this attracts a great deal of negative publicity, particularly from fishermen and the general public.

A crucial first step to help understand the ecological role of viruses in marine environments is their isolation and characterization. This study focuses on the basic characterization of a new *P. globosa*-specific virus isolate collected from a *Phaeocystis* spp. bloom off the coast of Plymouth in the English Channel. We determine its phylogenetic affiliation based on a fragment of the DNA polymerase gene, a well-known diagnostic marker for determining genetic relatedness in large dsDNA algal viruses (Chen & Suttle, 1995).

MATERIALS AND METHODS

Study site and sample collection

Surface seawater was collected from Station L4 (50°15′N 04°13′W), south of Plymouth, UK, in the English Channel on 8 May 2001. It was collected as part of a depth profile, using a rosette of 5-l Niskin bottles connected to a stainless steel cable operated by an on-deck winch from on board the RV 'Squilla'. Seawater was decanted into a 2-l polycarbonate bottle and transported back to the laboratory in a cool box within 2 h. Samples for virus isolation were pre-filtered through a 0.2 μm Supor (Gelman) filter and stored at 4°C in the dark.

Host culture

Unicellular *Phaeocystis globosa* strain PLY64 was obtained from the Plymouth Culture Collection (PCC) of Marine

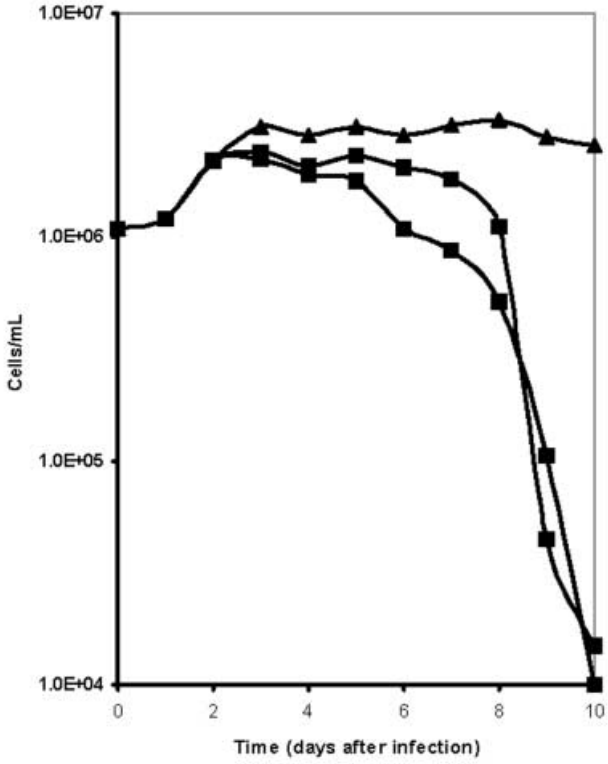

Figure 1. Infection kinetics of PgV-102P propagated on duplicate cultures of *Phaeocystis globosa* strain PLY64 (-■-) compared to a non-infected control (-▲-).

Algae (www.mba.ac.uk/education/education_outreach.php?culturecollection). It was grown in Erdschreiber medium (Bruce et al., 1940) prepared from 95% filtered (GF/F) seawater (diluted with distilled water) containing 0.02% (w/v) $NaN0_3$; 0.002% (w/v) Na_2HPO_4; 5% (v/v) soil extract (prepared from 1 kg finely sieved loam soil autoclaved in 1.4-l distilled water, the soil extract is the clear liquid remaining after being left to settle); the salts, soil extract and seawater are autoclaved separately and added together once cooled. Stock cultures, typically 50 ml volume in 125 ml glass conical flasks, were incubated at 15°C in a cooled illuminated incubator (Sanyo) set on a 16L:8D cycle.

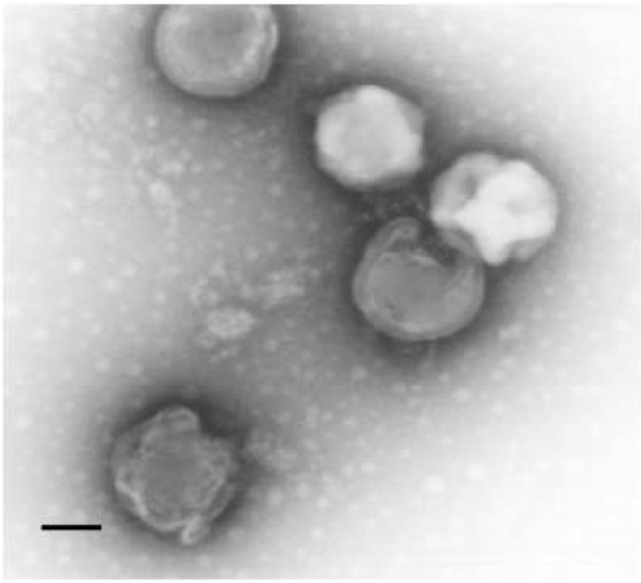

Figure 2. Transmission electron microscope (TEM) analysis of PgV-102P concentrated from a freshly lysed *Phaeocystis globosa* strain PLY64 culture. Scale bar: 50 nm.

Virus isolation and infection kinetics

Virus lysates were obtained by adding 1 ml of 0.2 μm-filtered seawater to 50 ml exponentially-growing host *P. globosa* strain PLY64. Once clearing of the host culture was observed, the lysate was passed through a 0.2 μm syringe filter (Gelman). A single virus clone, designated PgV-102P (*Phaeocystis globosa Virus* strain 102 Plymouth), was obtained by serial dilution to extinction three times. The highest dilution to lyse the host culture was passed through a 0.2 μm syringe filter (Gelman) for use in subsequent inoculations. Infection kinetics of PgV-102P were determined by adding 0.5 ml of the virus lysate to exponentially growing *P. globosa* strain PLY64 host cultures (multiplicity of infection not determined) and following their growth compared to non-infected controls. *Phaeocystis globosa* in the cultures were enumerated in a Neubauer improved haemocytometer under a light microscope.

Virus concentration and TEM examination

Cultures of exponentially growing *P. globosa* strain PLY64 (50 ml) were inoculated with 0.5 ml of fresh virus strain PgV-102P lysate. Lysed cultures were passed sequentially through 0.8 μm and 0.2 μm filters to remove cellular debris. Viruses were concentrated by centrifuging 25 ml of filtrate in a Macrosep centrifugal concentrator with a 30 kD MW cut off (Pall Filtron), down to approximately 0.5–1 ml. Virus concentrates were subsequently stored at 4°C in the dark.

For examination of viruses from concentrates, a drop was added to a formvar-coated copper grid and left for approximately 2 h. Excess liquid was then wicked off and the grid was left to air dry. All grids were post-stained with a saturated solution (approximately 3% w/v) of uranyl acetate. Transmission electron microscope (TEM) analysis was conducted on a Jeol JEM 200CX TEM operated at 120 kV using magnifications ranging from ×20,000 to ×100,000.

Pulse-field gel electrophoresis (PFGE)

PgV-102P genome size was determined by PFGE. A 100 μl aliquot of virus concentrate was added to an equal volume of 2% molten agarose in 0.5×TBE (90 mM Tris–borate, 1 mM EDTA, pH 8) and allowed to set in a well-shaped plug. Each plug was incubated at 55°C for 1 h, then overnight at room temperature in a lysis buffer (0.5 M EDTA, pH 8; 1% SDS and 100 mM Tris-HCl, pH 9) containing 0.5 mg/ml proteinase K. Plugs were then dialysed three times over 1 h in TE (10 mM Tris-HCl–1 mM EDTA, pH 8) at 4°C. Plugs were analysed by electrophoresis for 18 h at 14°C in 1% agarose (0.5×TBE) at 200 V with a pulse ramp of 20 s to 40 s using a DR-II Cell system (Bio-Rad). After electrophoresis, the gels were stained for 30 min in 0.2 g ml^{-1} ethidium bromide (0.5×TBE), destained for 30 min in 0.5×TBE and

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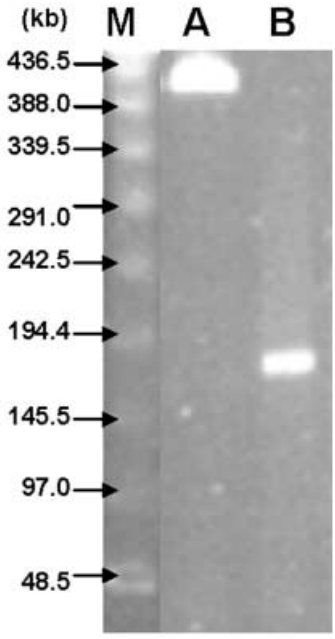

Figure 3. Pulsed field gel electrophoresis (PFGE) analysis of PgV-102P (Lane B) indicating a genome size of approximately 176 kbp. Phage Lambda concatemers (BioRad) (Lane M) and virus strain EhV-86 (Lane A) with a genome of 407 kb (Wilson et al., 2005a) were added as size markers.

visualized using the Gel Doc system with Quantity One software version 4.2.1 (Bio-Rad) using phage λ concatemers as molecular size markers (Bio-Rad).

DNA extraction, PCR amplification and cloning

DNA was extracted from PgV-102P by initially treating a concentrate with proteinase K (5 mg/ml) in lysis buffer (20 mM EDTA, pH 8 and 0.5% SDS (w/v)) at 65°C for 1 h. Aliquots of phenol (0.1×volume) were added to the samples, after which the DNA was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated with the addition of 0.5×volume 7.5 M ammonium acetate, pH 7.5 and 2.5×volume absolute ethanol.

Two degenerate oligomers, AVS1 and POL, corresponding respectively to highly conserved amino acid sequences, EGATVLDA and YGDTDS, of the DNA pol gene (Chen & Suttle, 1995), were used to amplify the DNA *pol* fragments from the virus isolates. Polymerase chain reactions (PCRs) were set up as follows: 100 ng of virus DNA was added to a 50 μl reaction mixture which contained 1 U *Taq* DNA polymerase (Promega), 1×PCR reaction buffer (Promega), 0.25 mM dNTPs, 3 mM $MgCl_2$, 30 pmol of each primer. The PCR was conducted in a PTC-100™ cycler (MJ Research) with an initial denaturing step of 95°C (3 min) then denaturing at 95°C (30 s), annealing at 48°C (60 s), and extension at 74°C (90 s). The PCR fragments were cloned using a TA Cloning Kit and the bacterial clones were screened for inserts according to manufacturer's instructions (Invitrogen Corporation).

DNA sequencing and sequence analysis

Sequencing was performed with the SequiTherm EXCEL II DNA Sequencing Kit-LC (Epicentre Technologies) with a LI-COR automated DNA sequencer (LI-COR). Sequence data were analysed using e-Seq release 1.1 software (LI-COR). DNA *pol* sequence data for PgV-102P obtained were lodged in the GenBank database under sequence Accession reference DQ401030. Homology searches with both DNA and protein sequences were carried out using the BLAST algorithm provided by the Internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

Phylogenetic tree construction

Amino acid sequences of DNA pol genes were obtained from the GenBank database. Amino acid sequence between the degenerate primer sequences of AVS1 and POL were aligned using ClustalW (http://www.clustalw.genome.ad.jp/). Phylogenetic trees were constructed using the various programs in PHYLIP (Phylogeny Inference Package) version 3.64 (Felsenstein, 1995) and the robustness of the alignments was tested with the bootstrapping option (SeqBoot), only bootstrap values above 90% being included in the analysis. Genetic distances, applicable for distance matrix phylogenetic inference, were calculated using the Protdist program in the PHYLIP package. Phylogenetic inferences based on the distance matrix (Neighbour) and parsimony (Protpars) algorithms were applied to the alignments. In both trees, the best tree or majority rule consensus tree was selected using the consensus program (Consense). The trees were visualized and drawn using the TREEVIEW software version 2.1 (Page, 1998).

RESULTS

Virus isolation and characterization

A 1 ml aliquot of 0.2 μm-filtered surface seawater from Station L4 (50°15′N 04°13′W), collected on 8 May 2001,

Figure 4. Multiple alignment of translated DNA *pol* sequences from a range of *Phaeocystis globosa*-specific viruses (PgV prefix), *Chrysochromulina brevifilum*-specific viruses (CbV prefix) and PgV-102P.

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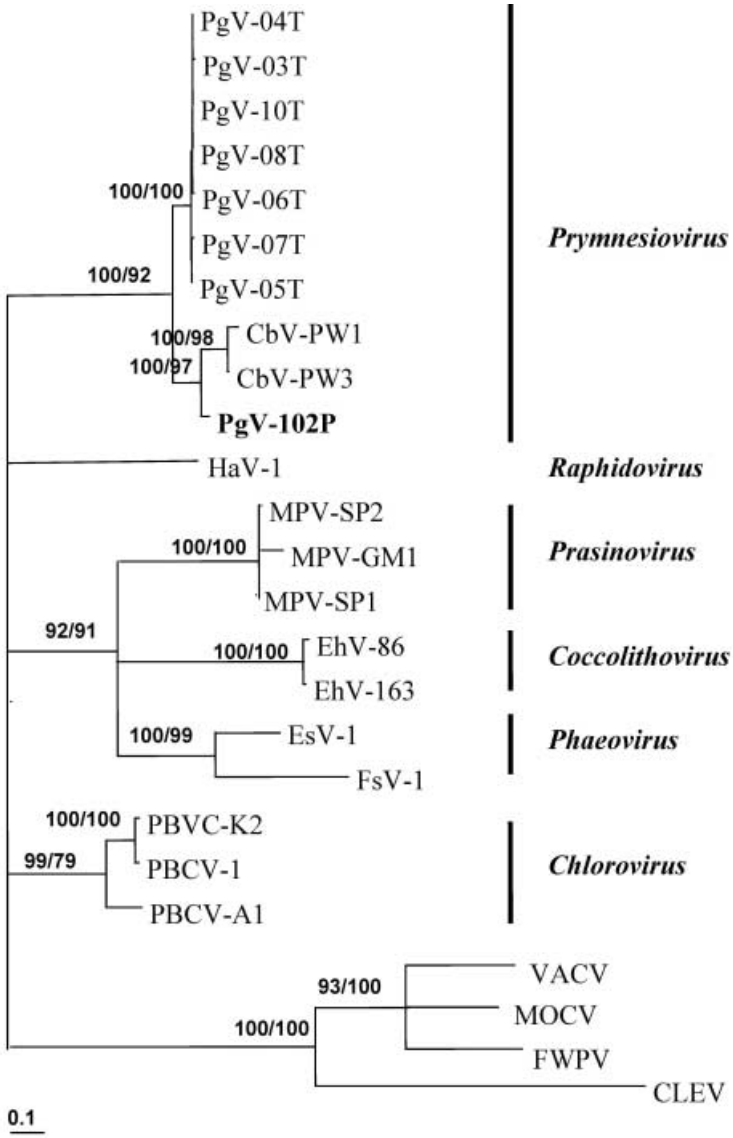

Figure 5. A phylogenetic inference based on a distance matrix algorithm between translated DNA *pol* gene fragments from the family of algal viruses, Phycodnaviridae (Neighbor, in PHYLIP version 3.64 (Felsenstein, 1995). Translated DNA *pol* gene fragment from the family Poxviridae (VACV, MOCV, FWPV & CLEV) were used to root the tree. The alignment was performed (ClustalW) on the region spanning the highly conserved regions I and IV (Villarreal & DeFilippis, 2000) of the DNA *pol* genes. Virus isolate PgV-102P forms a new clade within the genus *Prymnesiovirus*. Accession numbers for each of the virus *pol* sequences are: AY345137 (PgV-04T); AY345136 (PgV-03T); AY345142 (PgV-10T); AY345141 (PgV-08T); AY345139 (PgV-06T); AY345140 (PgV-07T); AY345138 (PgV-05T); AAB49740 (CbV-PW1); AAB49739 (CbV-PW3); DQ401030 (PgV-102P); AB194136 (HaV-1) (NB, the intein sequence (Nagasaki et al., 2005) was removed for our analysis); U32976 (MPV-SP2); AAB49742 (MPV-GM1); AAB66713 (MPV-SP1); AF453858 (EhV-86); AF453861 (EhV-163); AF204951 (EsV-1); AF013260 (FsV-1); AB011500 (PBCV-K2); A42543 (PBCV-1); AAB49748 (PBCV-A1); A24878 (VACV); NPO43990 (MOCV); NPO39057 (FWPV); S25855 (CLEV). Numbers at nodes indicate bootstrap values retrieved from 100 replicates for both the parsimony and neighbour-joining analyses. The bar depicts 1 base substitution per 10 amino acids.

was enough to cause lysis (indicated by clearing of the culture) of exponentially growing *Phaeocystis globosa* strain PLY64, after 8-d incubation. This lysate was used to obtain a clonal isolate that we designated PgV-102P (*Phaeocystis globosa* Virus strain 102 Plymouth). In a rudimentary host range experiment, PgV-102P only infected *P. globosa* strain PLY64; it did not infect *P. globosa* strain PLY147 or *Emiliania huxleyi* strains CCMP374; CCMP1516 and L (data not shown).

Complete lysis of *P. globosa* strain PLY64 host cultures by PgV-102P was observed 10 d after addition of fresh virus lysate to the culture (Figure 1). The TEM analysis of PgV-102P in concentrates revealed virus-like-particles (VLPs) with diameters of 98 nm (±5 nm) (Figure 2). Most VLPs contained electron dense cores surrounded by a capsid and their hexagonal shape suggests an icosahedral symmetry. The PFGE analysis of concentrated PgV-102P revealed a genome of approximately 176 kbp (±5 kbp) (Figure 3).

Phylogeny based on DNA pol

The PCR primers developed by Chen et al. (Chen et al., 1996) were used to amplify the DNA polymerase (*pol*)

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gene fragments from PgV-102P. The PCR products of the expected size (approximately 550 bp) were amplified from the PgV-102P DNA extracted from virus concentrate (data not shown). Sequence analysis and subsequent database searching of cloned PgV-102P PCR product revealed a high sequence identity to DNA polymerases isolated from members of the family of algal viruses known as Phycodnaviridae and DNA polymerases of eukaryotic origin (data not shown). An alignment of the translated PgV-102P DNA *pol* fragment, with other known PgV sequences, revealed a 13 amino acid insertion (Figure 4) that matched more closely to DNA *pol* sequences from *Chrysochromulina brevifilum*-specific viruses CbV-PW1 and CbV-PW3. Significantly, this had implications for the phylogeny of PgV-102P; it did not group within the *P. globosa* virus clade, instead it formed a separate clade within the Prymnesioviruses more closely related to the *C. brevifilum*-specific viruses (Figure 5).

DISCUSSION

Taxonomic sampling of marine phytoplankton has been conducted in the western English Channel for over 40 years and *Phaeocystis* sp. blooms are known to occur annually, usually sometime between the months of April and June (Southward et al., 2005; Gerald Boalch, personal communication; also see sampling database at www.pml.ac.uk/L4). Termination of these blooms is often associated with foam on the surface of the seawater, usually in late May/early June each year, probably caused by exopolysaccharides being whipped into a lather following disaggregation of colonial forms of *Phaeocystis* sp. Studies in this area of the English Channel indicate that bloom termination is not caused by herbivore grazing; indeed, it has been suggested that *Phaeocystis* sp. can actually have a negative effect on copepods (Bautista et al., 1992); moreover it has been shown that *Phaeocystis* colonies become nutrient-depleted, releasing single cells and exopolysaccharides contributing to bloom decline (Davies et al., 1992). These authors put bloom decline down to single cells being transported to deeper layers during surface mixing; this was probably true to a large extent. However, with numerous studies now documenting the existence of *Phaeocystis*-specific viruses and their ability to control *Phaeocystis* blooms (Jacobsen et al., 1996; Baudoux & Brussaard, 2005; Brussaard et al., 2005a), it is perhaps not surprising that in the current study, a new virus was isolated in the English Channel with relative ease. Consequently, virus lysis is likely to be an important factor in *Phaeocystis* bloom demise in the English Channel. As *Phaeocystis* colonies start to break up, released single cells will become more susceptible to virus infection exacerbating bloom demise. It was no coincidence that the new *Phaeocystis* virus strain PgV-102P was isolated from surface seawater collected during a *Phaeocystis* bloom (bloom data not shown).

After viruses were added to exponentially-growing host *P. globosa* strain PLY64, there was an initial drop in cell numbers after 2 d but the culture did not start to noticeably crash until 8 d after infection (Figure 1). This seems a long time and may indicate a less than optimal host strain, or possibly, the host was not growing optimally. Adding viruses earlier in the exponential growth phase gave similar results (data not shown), therefore the length of time taken to crash did not appear to be linked to adding virus so close to stationary phase growth.

Basic characterization of PgV-102P revealed it is a lytic virus (Figure 1) from the family of algal viruses Phycodnaviridae (Figure 5). Currently, the family Phycodnaviridae consists of six genera: *Chlorovirus*, *Coccolithovirus*, *Prasinovirus*, *Prymnesiovirus*, *Phaeovirus* and *Raphidovirus* (Wilson et al., 2005b). Viruses in this family infect freshwater or marine algae and they all contain large dsDNA genomes ranging from 100 to 560 kb and have a capsid diameter in the range of 100–220 nm. The genome and capsid diameter of PgV-102P clearly fall in the bottom end of this range (Figures 2 & 3) and are similar is size to Group II *P. globosa* viruses (PgVs) isolated from the southern North Sea in the years 2000–2001 (Baudoux & Brussaard, 2005). Phylogenetic analysis of DNA polymerase gene fragments places PgV-102P firmly within the genus *Prymnesiovirus* (Figure 5) which are loosely defined as viruses that infect hosts belonging to the algal class Haptophyceae, also referred to as the Prymnesiophyceae (with the notable exception of viruses that infect *Emiliania huxleyi* (Schroeder et al., 2002)). With morphological and genome characteristics so similar to Group II PgVs, it was perhaps surprising that the PgV-102P DNA *pol* contained a 13 amino acid insertion (Figure 4). Previous analysis of Group I and II PgVs revealed they formed a distinct monophyletic group (Brussaard et al., 2004) despite the fact these two PgV groups are morphologically very different (Baudoux & Brussaard, 2005). PgV-102P actually clusters with DNA *pol* sequences from *Chrysochromulina brevifilum*-specific viruses CbV-PW1 and CbV-PW3 which also contain this insertion (Figures 4 & 5). These viruses were isolated from coastal waters in the Gulf of Mexico and have a particle size 145–170 nm (Suttle & Chan, 1995), hence are much larger than PgV-102P, and indeed are actually closer in size to Group I PgVs (150 nm). Group II PgV strain PgV-03T has previously been shown to form a monophyletic group with HaV01 and CbV-PW1 (Nagasaki et al., 2005), however, these workers used a much less stringent bootstrapping value than the current study. Here we have used many more PgV strains in the analysis and only included bootstrap values of $>90\%$, hence the tree collapsed in the area around HaV (Figure 5). Consequently, this strengthens HaVs position within in its own genus (*Raphidovirus*) (Wilson et al., 2005b).

Comparison of these data merely demonstrates the massive diversity of marine viruses. We have a situation of two very different looking viruses (Groups I & II PgVs) isolated from the same area (Baudoux & Brussaard, 2005) that cluster together phylogenetically; two very different viruses that infect very different hosts isolated from opposite sides of the Atlantic Ocean (PgV-102P and CbVs) that cluster together; and finally some very similar-looking viruses that infect the same species, isolated from similar regions yet cluster in different groups. Such a confusing message simply points to the limitations of DNA *pol* as a phylogenetic marker. It is ideal for determining what family or even genus an algal virus belongs to, however, DNA *pol* does not have sufficient resolution when looking for relationships within virus genera, simply because it is such a conserved gene within the

Phycodnaviridae, consequently, it should be used with caution. With the massive diversity observed in marine viruses and so few isolates (and sequences) available, it is unlikely that a better single marker will become available. It is becoming apparent that phycodnaviruses are incredibly genetically diverse: of the three that have been fully sequenced they only share 16 core genes (DNA *pol* is one of these) from a total combined pool of approximately 1000 genes (Allen et al., 2006). Ideally, different genetic markers should be identified, such as the major capsid protein gene (Schroeder et al., 2003) or even from a combination of core genes to further resolve relationships within genera of the Phycodnaviridae. It is an ancient virus lineage and as new viruses are isolated the Phycodnaviridae is certain to reveal new genera and complex relationships within these genera; consequently, characterization of new algal viruses is an important area for further study.

This study was funded by the Natural Environment Research Council of the United Kingdom (NERC) grant no. GR8/03744 awarded to W.H.W. D.C.S. is a Marine Biological Association of the UK Research Fellow. W.H.W. is supported through the NERC-funded core strategic research programme of the Plymouth Marine Laboratory. We also acknowledge the help of the skipper and crew of RV 'Squilla'.

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Submitted 20 February 2006. Accepted 8 March 2006.