Characterisation of three novel giant viruses reveals huge diversity among viruses infecting Prymnesiales (Haptophyta)

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ABSTRACT

We have isolated three novel lytic dsDNA-viruses from Raunefjorden (Norway) that are putative members of the *Mimiviridae* family, namely *Haptolina ericina* virus RF02 (HeV RF02), *Prymnesium kappa* virus RF01 (PkV RF01), and *Prymnesium kappa* virus RF02 (PkV RF02). Each of the novel haptophyte viruses challenges the common conceptions of algal viruses with respect to host range, phylogenetic affiliation and size. PkV RF01 has a capsid of ∼310 nm and is the largest algal virus particle ever reported while PkV RF01 and HeV RF02 were able to infect different species, even belonging to different genera. Moreover, PkV RF01 and HeV RF02 infected the same hosts, but phylogenetic analysis placed them in different groups. Our results reveal large variation among viruses infecting closely related microalgae, and challenge the common conception that algal viruses have narrow host range, and phylogeny reflecting their host affiliation.

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Introduction

Photosynthetic microorganisms in the ocean form the basis of the marine food web, accounting for more than half of the primary production on Earth (Field et al., 1998). Microalgal diversity, abundance and dynamics are to a large extent regulated by physico-chemical factors, but also by grazers and parasites. Viruses are especially important in the demise of algal blooms (Bratbak et al., 1993; Nagasaki et al., 1994; Brussaard, 2004b; Brussaard et al., 2005; Short, 2012), where viral lysis can account for 25–100% of the algal mortality (Short, 2012). Viruses infecting non-blooming algae are also well known (Suttle and Chan, 1995; Larsen et al., 2001), but the ecological significance of these viruses remains enigmatic since it is thought that the host population has to exceed a certain threshold density to sustain a virus population (Wiggins and Alexander, 1985; Murray and Jackson, 1992; Suttle and Chan, 1994; Wommack and Colwell, 2000).

Haptophytes are found worldwide and constitute an important part of the autotrophic pico- and nano-plankton (Thomsen et al., 1994;

Moon-van der Staay et al., 2000, 2001; Liu et al., 2009; Shi et al., 2009). They often appear in low numbers in the ocean and many haptophycean species usually co-occur (Leadbeater, 1972; Estep and Macintyre, 1989). Some, like *Phaeocystis* spp. and *Emiliania huxleyi* (Larsen et al., 2004; Leblanc et al., 2009), form extensive recurrent blooms and viruses infecting these hosts have been thoroughly studied (Jacobsen et al., 1996; Castberg et al., 2002; Wilson et al., 2002; Baudoux and Brussaard, 2005; Wilson et al., 2006). Other haptophytes, e.g. *Haptolina*, *Chrysochromulina* and *Prymnesium*, usually occur at low abundances, but occasionally form blooms and may produce toxins that are harmful to fish and other organisms (reviewed by Edvardsen and Paasche, 1998; Gjosaeter et al., 2000; Dahl et al., 2005). Host-virus associations within these genera have been less well-studied, although viruses infecting *H. brevifila* and *H. ericina* (formerly known as *Chrysochromulina brevifilum* and *Chrysochromulina ericina*) have been isolated (Suttle and Chan, 1995; Sandaa et al., 2001).

So far, viruses infectious to at least 22 different algal species have been isolated (Nagasaki and Bratbak, 2010). The majorities of the algal viruses, and all isolated haptophyte-viruses, have dsDNA genome and belong to the *Mimiviridae* or *Phycodnaviridae* families. Phycodnaviruses are large, algae-infecting, icosahedral viruses with genomes between 160 and 560 kbp (Wilson et al., 2009). The *Mimiviridae* family comprises extremely large viruses such as

Mimivirus, with genome-sizes up to 1.2 Mbp, and more than 900 coding proteins (Raoult et al., 2004; Arslan et al., 2011). Most of these are isolated from freshwater samples on *Acanthamoeba* spp, but *Cafeteria roenbergensis virus* (CroV) (Fischer et al., 2010) and *Megavirus chilensis* (Arslan et al., 2011) have their origin in marine waters. Several metagenome studies have concluded that sequences belonging to *Mimiviridae* are abundant and diverse in the ocean (Ghedin and Claverie, 2005; Larsen et al., 2008; Monier et al., 2008; Claverie et al., 2009; Hingamp et al., 2013).

Mimiviridae belong to the monophyletic virus group NCLDV (Nucleocytoplasmic Large DNA Viruses), together with six other families of viruses infecting eukaryotes (*Phycodnaviridae*, *Poxviridae*, *Asfaviridae*, *Asco-* and *Iridoviridae* and the proposed *Marseilleviridae*). NCLDV is proposed to be reclassified as a new order, the Megavirales, the first order in a putative fourth domain of life, the capsid encoding organisms (Colson et al., 2012). The antiquity of the NCLDV-group together with the exceptional gene repertoire of viruses in this group has led to several hypotheses on their origin, evolution and possible vital role in the evolution of cellular life (Forterre and Prangishvili, 2009; Forterre, 2011).

The few algal viruses so far isolated likely only scratch the surface of the marine viral diversity. It is anticipated that all the ~350,000 marine algal species might have one or more specific virus (Short, 2012), and viral metagenome studies have highlighted the existence of a huge diversity of marine viruses (Angly et al., 2006; Kristensen et al., 2010), with the NCLDV as the second most abundant virus group after bacteriophages (Kristensen et al., 2010). Most sequences revealed from metagenome studies represent uncultured and completely unknown viruses, with 50–90% of the sequences without significant hits in the database (Angly et al., 2006; Kristensen et al., 2010). The diversity found within the *Phycodnaviridae* and *Mimiviridae* family further substantiate this (Chen et al., 1996; Larsen et al., 2008; Park et al., 2011), and underlines the need for culturing new viruses to gain more information about virus-host interactions and the ecological importance of algal viruses. Knowledge of host range, infectious behavior and the effect of viral infections on the host population is needed. In addition new viral isolates will provide valuable information about resistance mechanisms and viral evolution. We have isolated three new viruses infectious to members of the microalgal division Haptophyta, from Raunefjorden, Norway, and characterized these viruses based on morphology, phylogeny, molecular characteristics and infectious behavior.

Results

Isolation and confirmation of lytic viral particles

Among the ten phytoplankton strains used for virus screening, lysis occurred in eight, at several occasions with viral concentrates from Raunefjorden and Oslofjorden (Supplementary Table 1). Three lysates were chosen for purification and characterization. These were: HeV RF02 isolated on *Haptolina ericina* strain UIO028, PkV RF01 isolated on *Prymnesium kappa* strain UIO033, PkV RF02 isolated on *P. kappa* strain UIO034 (Table 1). After dilutions to extinction and CsCl purification, viruses still lysed their specific host cultures, while controls were not lysed, confirming the link between host cells lysis and the specific virus added. This was also confirmed by detection of viral particles by TEM in infected host cells and similar free viruses in purified lysates (Fig. 1).

Morphology and infectious behavior

Morphological observations of free and intracellular (thin sectioned) viruses by TEM show that all viruses are tailless and have icosahedral capsids that are assembled in the cytoplasm

Table 1

Host range of the viral isolates. + culture lysed, − culture not lysed, ND = Not determined.

Species	Strain code	Lysed by		
		HeV RF02	PkV RF01	PkV RF02
Haptolina hirta	UIO031	−	−	−
Haptolina ericina	UIO025	+	+	−
Haptolina ericina	UIO026	+	+	−
Haptolina ericina	UIO027	+	+	−
Haptolina ericina	UIO028	+	+	−
Prymnesium kappa	UIO032	−	−	+
Prymnesium kappa	UIO033	+	+	−
Prymnesium kappa	UIO034	−	−	+
Prymnesium polylepis	UIO036	−	−	−
Prymnesium polylepis	UIO037	−	−	−
Prymnesium polylepis	UIO038	−	−	−
Immantonia rotunda	UIO138	−	−	−
Emiliania huxleyi	UiB	−	−	−
Chrysochromulina leadbeatri	UIO035	−	−	−
Chrysochromulina rotalis	UIO044	−	−	−
Phaeocystis pouchettii	UiB - AJ01	−	−	−
Pyramimonas orientalis	UiB-IFM	−	−	−

(Fig. 1). The HeV RF02-virion appeared slightly elongated with a size of $190 \pm 20 \times 160 \pm 10$ nm (Fig. 1(B)). The PkV RF02-virion was 160 ± 30 nm (Fig. 1(D)) and the size of PkV RF01 was 310 ± 30 nm (Fig. 1(F)). All size measurements are of virus particles inside embedded and thin sectioned cells and the values reported are mean $\pm$ std ($n = 10$).

Sixteen different haptophyte strains together with one *Pyramimonas* strain (Chlorophyta) were infected with the isolated viruses to screen for host range. HeV RF02 and PkV RF01 showed identical host range and lysed all strains of *H. ericina* as well as one of the three *P. kappa* strains tested while PkV RF02 lysed the two other *P. kappa* strains tested (Table 1).

The lytic cycle of the viruses was investigated to determine latent time and burst size (Fig. 2). PkV RF02 and HeV RF02 had the shortest latent periods (12–18 h) and highest burst size (400–775, respectively) of the three viral-isolates (Table 2, Fig. 2(A) and (B)). PkV RF01 had a slightly longer latent period of 24–32 h and a much lower burst size of 80 (Fig. 2(C), Table 2). PkV RF02 lysed all host cells in the culture, while HeV RF02 and PkV RF01 lysed only ~90% of the hosts. The small fraction of cells that survived the virus infection was resistant to lysis in subsequent infection with the same viral strain. The uninfected controls continued to grow throughout the experimental period (Fig. 2) and showed no sign of lysis.

Genome characterization and phylogenetic analysis

HeV RF02 and PkV RF02 had linear dsDNA genomes (result from enzymatic digestion see Supplementary Fig. 1), of 530 kbp and 507 kbp respectively (Fig. 3). The nucleic acid extraction and preparation of PkV RF01 was done in the same way as for the two other viruses. Virus-plugs were prepared multiple times from different lysates and run on PFGEs with settings optimized for small (pulses of 1–8 s), large (pulses of 20–40 s) and very large viruses (pulses of 25–60 s). In spite of several PFGE runs with different settings we were not able to detect the genome of PkV RF01. The reason could be that the virus comprises a circular genome, and it is known that large sized circular forms of DNA do not migrate into pulsed-field gels (Schwartz and Cantor, 1984).

MCP gene from HeV RF02 was amplified using the MCP primers, the other viruses MCP were not amplified by the specific primers. The sequenced MCP-gene from HeV RF02 (KJ558369) was
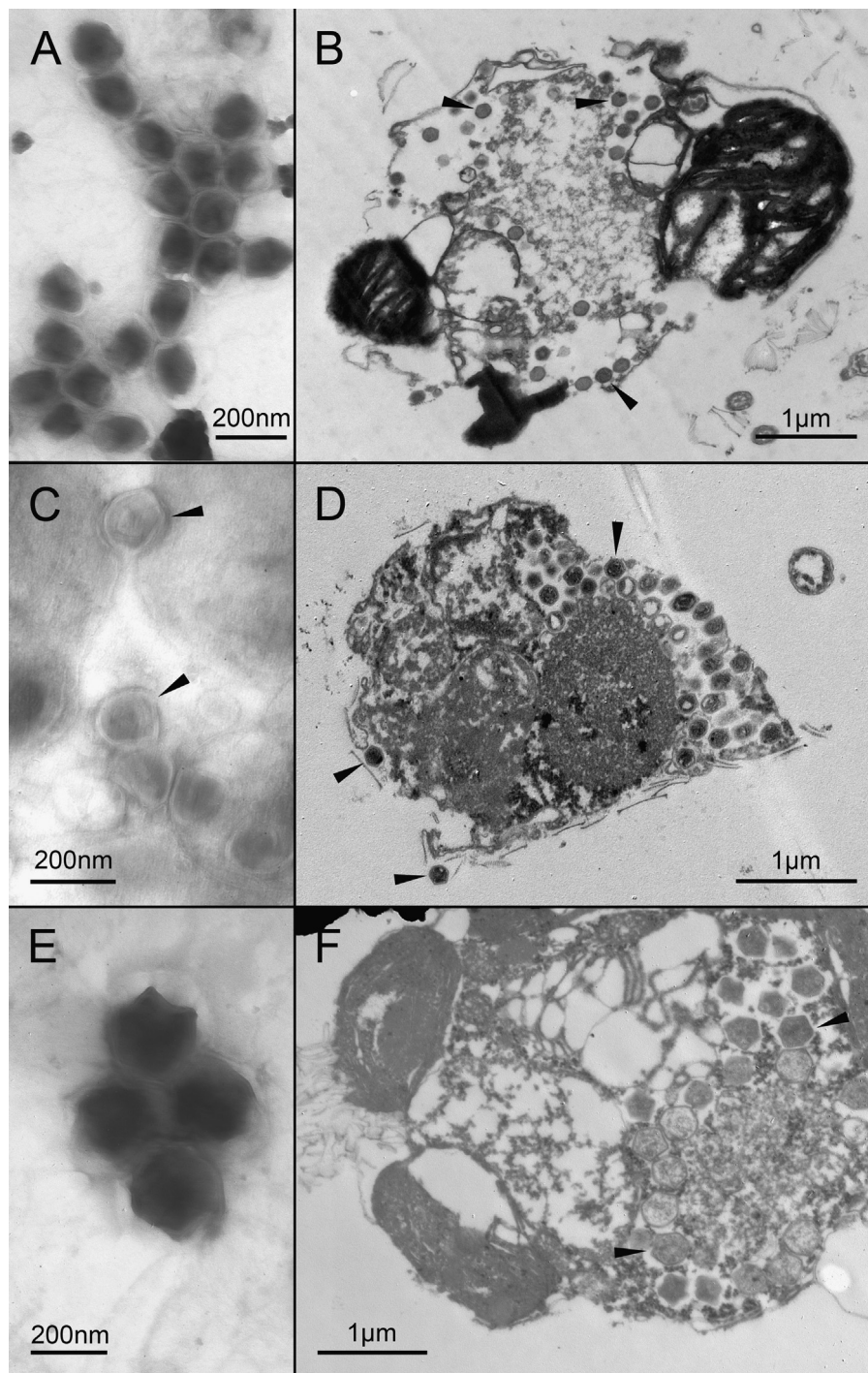
Fig. 1. Transmission electron micrographs of viral lysates (A), (C), and (E) and thin sections showing virus infected cells (B), (D), and (F). HeV RF02 particles in lysate (A) and *H. ericina* UIO028 infected with HeV RF02 (B). PkV RF02 particles in lysate (E) and *P. kappa* UIO034 infected with PkV RF02 (D). PkV RF01 particles in lysate(E) and *P. kappa* UIO033 infected with PkV RF01 (F). Arrows point to the virions.

449 bp. The translated HeV RF02 MCP sequence showed 81% similarity to *Phaeocystis* viruses (PpV01 (A7U6F0) and PgV 12T (AET73005)), and formed a clade with relatively high level of branch support with *Mimiviridae*-related haptophyte viruses in the tree (Fig. 4).

Predicted proteins of PkV RF02 and PkV RF01 included 5 DNA fragments for each virus with significant amino acid (aa) sequence similarity to MCPs and 8 DNA fragments (5 for PkV RF01 and 3 for PkV RF02) with significant aa sequence similarity to DNA polB found in other large viruses. Two of these sequence fragments (PkV

RF02_00023 (1001 bp, KJ558371), PkV RF01_00046 (783 bp, KJ558372)) aligned within the same region as the reference sequences and were used for the phylogenetic analysis. The ORF PkV RF02_00023 showed 92% aa similarity to *Phaeocystis* infecting viruses (*E*-value 0.0) and 76% aa similarity to Organic Lake phycodnavirus 2 (*E*-values of $3e-154$) (ADX06358). The sequence clustered together with the *Mimiviridae*-related algal viruses, with relatively high level of branch support. The ORF PkV RF01_00046 showed 93% aa sequence (*E*-value $2e-42$) similarity to *Pyramimonas orientalis* virus (PoV01B) (A7U6E9) and 82% (*E*-value $9e-38$) to an unknown
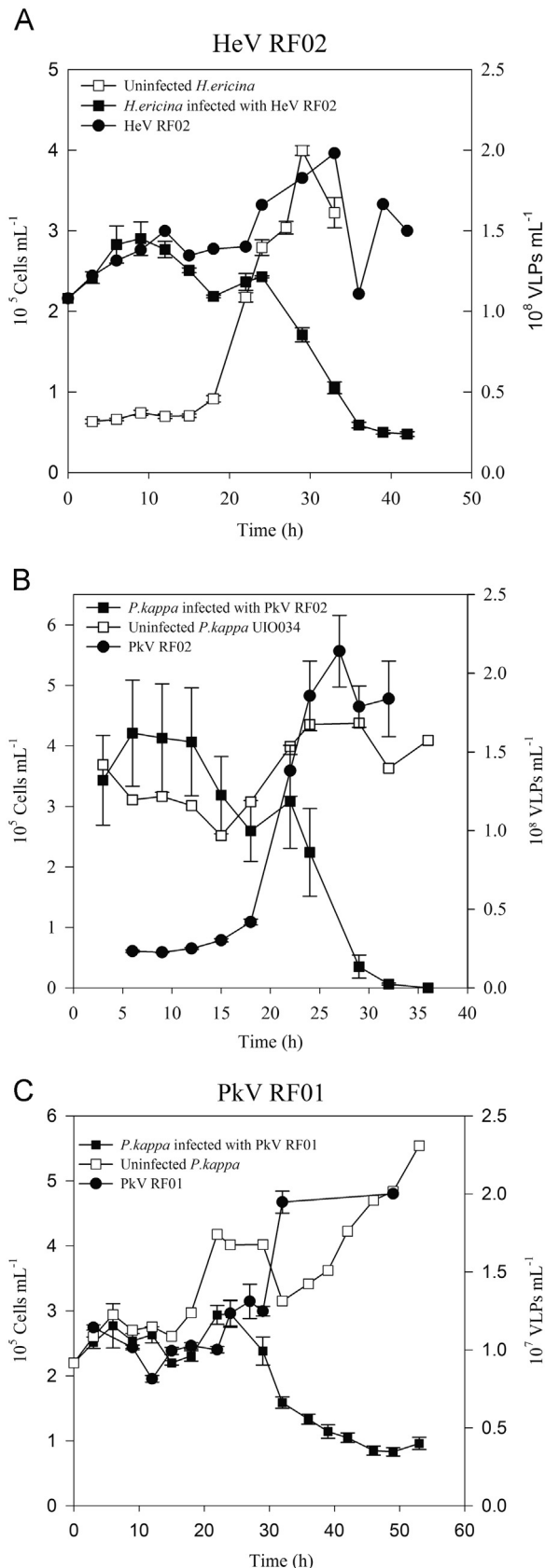
Fig. 2. Viral infection cycle of (A) HeV RF02 propagated on *H. ericina* UIO028, (B) PkV RF02 propagated on *P. kappa* UIO034, (C) PkV RF01 propagated on *P. kappa* UIO033. The graphs show the average number of viral particles (circle) and host-cells (infected cells–filled squares, uninfected cells–open squares) from triplicate experiment. Error bars represent the Standard error of the means across the replicates.

phycodnaviruses (AGI16597). This ORF also clustered with the *Mimiviridae*-related algal viruses, but in another branch together with PoV01B (Fig. 4).

Two of the longest DNA polB ORFs (PkV RF02__00142 (493 bp, KM065803), PkV_RF01__00225 (467 bp, KM065802) aligned in the same region as other DNA polB sequences from NCLDV and was used for phylogenetic comparison. The ORF PkV_RF01__00225 showed 81% aa similarity to *P. orientalis* virus (PoV01B) with an *E*-value of 3e−45. The other ORF, PkV RF02__00142 showed an 85% similarity to both *Phaeocystis globosa* virus (PgV) and *Phaeocystis pouchetii* virus (PpV) with *E*-values of 9e−77 and 3e−76, respectively. Both clustered within the *Mimiviridae* family together with PoV (PkV RF01) and PgV (PkV RF02) (Supplementary Fig. 2), with relatively high level of branch support. However, PkV RF01 and PoV made a separate branch closer to the amoeba-infecting mimiviruses (e. g. *Megavirus chilensis*, *Acanthamoeba castellanii mamavirus*) than PkV RF02 that clustered together with algal mimiviridae-like viruses. DNA pol from HeV RF02 was not accessible as the DNA pol primers (Chen and Suttle, 1995) did not amplify the DNA pol gene in our viruses (data not shown).

The density of the viruses was between 1.315 (PkV RF01) and 1,339 (HeV RF02) (Table 2). The viruses had between 6 and 10 main proteins detectable on the SDS-PAGE gel, with sizes ranging from 14 to 121 kDa (Supplementary Fig. 3).

Discussion

Our three novel viruses all challenge the common conceptions about algal viruses, especially with respect to host range and phylogenetic affiliation but also to size as one of our viruses (PkV RF01) is the largest algal virus particle ever reported. The viruses infect both *P. kappa* and *H. ericina* formerly known as *Chrysochromulina kappa* and *Chrysochromulina ericina* respectively (Edvardsen et al., 2011). These hosts belong to the Haptophyta, which is an important group of single-celled plankton that is ubiquitous in the world's oceans (Liu et al., 2009).
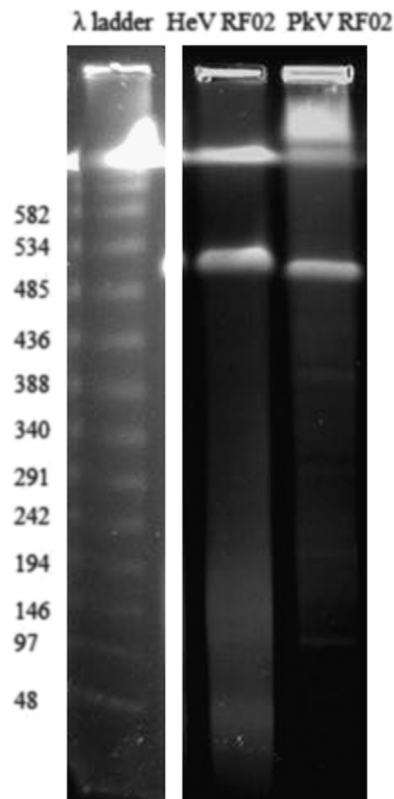
All our three novel viruses show similarities to the nucleocytoplasmic large DNA viruses (NCLDV) (Iyer et al., 2006). The size, icosahedral shape and location of the virions inside the cell, as well as the MCP and DNA polB-phylogenies suggest placement within the *Mimiviridae* family (La Scola et al., 2003) together with other algal viruses like PgV-16 (Santini et al., 2013), PpV (Jacobsen et al., 1996), CeV and PoV (Sandaa et al., 2001). In agreement with our analysis, recent results has shown that a brown tide virus, AaV, also cluster within the *Mimiviridae* family together with other algal viruses like CeV, PgV-16T and Organic Lake Phycodnavirus 1 and 2 (Moniruzzaman et al., 2014). The placements of these algal viruses within the *Mimiviridae* family supports the suggestion of Yutin et al. (2013), that a substantial expansion of this family is needed, also including viruses with much smaller genomes that those of the original member of the *Mimiviridae* (Moniruzzaman et al., 2014).

The morphology, phylogeny, life cycle and genome size of PkV RF02 and HeV RF02 resemble other isolated large dsDNA viruses infectious to microalgae (Sandaa et al., 2001; Castberg et al., 2002; Baudoux and Brussaard, 2005; Sandaa, 2008; Moniruzzaman et al., 2014). The PkV RF01, in contrast, has a capsid diameter of ~310 nm and is thus even larger than the giant *Cafeteria roenbergensis* virus (CroV) which is 300 nm in diameter (Fischer et al., 2010). The large capsid size of PkV RF01 suggest that it contains a larger genome than any of the other cultured algal viruses, which typically have capsids around 200 nm containing genomes between approx. 300 and 500 kbp (Wilson et al., 2009). Phylogeny, based on both MCP and DNApolB, support the placement of PkV

Table 2
Characteristics of the isolated viruses. ND=not determined.

Virus	Sample location	Host	Capsid size (nm)	Genome size (kbp)	Latent period (h)	Burst size mean (range)	Density
HeV RF02	Raunefjorden	*H. ericina* (all tested strains), *P. kappa* UIO033	$190 \pm 20 \times 160 \pm 10$	530	14–18	775 (683–933)	1.339
PkV RF01	Raunefjorden	*H. ericina* (all tested strains), *P. kappa* UIO033	310 ± 30	ND	24–32	80 (34–253)	1.315
PkV RF02	Raunefjorden	*P. kappa* UIO034 and UIO032	160 ± 30	507	12–16	400 (305–471)	1.329

Fig. 3. Viral genome size determined by pulsed-field gel electrophoresis.

RF01 in another group or as a separate branch in the group of mimiviridae-like algal viruses together with the *Pyramimonas orientalis* virus which is the so far largest algal virus described with a capsid of 220×180 nm and a genome of 560 kbp (Sandaa et al., 2001). The phylogenetic difference of PkV RF01 from the other algal viruses in the mimiviridae-like group was most pronounced in the analysis of DNApolB where PkV RF01 and PoV 01 made a new branch within the cluster of CroV (Fischer et al., 2010), *Acanthamoeba polyphaga Mimivirus* (La Scola et al., 2003), *Acanthamoeba castellanii Mamavirus*, *Megavirus chilensis* (Arslan et al., 2011) and *Acanthamoeba polyphaga* Moumouvirus (Yoosuf et al., 2012). This result indicate that PkV RF01 is as divergent from the other mimivirdae-like algal viruses as CroV, and may therefore enhance our understanding on the evolutionary history of giant viruses (Colson et al., 2011; Yutin et al., 2013)

In most cases phytoplankton viruses have demonstrated narrow host range, being species specific or even strain specific (Nagasaki and Bratbak, 2010; Short, 2012), but we found that two of our three isolated viruses were able to infect and lyse different species, even belonging to different genera (i.e. *Haptolina* and *Prymnesium*). Algal
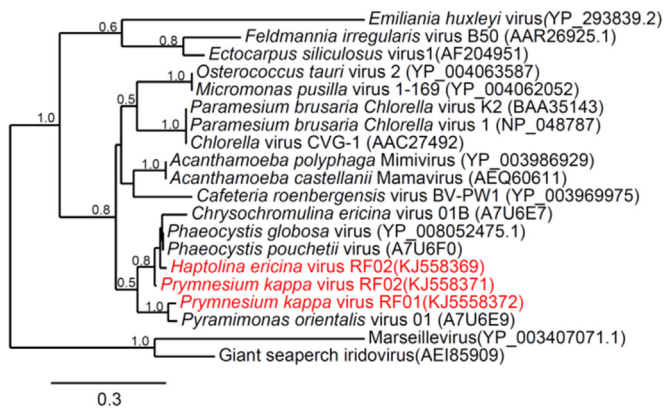
viruses infecting different genera is only known from CbV-PW1 infecting *Haptolina brevifila* (former *Chrysochromulina brevifilum*) and *Chrysochromulina strobilus* (Suttle and Chan, 1995) and from viruses infecting macroscopic brown algae (e.g. EsV, FirrV) (Kapp, 1998). The fact that *Megavirus chilensis* was isolated from a marine sample using a freshwater amoeba as host (Yoosuf et al., 2012) indicate that also this and related viruses are able to infect several host species. Our current results add further evidences that cross-species infectivity is more widespread than previously anticipated, and in the case of viral nomenclature, this generalistic infection behavior challenges the tradition of naming algal viruses based on the name of the host it infects.

The hosts of the viruses described in our study are usually present in low numbers in the ocean or are occasional bloomers, and cross-species infectivity might be an adaptation by viruses to survive with their low-abundance hosts. The ubiquity of viruses infecting low-abundance hosts has been difficult to explain since virus abundance is directly linked to host availability. Other possible explanations for the presence and repeated isolation of viruses infecting low abundance hosts might be an increased resistance to degradation or an ability to chronically or latently infect their hosts, although all microalgal viruses isolated so far are lytic (Short, 2012).

The common conception is that related algal hosts are infected by related algal viruses as reflected by the virus genus names (*Chloroviruses*, *Prasinoviruses*, *Phaeoviruses*, *Prymnesioviruses*, *Coccolithoviruses*, *Raphidoviruses*) (Wilson et al., 2006, 2009; Moreau et al., 2010; Clerissi et al., 2012). The finding that PkV RF01 did not cluster with other Prymnesiales-infecting viruses (HeV RF02, PkV RF02, CeV 01B) and moreover that the MCP fragment of HeV RF02 and PkV RF02 showed only 74% and 77% (NCBI, Blast) homology with CeV-01B, challenge this common assumption. Possible explanations for the phylogenetic relationship reported here, may be recent host switches or perhaps a broader host range than commonly observed.

The large genomes of PkV RF02, HeV RF02 and most likely also PkV RF01 classify these viruses as "giant viruses" (or giruses), which is loosely defined as viruses with genome size larger than 280 kb (Claverie et al., 2006). The discovery of such large viruses has challenged many widely accepted postulates about viruses. Giant viruses have genomes that are larger than the smallest bacterial genomes, and commonly contain genes previously associated exclusively to cellular organisms (such as mismatch repair and genes involved in transcription and translation). Giant viruses even have virophages, viruses parasitizing the viral machinery in the cell. All of these facts together with the sequence information of *Acanthamoeba polyphaga mimivirus* (Raoult et al., 2004) and related viruses (Arslan et al., 2011; Colson et al., 2011; Yoosuf et al., 2012) have revived the rather philosophical debate about viruses being living organisms and whether or not they should be included in the tree of life (Claverie and Abergel, 2010; Forterre, 2010). As a result a fourth domain of life has been proposed comprising all viruses, based on a general division

Fig. 4. Phylogenetic analysis showing the relationship between viruses belonging to the families *Phycodnaviridae* and *Mimiviridae*, based on alignment of a part of MCP. Maximum likelihood and neighbor joining method yielded trees with identical topology. Sequences from viral isolates described here are highlighted in read. Scale bar represents 0.3 substitutions per site.

of the living world into capsid-encoding organisms and ribosome-encoding organisms (Boyer et al., 2010; Colson et al., 2012). This view has been strongly contradicted by other researchers who promote the traditional view of viruses as inert particles, only capable of replication and evolution by cells (Moreira and Lopez-Garcia, 2009; Van Regenmortel, 2010; Williams et al., 2011). Hence the study of these large and ancient viruses, possibly bridging the gap between the living and non-living world, that might have had important roles in the origin of life, has implication that affects existential questions that is of interest to all biologists. The three novel giant viruses described here might shed a new light on this debate.

Conclusions

The three novel viruses characterized here all show some similarities to viruses in the *Mimiviridae* family, but highlight the diversity of viruses infecting algae. PkV RF02 and HeV RF02 have genomes of 507 and 530 kbp respectively, which is similar to the largest algal viruses isolated. The huge capsid of PkV RF01, ~310 nm, makes it the largest algae-infecting virus ever isolated. Our findings suggest that cross-species infectivity might be more widespread among algae infecting viruses than previously reported. Phylogenetic analyses of MCP and DNApolB from the viruses challenge the common conception that there is a strong correlation between host relatedness and virus relatedness. These isolates highlight the importance of virus-isolation in understanding virus-host interactions and the role of viruses in natural communities, as well as for interpretation of metagenome data.

Material and methods

Isolation of viruses

Sea-water samples were obtained at 5 m depth from Raunefjorden (60°16.2'N, 5°12.5'E), 30 km south of Bergen, and from outer Oslofjorden, station OF2 (59°18.7'N, 10°69.2'E), Norway, collected between May 2009 and Aug 2011. Twenty litres of water were filtered through 0.45 μm pore sized low protein binding filter (145 mm, Durapore, Millipore). The virus-containing filtrates were further concentrated to a final volume of approx. 50–100 mL by tangential flow filtration using a QuixStand benchtop system equipped with a 100,000 pore size (NMWC) hollow fiber cartridge (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

The microalgae cultures (Table 1) were obtained from University of Oslo (UiO) and University of Bergen (UiB). The algae were grown in liquid IMR/2 medium (Eppley et al., 1967) made with 70% aged seawater and 30% distilled water (25 PSU) and added selenite (10 nM, final concentration). The cultures were kept at 14 °C, except for *Phaeocystis pouchettii* which was grown at 8 °C. Light was supplied at 16:8 h light: dark cycle and with irradiance of 100 μmol photons m^{-2} s^{-2}, using white fluorescent tubes.

Exponentially growing microalgal cultures (25 mL) were infected with 1 mL of viral concentrate and inspected visually for lysis. Uninfected cultures were used as controls. Lysed algal cultures and controls were checked for viruses by flowcytometry (FCM) (see procedure below). Lysed cultures were centrifuged to remove algal debris and some bacteria (5500 rpm, 15 min). Clonal virus-cultures were obtained by dilution to extinction three times; viral lysate was diluted 10–10^9 times, the lysed culture with highest dilution of virus was used for the next dilution and infection series. Viral particles were further purified by CsCl gradient centrifugation and checked for viruses by FCM and infectivity by infection of their specific host cultures.

Host range, infection cycle and burst size

Host range of the viral isolates was determined by infecting duplicate exponentially growing microalgal cultures (25 mL) with fresh viral lysate. The cultures that had lysed within two weeks were scored positive. Proliferation of viruses in all cultures was checked by FCM (procedure, see below). Infection cycle and burst size were determined in two independent experiments. Triplicate exponentially growing cultures were infected with viruses (virus: algae ratio of > 50), an equal amount of boiled (inactivated) virus lysate or fresh medium was added to the control cultures. Samples were taken every third hour for 36 h, and then every day until lysis was observed. Latent period was defined as the time from addition of viral lysate until the number of free viruses started to increase.

Counts of algae and viral particles were carried out by FCM (FACSCalibur Becton and Dickinson), according to Marie et al. (1999) and Brussaard (2004a). Algae were counted in fresh unpreserved samples with the trigger set on red fluorescence and using a flow rate giving 50–800 events/sec. For virus enumeration the samples were fixed in 1% glutaraldehyde for 30 min at 4 °C and snap frozen in liquid N$_2$. The samples were thawed immediately before counting, diluted and stained with 1x SYBR green I (Invitrogen) for 10 min at 80 °C. The samples were analyzed for 60 s with the trigger set on green fluorescence and using a flow rate giving 50–800 events/min.

Morphology

Fresh viral lysates from purified CsCl viral particles were harvested onto electron microscope grids by ultracentrifugation and stained with uranyl acetate as described in Bratbak and Heldal (1993). In addition, samples for thin sectioning of infected algae were fixed in 2.5% glutaraldehyde. Briefly, the fixed microalgae were pelleted by centrifugation (13,000 rpm, 3–5 min) and washed in 0.1 M Na-cacodylate-buffer (three times 15 min). Cells were post fixed in 1% OsO$_4$ for 60 min and washed in 0.1 M Na-cacodylate-buffer (2 times 10 min), followed by dehydration in increasing concentration of ethanol (30%, 50%, 70%, 96%, 100%), and thereafter in 100% propylene oxide. The cells were embedded in Agar 100 Resin (Agar Scientific, Essex, UK). Embedding, staining and thin sectioning were done at Molecular Imaging Center at UiB. Transmission electron microscopy was performed on a JEOL JEM 1100 80 kV. Ten virus particles from two fields of vision were measured to estimate the average size of the viral particles.

Genome characterization and phylogenetic analysis

Genome sizes of the viruses were determined by pulsed field gel electrophoresis (PFGE) as described earlier (Wommack et al., 1999; Sandaa et al., 2001a). In short, the viral lysates were pelleted by ultracentrifugation (25,000 rpm in Beckman optima L90K ultracentrifuge). The pellets were resuspended in SM-buffer (0.1 M NaCl, 8 mM $MgSO_4 \times 7H_2O$, 50 mM Tris–HCl, 0.005% Glycerin) (Wommack et al., 1999) and mixed 1:1 with agarose (1.5%, InCert, FMC Bioproducts) to make virus plugs. The plugs were treated with lysis buffer (1 mg/mL proteinase K, 1% SDS, 250 mM EDTA, pH 8.0) o.n. at 30 °C and thereafter washed three times (30 min each) in TE 10:1 (10 mM Tris-Base, 1 mM EDTA, pH 8.0) and kept in TE 20:50 (20 mM Tris Base 50 mM EDTA, pH8.0) at 4 °C until use. The plugs were loaded on 1% agarose gels (Seakem GTG agarose) together with DNA size standards (CHEF lambda ladder and 5 kb ladder (DNA size standard, Bio-Rad)). The gels were run in $0.5 \times$ TBE buffer ($1 \times$ TBE: 89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0), at 6 V for 22 h at 14 °C, with pulses of 1–8 s (separating genomes from 5–200 kbp), 20–40 s (separating genomes from 50–600 kbp) and 25–60 s (separating genomes up to 1 Mbp). The gels were stained in 1x SYBR green I (Invitrogen) for 45 min and washed for 15 min in 1xTBE. Gels were visualized in a Bio-Rad ChemiDoc system, and analyzed by the software Image-Lab3 (Bio-Rad).

The viral genomes were subjected to enzymatic digestion to determine the nature of the viral genomes (double- or single-stranded, DNA or RNA). For DNA digestion, the virus-plugs, prepared as for PFGE, were submerged in reaction buffer (20 mM Tris–HCl pH 8.3 with 2 mM $MgCl_2$) for 4 h (4 °C). The reaction buffer was removed and buffer containing 0.5 µl DNase (15 mg/mL, 20.1 units/µg, Sigma) was added. DNA was digested for 18 h at room temperature. To digest both single stranded and double stranded RNA, the virus-plugs were placed in 0.01x SSC buffer (1xSSC: 0.15 M NaCl, 0.015 M Sodium citrate, pH 7.0) for 4 h. The buffer was exchanged with buffer containing 3 U RNaseA (Sigma) and the RNA was digested overnight at 37 °C. To digest ssRNA (and not dsRNA), the above procedure was carried out under high salt conditions, i.e. 2x SSC-buffer. All plugs were run on PFGE as described above. Control plugs without enzyme was included for all conditions.

Primers developed by Larsen et al. (2008) were used to amplify and sequence part of the gene encoding major capsid protein (MCP) (MCPforward: 5′-GGY GGY CAR CGY ATT GA-3′ and MCPreverse: 5′-TGI ARY TGY TCR AYI AGG TA-3′). The PCR reactions contained: 0.625 U HotStarTaq DNA polymerase (Qiagen), 1x PCR buffer, 0.2 mM of each dNTP, 0.5 µM of each of the primers and 1 µL template in a reaction volume of 25 µL. Viral lysate that had been heated (90 °C) for 22 min was used as template. The following PCR-program was used: initial activation at 95 °C (15 min), a touchdown PCR of 20 cycles: denaturation at 94 °C (30 s), annealing at 60 °C and decreasing 0.5 °C per round (30 s) and extension at 72 °C (30 s), followed by 35 cycles with fixed annealing temperature at 45 °C, and a final elongation of 72 °C for 7 min. The PCR products and ladder (Mass ruler™DNA ladder, Fermentas) were electrophoresed in 1xTAE on agarose gels (1.5%) at 200 V for 20 min. The gels were stained with EtBr and visualized in ChemiDoc (Bio-Rad). The amplified fragments were sequenced with Big Dye v3.1 at the sequencing facility at UiB.

Phylogeny was analyzed from translated partial MCP sequences, amplified by PCR (HeV RF02) and from partial genome sequencing by Solid (PkV RF01, PkV RF02). Alignments of the sequenced DNA fragments together with MCP and DNA polB sequences from cultured mimi- and phycodna-viruses were performed using ClustalW (v2.0.3) Larkin et al. 2007). Further, phylogenetic relationships of MCP and DNA polB of the selected sequence fragments, PCR sequences and references were inferred from multiple alignments using ClustalW (v2.0.3) alignment tool (Larkin et al., 2007) hosted at http://phylogeny.fr (Dereeper et al., 2008, 2010). Further alignment refinement of MCP was conducted by GBlocks 0,91b (Castresana, 2000), and phylogeny was analyzed using the BioNJ/Neighbor (PHYLIP) 3.66 program with 1000 bootstraps (Felsenstein, 1989; Elias and Lagergren, 2007). Trees were edited using the TreeDyn program (Chevenet et al., 2006). DNA polB protein sequences were analyzed using the maximum-likelihood method and phylogenetic trees were constructed with the PhyML 3.0 program (Anisimova and Gascuel, 2006; Guindon et al., 2010). Supports for clades were estimated by the approximate likelihood-ratio test with SH-like settings. The MCP and DNA polB sequences from this study have been deposited in the GenBank database (accession no KJ558369, KJ558371, KJ558372 for MCP and KJ55837, KJ558372 for DNA polB).

Acknowledgments

Appendix A.   Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.014.

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