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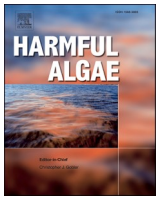

Discovery of novel and known viruses associated with toxigenic and non-toxigenic bloom forming diatoms from the Northern Adriatic Sea

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ABSTRACT

Algal blooms impact trophic interactions, community structure and element fluxes. Despite playing an important role in the demise of phytoplankton blooms, only few viruses infecting diatoms have been cultured. *Pseudo-nitzschia* is a widespread diatom genus that commonly blooms in coastal waters and contains toxin-producing species. This study describes the characterization of a novel virus infecting the toxigenic species *Pseudo-nitzschia galaxiae* isolated from the northern Adriatic Sea. The ssRNA virus PnGalRNAV has 29.5 nm ± 1.2 nm icosahedral virions and a genome size of 8.8 kb. It belongs to the picorna-like *Marnaviridae* family and shows high specificity for *P. galaxiae* infecting two genetically and morphologically distinct strains. We found two genetically distinct types of this virus and screening of the global virome database revealed matching sequences from the Mediterranean region and China, suggesting its global distribution. Another virus of similar shape and size infecting *Pseudo-nitzschia calliantha* was found, but its genome could not be determined. In addition, we have obtained and characterized a new virus that infects *Chaetoceros tenuissimus*. The replicase protein of this virus is very similar to the previously described ChTenDNAV type-II virus, but it has a unique genome and infection pattern. Our study is an important contribution to the collective diatom virus culture collection and will allow further investigation into how these viruses control diatom bloom termination, carbon export and toxin release in the case of *Pseudo-nitzschia*.

1. Introduction

Diatoms are an important group of photoautotrophic organisms known for dense blooms (especially in coastal seas) and are responsible for up to 40 % of marine primary production (Nelson et al., 1995; Armbrust, 2009) although recent estimates put this value closer to 25 % (Behrenfeld et al., 2021). Diatoms primarily sustain the coastal pelagic food web, while their relatively heavy silica frustules aid in the export of organic carbon from the euphotic zone to the deep sea (Tréguer et al., 2018). Despite their important ecological role, our knowledge of diatom viral ecology is far from complete. Viruses are key drivers of biodiversity and global biogeochemical fluxes (Brussaard et al., 2008; Brockhurst et al., 2014; Laber et al., 2018; Kranzler et al., 2021). They are responsible for significant phytoplankton mortality (Bratbak et al., 1993; Mojica et al., 2016; Biggs et al., 2021) and are thought to regulate the decline of phytoplankton blooms (Bratbak et al., 1993; Baudoux et al., 2006; Brussaard et al., 2007; Sheik et al., 2013; Kranzler et al., 2019). Viral lysis of host cells results in the flow of particulate organic matter being diverted from higher trophic levels towards the microbial loop (Lønborg et al., 2013), reducing the efficiency of trophic transfer and the biological organic carbon pump (Brussaard et al., 2008). At the same time, viruses infecting phytoplankton, including diatoms, are thought to contribute to the formation of aggregates of their lysed host cells, thereby promoting sedimentation of the cells and thus increasing carbon export to the oceans (Laber et al., 2018; Nissimov et al., 2018; Yamada et al., 2018).

With the increasing availability of high-throughput sequencing (HTS) technologies and data mining approaches, novel algal viruses,

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including diatom-associated viruses, are being discovered (Charon et al., 2020, 2021). Nevertheless, despite the large number of diatom species in the marine environment (Guiry, 2012; Malviya et al., 2016), the extensive knowledge of diatom viral ecology comes mostly from just a few virus systems. Both centric and pennate diatom viruses have been isolated, but the majority of known diatom viruses infect *Chaetoceros* species (Bettarel et al., 2005; Nagasaki, 2008; Tomaru et al., 2008, 2009, 2011a; Tomaru et al., 2011b; Kimura and Tomaru, 2013, 2015) and most were isolated in Japanese waters. Other diatom genera infected with viruses brought into culture are *Rhizosolenia* (Nagasaki et al., 2004), *Guinardia* (Arsenieff et al., 2019), *Nitzschia* (Toyoda et al., 2019), *Haslea* (Gastineau et al., 2020) and *Thalassionema* (Tomaru et al., 2012). Apart from the *Chaetoceros* viruses, little or no further research has been done on the ecological role and impact of these viruses.

Characterization of new diatom viruses from new sites is desirable to better understand the diversity of these viruses and their impact on diatom physiology, population dynamics, and carbon flux. The northern Adriatic Sea and the Gulf of Trieste (GoT) are among the most thoroughly studied coastal regions in the world. Several long-term ecological monitoring stations provide data series documenting the ever-changing and transitional nature of this area (Brush et al., 2021). These data range from phytoplankton (Mozetič et al., 2012; Vascotto et al., 2021), harmful algal blooms (Mozetič et al., 2019; Francé et al., 2021), carbon and nutrient regimes (Brush et al., 2020), oceanographic and climatic conditions (Boicourt et al., 2020), to invasive species and critical habitats (Palinkas et al., 2020) and even heavy metal pollution such as mercury (Faganeli et al., 2003). However, little is known about the microalgal viruses and their interaction in this region, as studies have only investigated the effects of viruses on bacterial mortality (Bongiorni et al., 2005; Karuza et al., 2010).

Chaetoceros and *Pseudo-nitzschia* are two bloom-forming and ecologically important planktonic diatom genera (Leblanc et al., 2012; Trainer et al., 2012; Guidi et al., 2016; De Luca et al., 2019), with *Pseudo-nitzschia* also being one of the few diatom genera that are toxigenic (Bates et al., 2018). Certain species of this genus can produce the neurotoxin domoic acid, which is responsible for harmful algal blooms that can cause amnesic shellfish poisoning (ASP) (Bates et al., 1989; McCabe et al., 2016). In the GoT, where the present study was conducted, *Pseudo-nitzschia multistriata* H. Takano and *Pseudo-nitzschia galaxiae* Lundholm & Moestrup were found to be toxic (Turk Dermastia et al., 2022), while the wider Adriatic region harbours many other toxic *Pseudo-nitzschia* species, of which the most commonly reported is *Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle (Marić et al., 2011; Arapov et al., 2020b). While the amount of research on *Pseudo-nitzschia* is growing, little is known about its viruses. One study, conducted in the coasts of Washington State (USA), reported the infectivity of various environmental viral concentrates to *Pseudo-nitzschia* strains and suggested that the role of viruses in bloom regulation was highly relevant and of great importance (Carlson et al., 2016). Furthermore, Kranzler et al. (2019, 2021) investigated the diversity and abundance of cell-associated diatom viruses in the light of diatom ecophysiological states and showed that viral lysis strongly influences diatom abundance (including *Pseudo-nitzschia* species) under natural conditions. Here, we report the discovery and characterization of novel viruses infecting the toxigenic bloom-forming diatom species *Pseudo-nitzschia calliantha* (Marić et al., 2011; Arapov et al., 2020b) and *Pseudo-nitzschia galaxiae* (Turk Dermastia et al., 2022, 2023), and viruses infecting the non-toxigenic bloom-forming *Chaetoceros tenuissimus*.

2. Methods

2.1. Diatom cultures and environmental concentrates

Diatom cultures were isolated from phytoplankton net tows carried out during this study around the time of virus sampling or were kindly provided by the Institute of Oceanography and Geophysics (OGS) in Trieste. Cultures were grown from single cells or chains isolated by capillary flow with glass pipettes in combination with light microscopy. The cultures were taxonomically identified by light microscopy and sequencing of the *rbcL* marker gene conducted as in Turk Dermastia et al. (2020). A list of strains used in this study can be found in Supplementary Table 1. For some of the strains GenBank accession numbers are also provided. Cultures were maintained in 150 ml Erlenmeyer flasks filled with 30 ml L1 medium (Guillard and Hargraves, 1993) at 100 µmol photons m^{-2} s^{-1} under a 12:12 day:night cycle and at 18 °C. The cultures were carefully mixed every day. Every two weeks, 1 ml of the cultures was transferred to 30 ml of fresh medium.

Seawater (15 L) for virus concentration was collected in the GoT near the Slovenian long-term ecological research (LTER) site 00BF (45.5403 N, 13.557 E) from the chlorophyll-a (Chl-a) max layer (about 15 m) determined by CTD during three cruises on September 7, 2021, August 30, 2022, and August 17, 2023. It was filtered through GF/F (Whatman®) filters to remove large cells, followed by 0.22 µm filtration to remove bacteria. The volume of the filtrate was then concentrated approximately 100–300x using two parallel 30 kDA polyethersulfone (PES) tangential flow membranes (VivaFlow, Sartorius). The resulting concentrate was then further reduced to a final concentration of 1200x using Pierce™ protein concentrators (20 mL, 30 kDA, Thermo Scientific). An aliquot was stored at -80 °C and the remainder at 4 °C for immediate use.

2.2. Concentrate infectivity, virus isolation and host specificity

The concentrates from September 2021 (Conc-Sept21), August 22 (Conc-Aug22), and August 23 (Conc-Aug23) were used to infect different strains and species of diatoms (Table 1). Concentrates were added to exponentially growing diatom cultures in 24-well microplates (Nunc™, Thermo Scientific) at a ratio of 10:1 v/v diatom host to virus concentrate. Six control wells consisted of the diatom culture amended with sterile L1 medium at the same ratio, while the remaining 18 wells were inoculated with the concentrates. Lysis was monitored daily for up to two weeks using a multimode fluorometer (Spark, Tecan) to measure Chl-a autofluorescence. A decrease in the signal (clearing of the culture) indicated lysis. Cleared wells containing lysed diatom cultures were filtered through a sterile 33 mm 0.22 µm filter (Milex, Millipore) to obtain bacteria free lysate filtrates. When multiple wells containing the same diatom cultures were cleared, the lysates from these wells were filtered together. A portion of the lysate filtrate was used for a second round of infection, at a ratio of 10:1 v/v diatom host to filtered lysate using a fresh host culture in a 12-well microplate format (4 control wells, 4 sterilized lysate wells and 4 lysate wells). Another part of the lysate filtrate was sterilized with UV (Sylvania G30 W T8, UV-C) for 30 min under a laminar hood in an open 2 ml microtube. As an additional control (volume of the obtained filtered lysate permitting), 1 ml of the generated lysate filtrate was filtered through a 0.022 µm filter (Anodisc, Millipore) to remove potential viruses and added to fresh host cultures. This process was repeated up to five times to establish the transfer of infectivity between subsequent rounds of infection (we did not perform extinction-dilution isolations at this point). The absence of lysis with the treated lysate filtrates and the presence of lysis with the untreated lysate filtrates indicated that the infectious agent was a virus. The lysate filtrates containing potential viruses were stored at 4 °C and at −80 °C in the dark in native filtered medium. The infectivity of the lysate filtrates was tested again after several months by bringing a fresh healthy culture into contact with the stored lysate filtrates.

Lysate filtrates obtained from certain diatom strains (LysPn109, LysPn201, LysChV, LysP2D4, Table 1) were tested on strains intended for cross-host infectivity tests (Table 1). The selection of these strains was based on their viability in culture. The procedure was performed as described above, but in addition to the negative control wells treated with medium only, positive control wells were established, by inoculating the diatom strain from which the virus was originally isolated

Table 1
Details of strain infections, cross infections and processed lysates.

Strain	Species	Concentrate test[1]	Lysis in concentrate[2]	Lysis in filtered lysate[2]	Lysis in UV treated lysate[2]	RNA/DNA extracted	Sequencing	Filtered lysate ID[3]	Host specificity test (Filtered lysate that caused lysis)[4]
DIAVIR2	*P. calliantha*	Conc-Sept21	x	x		Yes	Failed	LysDIA2	No
DIAVIR6	*P. calliantha*	Conc-Sept21				No			No
DIAVIR10	*P. calliantha*	Conc-Sept21	x			No			No
DIAVIR11	*P. calliantha*	Conc-Sept21				No			No
DIAVIR13	*P.calliantha*	Conc-Sept21				No			No
DIAVIR14	*P. calliantha*	Conc-Sept21				No			No
DIAVIR7	*P. delicatissima*	Conc-Sept21				No			No
DIAVIR9	*P. manni*	Conc-Sept21	x			No			No
DIAVIR12	*P. manni*	Conc-Sept21	x			No			No
DIAVIR12	*P.manni*	Conc-Aug22				No			No
DIAVIR14	*P. calliantha*	Conc-Aug22	x	x		Yes	Failed	LysDia14	No
141,222	*P. calliantha*	Conc-Aug22				No			No
Pn109	*P. galaxiae*	Conc-Aug22	x	x		Yes	Successful	LysPn109	Yes (LysPn201)
Pn201	*P. galaxiae*	Conc-Aug22	x	x		Yes	Successful	LysPn201	Yes (LysPn109)
ChTen-Got	*C. tenuissimus*	Conc-Aug22	x	x		Yes	Successful	LysChV	No
Pn202	*P.calliantha*	Conc-Aug22				No			Yes (NEG)
Pn205	*P.calliantha*	Conc-Aug22				No			Yes (NEG)
Pn206	*P. calliantha*	Conc-Aug22				No			Yes (NEG)
Pn207	*P.calliantha*	Conc-Aug22				No			Yes (NEG)
Pn101	*P. delicatissima*	Conc-Aug22				No			Yes (NEG)
Pn102	*P. delicatissima*	Conc-Aug22				No			Yes (NEG)
Pn204	*P. delicatissima*	Conc-Aug22				No			Yes (NEG)
Pn208	*P. galaxiae*	Conc-Aug22	x	x		No			Yes (LysPn201, LysPn109)
P2D4	*P. galaxiae*	Conc-Aug23	x	x		Yes	Successful	LysP2D4	Yes (NEG)
Chsoc-Got	*C. socialis*					No			Yes (LysChV)

[1] Concentrate test column indicates the environmental concentrate to which the strain was exposed.
[2] An x in these columns indicates culture lysis took place.
[3] Filtered lysate ID indicates the name of the lysate containing viruses that was used in subsequent analyses.
[4] Host specificity test indicates whether host specificity experiments have been conducted and to which Filtered lysate the tested strain was susceptible.

with the lysate filtrate being tested.

2.3. Transmission electron microscopy (TEM)

The presence and morphology of virus particles in lysate filtrates (LysDIA2, LysPn109, LysPn201, LysChV) obtained by filtration (0.22 μm filter) was determined by TEM using a negative staining method. A small drop (25 μL) was applied to freshly glow discharged Formvar coated (Agar Scientific, Stansted, UK) carbon stabilized copper grids for 5 min. The grids were washed with a few drops of deionized water before negative staining with a 1 % (w/v) aqueous uranyl acetate solution (SPI Supplies, West Chester, PA, USA). All samples were analyzed with the TALOS L120C TEM (Thermo Fischer Scientific, Waltham, MA, USA) at 100 kV, and micrographs were captured with the Ceta 16 M CCD camera using Velox software (Thermo Fischer Scientific, Waltham, MA, USA) for micrograph acquisition and processing. A selected number of virus particles (n) were measured to obtain the average width and standard deviation. Thin-section microscopy was attempted but did not yield satisfactory results.

2.4. Nucleic acid extraction and virome analysis

Both DNA and RNA viruses were targeted, as they are both known to infect diatoms (Kimura and Tomaru, 2015; Arsenieff et al., 2022). Total nucleic acids were thus extracted from the filtered lysates using the QiaAMP MinElute Virus Spin Kit (Qiagen, Hilden, Germany). DNA viruses also produce RNA molecules in their infection process, and sequencing total RNA is thus frequently employed to detect and discover both RNA and DNA viruses. We checked RNA concentrations with the Qubit RNA HS Assay (Thermo Fisher Scientific) and the integrity with the 2100 Bioanalyzer (Agilent). The detected amount of RNA was largely below detection limits, so a random pre-amplification step was required to increase the concentration of nucleic acids. Preamplification was performed according to the protocol of Fernandez-Cassi et al. (2018) with slight modifications (Bačnik et al., 2020). The average fragment size after preamplification was between 500 and 1000 bp, measured on the TapeStation system (Agilent). We sent the pre-amplification products to Novogene (UK) where sequencing libraries were prepared from at least 200 ng of cDNA per sample using the TruSeq RNA sample preparation kit (Illumina, USA). Paired-end read sequencing (2 × 150 bp) was then performed using Illumina Novaseq (Novogene, UK). Negative isolation control was spiked with luciferase RNA (Promega, WI, USA) and also sent for sequencing to control contamination during the isolation process and crosstalk of samples during sequencing. The residues of the sequencing adapters and primer sequences were trimmed, and the resulting reads were checked for quality using the CLC Genomics WorkBench 23 (Qiagen, Hilden, Germany). *De novo* assembly of reads was performed using metaSPAdes 3.15.5 (Nurk et al., 2017). The *de novo* created contigs were queried for similarity with a complete NCBI database (non-redundant, accessed on July 16, 2022) using DIAMOND BLASTx (Buchfink et al., 2021). Viral contigs that we identified as potentially novel and related to the main lysing agent in the diatom lysates were initially classified as either DNA or RNA viruses. For the RNA viruses, large contigs assigned to *Riboviria* (Huson et al., 2016) were extracted and further assembled in CLC Genomics Workbench to generate whole or near whole genomes. Sequencing reads were then mapped to consensus genomes (similarity fraction = 0.9, length fraction = 0.9) to assess read abundance associated with the identified viruses using the "Map reads to reference" tool in CLC. SNP calling was performed in CLC using the "Low frequency variant detection" option with default parameters. For SNP analysis new read mappings were produced as reads were additionally trimmed by inspecting the mismatch fraction plots that show more mismatches in the 5′ and 3′ ends of the reads (Kutnjak et al., 2015). SNP profiles from different assemblies were compared to assess their concordance and allele frequency correlation. Because different assemblies had different

percentages of reads mapped to the reference genome, the reads were normalized so that the mapped read number was comparable. The concordance rate was computed as the ratio of shared SNPs to the total number of SNPs, considering the assembly with the fewer SNPs as the denominator. To quantify the similarity in allele frequencies of shared SNPs, the Pearson correlation coefficient was calculated.

For the DNA viruses, the contigs associated with the *Bacilladnaviridae* family according to DIAMOND blastX were selected for further analysis. Again, an additional assembly step was required to construct the complete circular genome of the ChTenDNAV type-II virus. A PCR assay was developed to determine the correct sequence that closed the circular genome. The primers designed were Cten_circF (GCTTCAGGATGACATAA GCACC) and CTen_circR (ACGAGGTTTCGACTCAA TGTA). AllTaq polymerase (Qiagen, Hilden) was used to amplify the products. The manufacturer's protocol was used and the Q solution was included in the master mix. Tm was 55 °C. Sanger sequencing was performed by Macrogen Inc.

2.5. Viruses in publicly available datasets

Using the palmprint sequence of the new RNA viruses, we searched the Serratus RNA viral palmprint database (Edgar et al., 2022) using the palmID tool (Babaian and Edgar, 2022) for similar or nearly identical sequences previously found in metatranscriptomic datasets (Supplementary Table 2). The SRA accessions (ERR1712137, SRR10482238, SRR10482249, SRR10482241, SRR10482235, SRR10800990) associated with the identified similar sequences in Serratus were downloaded (Supplementary Table 2). They were then *de novo* assembled with rnaviralSPAdes 3.15.5 (Meleshko et al., 2021) and DIAMOND blastx against the sequence of the newly identified PnGalRNAV sequences was used to identify highly similar contigs assembled from SRA datasets. The longest contig obtained was also included in the final phylogenetic analyses.

2.6. Phylogenetic analysis of the obtained viral genomes

For the RNA virus RdRp amino acid sequences, the most similar homologous reference RdRP amino acid sequences were retrieved from the National Center for Biotechnology Information (NCBI) using a blastp search against the NCBI nr database (June 2023) and used in the phylogenetic analysis along with the sequences used in Kranzler et al. (2019) and Shi et al. (2016), as well as the contigs obtained through SRA search.

The RdRp amino acid sequences were aligned using the l-INS-I algorithm of the MAFFT program (Katoh and Standley, 2013) and the alignments were trimmed manually and using the TrimAl automated 1 mode (Capella-Gutierrez et al., 2009). A maximum likelihood tree was constructed from the reference alignment (989 amino acids) using IQTree version 1.6.12 (Nguyen et al., 2015). The Le Gascuel (LG+$F+I+$G4) amino acid replacement model selected by IQTree ModelFinder was used, with 1000 bootstrap repeats. The trees were visualized in iTOL version 6.8.1 (Letunic and Bork, 2021).

For the DNA virus, the DNA polymerase sequences were used for phylogenetic analysis. The sequences were aligned and trimmed as described above. The evolutionary models were predicted using modeltestNG (Darriba et al., 2020). A maximum likelihood tree was created from the reference alignment using RAxML v. 8.2.12 (Stamatakis, 2014). The trees were visualized in FigTree v. 1.4.3. Representative sequences of viruses within this family were extracted from NCBI based on accessions included in the recently published taxonomy by Varsani and Krupovic (2022), which proposes a threshold of 75 % amino acid identity for the delimitation of different viral species within the *Bacilladnaviridae*. Also based on this taxonomy proposal, members of the family *Circoviridae* were selected as an outgroup because they belong to a different group within the phylum *Cressdnaviricota*. For pairwise sequence comparison with other *Chaetoceros tenuissimus* viruses, we used the SDT software (Muhire et al., 2014).

2.7. Replication monitoring of PnGalRNAV with RT-qPCR

To quantitatively monitor the replication of PnGalRNAV type-I, we developed a TaqMan RT-qPCR assay following MIQE guidelines (Bustin et al., 2009) based on the genomes assembled from LysPn201 and LysPn109. The assay was designed using PrimerExpress 2.0 software (Applied Biosystems) and consisted of a FAM-TAMRA probe (FAM'-TGGCGTGGAGGCATTCGATGG-TAMRA), forward primer (5′-ACATTGGGTAACATATGCATTTGC-3′) and reverse primer (5′-CATTATGGGATCCATTGGGTGTA-3′) to produce an amplicon of 77bp. The primers target the capsid protein of the novel virus (positions 7962–8016, GenBank: OR271362.1). Details of the assay design and validation are supplied in Supplementary Method 1.

To indirectly assess the infectivity of the discovered virus we quantified the genomic replication of the virus in the host culture over time. A non-axenic culture of *Pseudo-nitzschia galaxiae* (isolate Pn208, *rbcL* accession number OR482970) was grown in a 10-liter Duran® flask containing 8 L of L1 medium. When the culture reached exponential phase of growth, determined by cell counts on a Fuchs-Rosenthal counting chamber, the culture was distributed into six 1-liter Erlenmeyer flasks. Three of the 1-liter flasks were designated as controls and three were designated as treatments. Into the control vessels we added 30 µl of sterile medium. Into the treatment vessels we added 30 µl of a preharvested viral lysate of the PnGalRNAV obtained through previous infection cycles. The vessels were kept in a thermostatic chamber at 18 °C on a shaker (400 rpm) and a 12:12 hour light:dark cycle with a light intensity of ~100 µmol photons m^{-2} s^{-1}.The batch experiment was left to run for five days. An additional sampling step was performed after 9 days of incubation. 1 ml of the culture in duplicate was collected daily from each flask and filtered through sterile 0.22 µm Millex filters (Millipore, Merck). The filtrates, now technically void of bacteria and diatoms, were stored at −80 °C until RNA extraction with the QIAamp MinElute Virus Spin Kit. Virus RNA extracted from the pre-harvested viral lysate was used as positive RT-qPCR control and to generate a standard curve by analysis of 10-fold serial dilutions in pentaplicate (see Suplementary Methods - Fig. 1). Samples were applied in triplicate both undiluted and 10-times diluted. Both samples and standards were applied to one step RT-qPCR reaction in a Quant Studio 7 Pro qPCR system using FAST virus 1-step master mix (Thermo Scientific) with cycling conditions as recommended by the kit manufacturer. Negative controls of RNA isolation (NCI) and Negative controls of the qPCR reaction (NTC) were applied alongside the samples and standards. The threshold of amplification was manually set to 0.29 and quantification cycles (Cqs) were obtained using the Design and analysis 2.5.1 software (Thermo Scientific). The quantification of theoretical viral genome copy number in the samples was performed using the regression equation obtained from the standard curve, by assigning a theoretical concentration of 1 copy/µl to the dilution resulting in the highest Cq. (see Suplementary Methods, Fig. 1)

3. Results and discussion

3.1. Putative pseudo-nitzschia calliantha virus

We have successfully propagated a virus from Conc-Sept21 that infected the *Pseudo-nitzschia calliantha* strain DIAVIR2. Unfortunately, although the isolated nucleic acids were subjected to sequencing library preparation, sequencing was not successful, possibly due to a repeatedly failed pre-amplification step resulting in too low a cDNA concentration for sequencing. We were able to visualize the virus particles in the filtered lysate (LysDIA2) using TEM. This revealed a 32 ± 1.6 nm (n = 13) icosahedral virus (Fig. 1). Further attempts to propagate and grow the virus were unsuccessful as the host culture was lost and no further cultures of the same host species were available at that time. UV-treated or Anodisc-filtered lysates did not result in lysis of the culture. We are currently seeking new virus isolates capable of lysing *Pseudo-nitzschia*

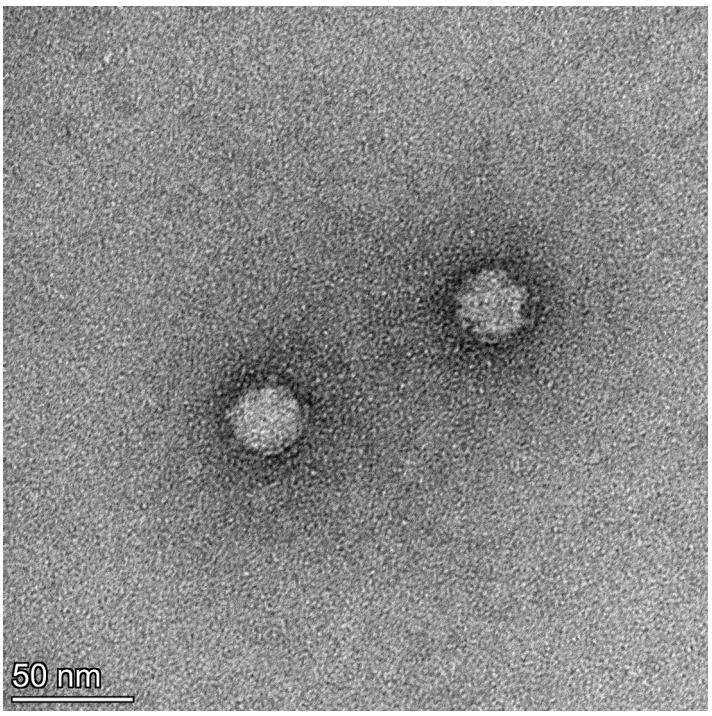

Fig. 1. Transmission electron micrographs of the putative *Pseudo-nitzschia calliantha* virus particles in LysDIAVRI2. Two undamaged virus particles of the putative *Pseudo-nitzschia calliantha* virus under higher magnification.

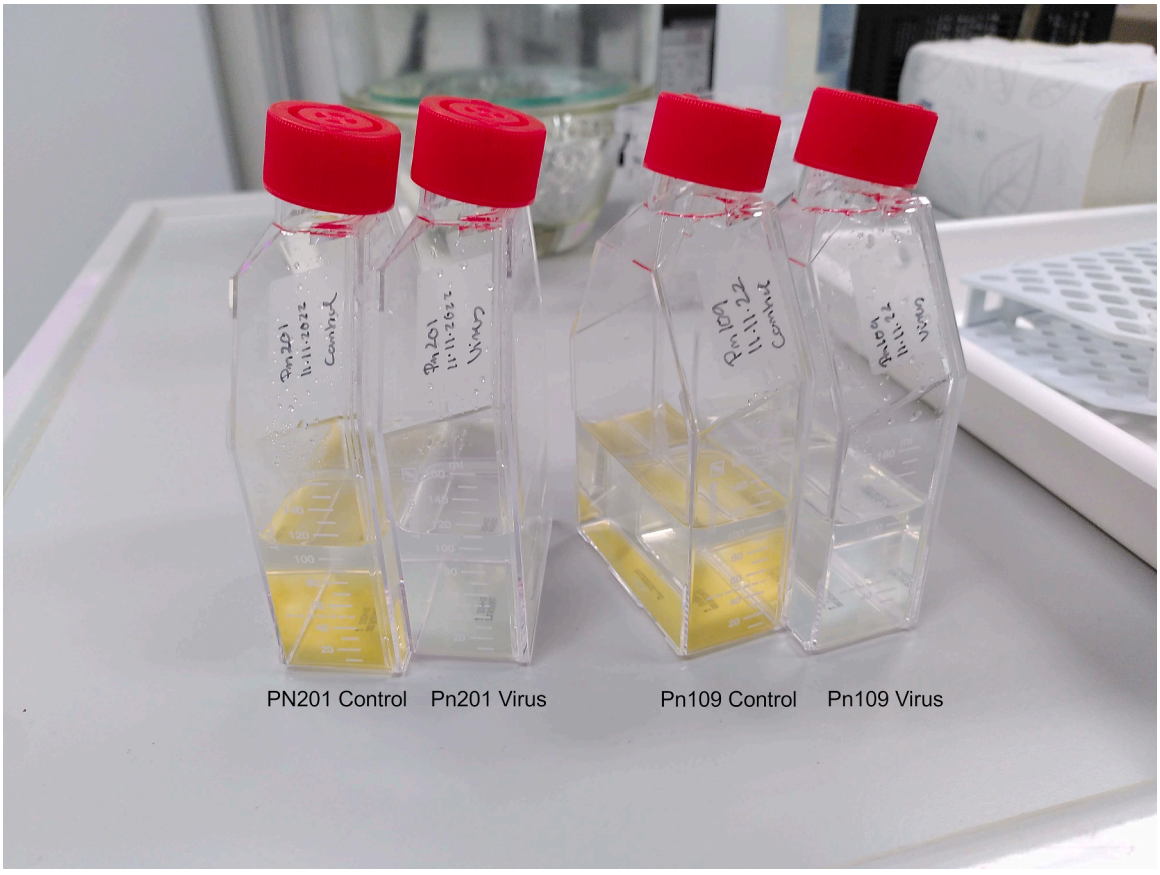

Fig. 2. Images showing uninfected and infected cultures of *Pseudo-nitzschia galaxiae* strains Pn201 and Pn109 five days after infection with lysates obtained from three rounds of infection following the initial screening of infectivity with environmental concentrates.

calliantha. This is one of the most common *Pseudo-nitzschia* species in the GoT and Adriatic (Arapov et al., 2020a; Turk Dermastia et al., 2020; Giulietti et al., 2021) and cultures of this species could serve as a suitable model system for viral infections, with potentially important ecological implications.

3.2. *Pseudo-nitzschia galaxiae RNA virus (PnGalRNAV)*

3.2.1. *Viral culture and host specificity*

Two viruses (from LysPn109 and LysPn201, Table 1) causing complete lysis of *Pseudo-nitzschia galaxiae* strains Pn109 and Pn201 were propagated from the environmental concentrate Conc-Aug22 (Fig. 2). Another virus (from LysP2D4, Table 1), causing complete lysis of *P. galaxiae* strain P2D4, was propagated from Conc-Aug-23. The viruses were transmissible after 0.22 μm filtration and caused lysis within 3 days of transmission, (based on Chl-a autofluorescence measurements). No lysis was observed after 0.02 μm filtration and UV sterilization of the lysate (Table 1). Genomic analysis, presented below, showed that lysates LysPn109 and LysPn201 contained an identical *Picornavirales* virus with a high read coverage, whereas LysP2D4 contained a similar virus based on the sequence of the RdRP gene. Negative contrast staining and subsequent TEM examination of LysPn109 and LysPn201 revealed large amounts of virus-like particles (Fig. 2), some of which appeared to be damaged and others intact. These particles had a size of 29.5 nm ± 1.2 nm ($n = 17$) and were icosahedral in shape, resembling other described *Picornavirales* (Sadeghi et al., 2021).

Filtered lysates remained infectious for at least 5 months when stored in the dark at 4 °C (data not shown). Persistence is expected in *Picornavirales* in controlled and stable environments, as they lack lipid membranes that make viruses more susceptible to degradation (Silverman and Boehm, 2021). Additionally, the persistence of diatom viruses, both in culture and in the natural environment, has been shown to be quite strong (Kimura and Tomaru, 2015).

To determine the specificity of the viruses, we exposed different *Pseudo-nitzschia* cultures to filtered lysates containing the viruses (Table 1, strains selected for host specificity tests). Lysis was only observed in cultures of *P. galaxiae*. LysPn109 and LysPn201 both caused cross-infection of their respective strains, but they also cause infection of strain Pn208. This strain belongs to a different morphotype (medium) and genotype than the strains from which the virus was propagated (both small morphotype; Supplementary Table 1, Supplementary Fig. 1). It is worth noting that *Pseudo-nitzschia galaxie* occurs in three different morphotypes (small, medium, large) that also differ genetically (Cerino et al., 2005; Turk Dermastia et al., 2020). Both diatom strains (Pn109 and Pn201) were isolated from the same sample and were genetically identical based on *rbcL* barcoding, suggesting that they were clonal isolates. However, neither LysPn109 nor LysPn201 caused infection of a strain of *P. galaxiae* belonging to the large morphotype (P2D4) that was isolated a year later from the same waters and a similar temporal window. The virus propagated from this strain (LysP2D4), was likewise noninfective for the small and medium *P. galaxiae* strains. No other *Pseudo-nitzschia* species was affected by these viruses, as no lysis was observed.

Replication of the virus was tested in *P. galaxiae* strain Pn208 with qPCR. The virus successfully replicated in the diatom culture, as demonstrated by a relative 11-fold increase in the measured capsid protein theoretical copy number, from 25,000 copies μL^{-1} at the start to close to 275,000 copies μL^{-1} 48 h post infection (Fig. 3A, Supplementary Fig. 4). At the time of the peak in virus copy number (based on one capsid protein per virus), the diatom host culture collapsed while the non-infected controls remained over 2×10^6 mL^{-1} (Fig. 3B). The control did decline somewhat later in the experiment (growth conditions may have become sub-optimal as the density of the diatoms was rather high and there was a significant depletion of nutrients; data not shown) but abundances were still at least 10-fold higher than for the infected cultures. The virus copy numbers decreased rapidly, although quantifiable amounts remained in the experimental flasks (also after the culture collapse). Considering the stable infectivity of the virus upon storage (Section 3.2.1.), the drop in capsid protein copy numbers was somewhat surprising. However, late batch culture conditions with relatively high concentrations of host cells undergoing lysis could be unfavorable for viruses since these can be high reactive oxygen species environments

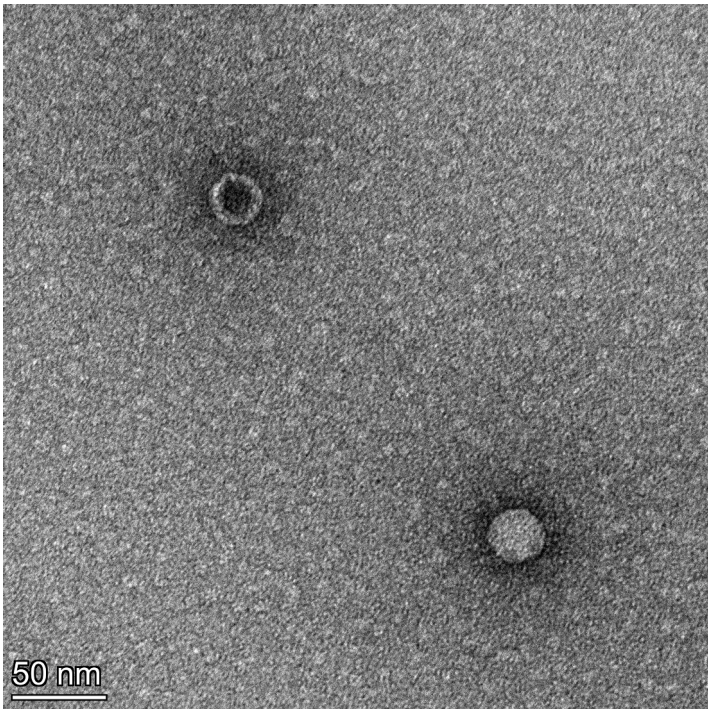

Fig. 3. Transmission electron micrographs of viral particles in filtered lysate (LysPn109). Virus particles under higher magnification. One of the particles (top) appears to have a destroyed protein coat, while the other is intact (bottom).

(Bidle, 2016). These conditions can also enhance virus removal during the 0.22 μm filtration upon sampling by sticking to cell debris, or aggregation of the viruses.

3.2.2. Genomic characterization

The genomes of the viruses were assembled independently from shotgun sequences of the DNA and RNA extracted from LysPn109, LysPn201. The assembled genome sequence, approximately 8.8 kbp in size, consisted of two open reading frames (ORFs), a larger one encoding the RNA-dependent RNA polymerase (RdRP) and another one encoding the structural coat protein. In the blastX analysis, the assembled genome sequence showed the highest amino acid identity with the non-structural (71 %) and structural (65 %) polyprotein sequences of Cylindrotheca closterium RNA virus type-II isolated from seawater in Hawaii (Schvarcz, 2018). Consistent with the blastX search, phylogenetic analysis (Fig. 4) revealed that the detected viral sequences clustered with Cylindrotheca closterium RNA virus type-II and other *Picornavirales* characterized by linear ssRNA genomes, which include many of the other described diatom viruses (Arsenieff et al., 2022). To determine the abundance of viral RNA in the sample, we mapped the reads back to the assembled genome. In LysPn201, the virus accounted for approximately 16 % of all reads in the sample, compared to only 2 % in LysPn109 (Supplementary Table 3). The partially annotated genome of the new virus was deposited in GenBank under accession number OR271362 and the name Pseudo-nitzschia galaxiae RNA virus type-I (PnGalRNAV type-I). The consensus sequence of both assemblies was identical. This was exacerbated by the fact that variant calling revealed similar sets of SNPs in both virus populations (Supplementary Tables 4, 5, Supplementary Fig. 2, concordance rate 0.5). The individual allele frequencies of the shared SNPs were highly correlated ($r = 0.97$, Supplementary Fig. 3). These results indicate that the viral population of PnGalRNAV was genetically diverse between and within the cultures but there was one prevailing viral population in the system resulting in identical consensus genome sequences. Genetic diversity within the same viral population is expected, as RNA viruses are known to be one of the fastest evolving biological entities that exist in a high similarity sequence spectrum (Domingo et al., 2012). This diversity quickly increases after genetic bottlenecks, where the intraspecific diversity is depleted, as was shown in mammalian RNA virus systems with single-cell sequencing (Combe et al., 2015). A clonal isolate may thus quickly evolve to represent a new viral population.

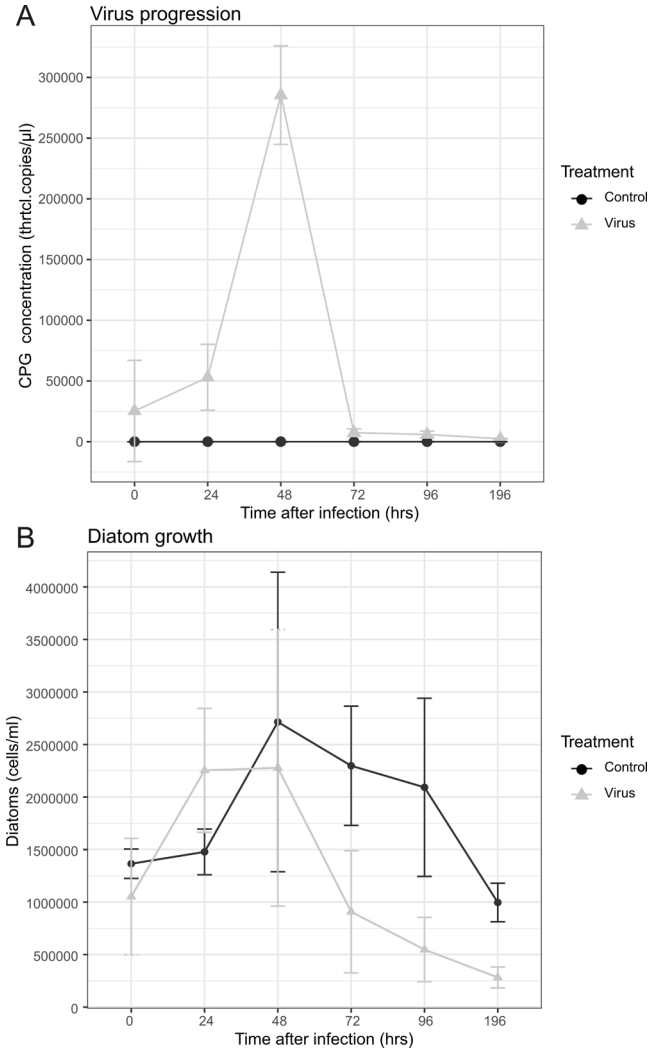

Fig. 4. Change in PnGalRNAV type-I capsid protein theoretical copy number (A) and in the growth of diatoms (B) over time after inoculation with either virus (gray) or mock (black). CPG stands for capsid protein gene. Theoretical copy number is estimated from the calibration curve, presented in Supplementary Methods (Fig. 1). Points represent mean values across the biological replicates ($n = 3$) while the error bars represent the standard deviation.

Another partial genome was assembled from shotgun sequences from RNA extracted from LysP2D4, resulting in a contig that spanned the entire RdRp gene. There was about 10 % difference between the amino acid sequences of this and the previously described virus, which could explain the fact that this virus was not infective for the other diatom strains. Notwithstanding, these viruses are likely just different strains of the same virus, as their sequences are quite similar. However, there is no set taxonomic rules for viral species determination in *Marnaviridae* sp. (Koonin et al., 2019). Knowing this, we describe this virus as Pseudo-nitzschia galaxiae RNA virus type-II (PnGalRNAV type-II) because of its distinct host preference. The RdRP of this virus was deposited in GenBank under the accession PP728770.

3.2.3. Virus in publicly available datasets

We investigated whether the sequences of viruses infecting *Pseudo-nitzschia galaxiae* could be contained in publicly available sequencing datasets that were not primarily analysed for viruses. Using the Serratus RNA viral palmprint database (Edgar et al., 2022) and the palmID tool (Babaian and Edgar, 2022), we identified similar or nearly identical sequences (Supplementary Table 2). The highest reported palmprint identities (93 % for PnGalRNAV type-I and 100 % for PnGalRNAV type-II) were associated with a TARA Oceans sample from the Catalan coast in the Mediterranean Sea and several samples from China from marine or intertidal areas (Bohai Sea, Xiangshan Bay). Performing *de novo* assembly of the identified datasets, we were able to obtain larger contigs with up to 1200 amino acids that were very similar to PnGalRNAV type-I (91 %) and even more to PnGalRNAV type-II (98.5 %) based on blastX analysis. The selected longest contig (from SRR10800990, Bohai Sea) was included in the phylogenetic analysis (Fig. 5). It clustered with the viruses from GoT, very close to PnGalRNAV type-II. Although *P. galaxiae* is globally distributed (Bates et al., 2018, and references therein), its virus was not commonly encountered, at least not in metatranscriptomic SRA datasets surveyed by the Serratus database. However, understanding the nature of metatranscriptomic data is key here, as the discovery of viruses, especially when present in low numbers, is highly dependent on the method of sample collection and sequencing depth. In addition, as indicated by our data, *P. galaxiae* infecting viruses are diverse which may be reflected also in the global diversity. Lastly, to our knowledge no comprehensive survey of public datasets solely for diatom viruses has been conducted and was also out of the scope for this paper but such efforts may be pursued in the future as the number of reference diatom virus genomes increases.

3.2.4. Description of PnGalRNAV and its importance

Based on genetic and morphological features, experimentally demonstrated associations of the virus with *P. galaxiae*, supported by a

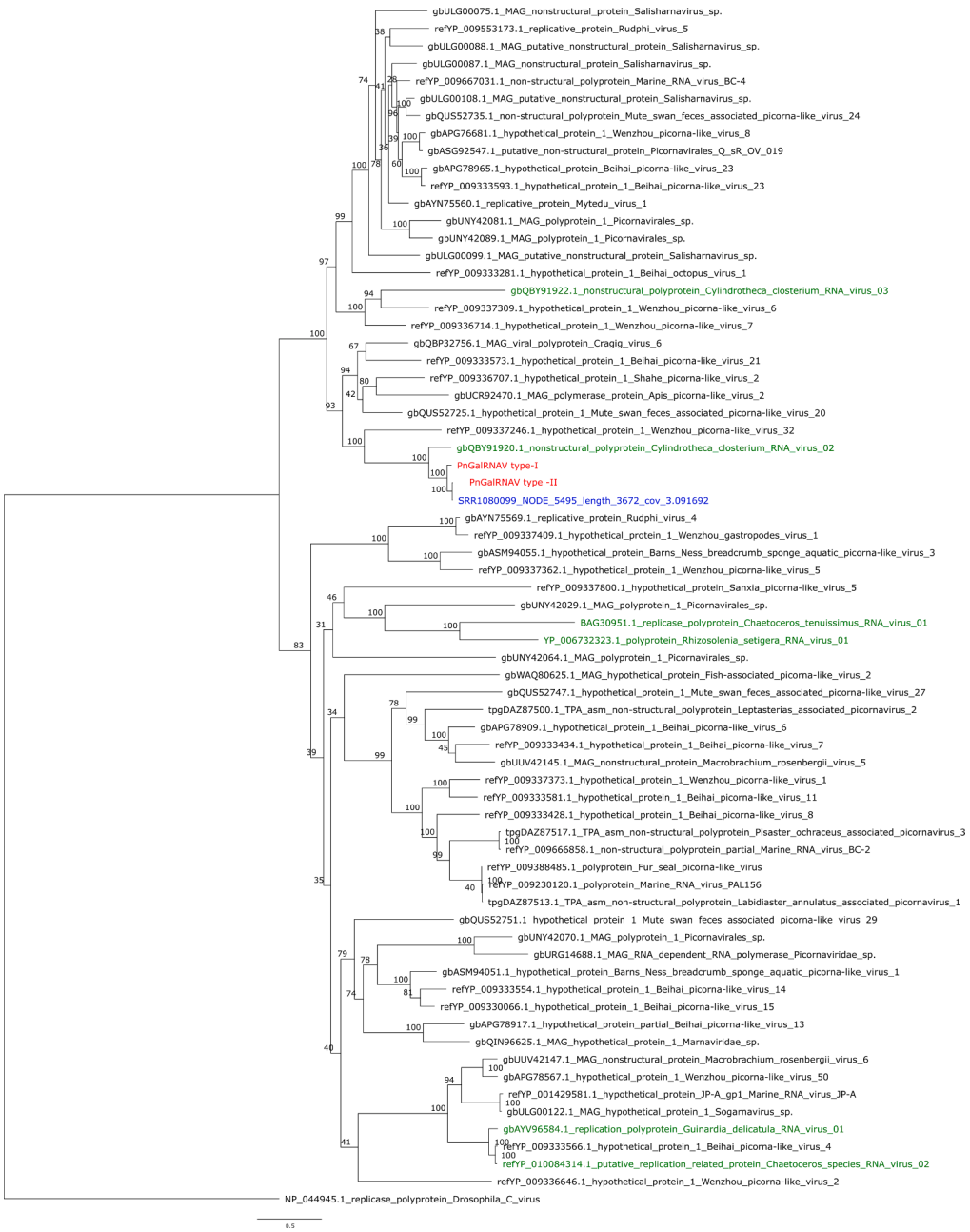

Fig. 5. Phylogenetic tree of *Picornavirales* RNA-dependent RNA polymerase (RdRp) including sequences of new viruses infecting *Pseudo-nitzschia galaxie*. The tree was generated with the maximum likelihood approach (LG+$F + I +$ G4, 1000 bootstraps) based on the alignment (989 amino acids) of a conserved segment of RdRp domain generated with MAFFT and trimmed with TrimAI using representative selected sequences of phylogenetically related viruses. Branch length represents the average number of amino acid substitutions per site. Known diatom viruses are shown in green, the sequence assembled from the SRA run SRR1080099 in blue and the novel PnGalRNAV in red. The tree was rooted with the Drosophila C virus, a sister virus of the members of *Picornaviridae*.

high number of viral sequences in the analysed datasets, qPCR results, and sequence similarities with viruses previously described in diatom hosts, we propose a new virus named PnGalRNAV (Pseudo-nitzschia galaxiae RNA virus). PnGalRNAV is a typical representative of the of the *Marnaviridae* family (Lang et al., 2021), based on its ssRNA genome organization with two ORFs and a conserved morphology with icosahedral capsid structure without obvious lipid membranes. Its genome sequence is similar (~70 % pairwise identity) to that of the previously described Cylindrotheca closterium RNA virus (Culley et al., 2014; Schvarcz, 2018). This suggests that the evolutionary histories of these two viruses are linked to the evolutionary histories of their hosts, as *Cylindrotheca* is also closely related to *Pseudo-nitzschia* (Lundholm et al., 2002). PnGalRNAV is the fifth characterized pennate diatom virus and the first diatom virus described from the Mediterranean (Arsenieff et al., 2022). Based on our data, there are at least two types of this virus that infect different morphological and genetical strains of *P. galaxiae.* The PnGaLRNAV type-I virus is infective for the small and medium *P. galaxiae* morphotypes, whereas the type-II virus is only infective for the large morphotype. Intraspecific infectivity of diatom ssRNA viruses has also been demonstrated in certain viruses infecting *Chaetoceros* species (Kimura and Tomaru, 2015; Tomaru and Kimura, 2020). *Pseudo-nitzschia galaxiae* is very common in the GoT, although it is often underrepresented in phytoplankton inventories due to its small size and misidentification (Turk Dermastia et al., 2023). It occurs in several sympatric, microdiverse populations with high relative abundance (Turk Dermastia et al., 2023). The high genetic and morphological

diversity of this species is an outlier within the *Pseudo-nitzschia* genus. Virus-mediated evolutionary differentiation is one of the possible explanations for this phenomenon. Models and limited experiments mostly in bacteriophage systems point to virus-mediated coevolution either through arms-races or through frequency-dependent selection for rare host and parasite (virus) genotypes (Frada et al., 2008; Martiny et al., 2014; Ignacio-Espinoza et al., 2020). It has also been documented in a few algal virus systems (Frickel et al., 2018; Wang et al., 2023). However, coevolution is likely not the only factor in our case as we have seen that PnGalRNAV type-I was able to lyse both morphologically and genetically distinct strains of *P. galaxiae*, albeit more closely related to each other than to the large morphotype (Supplementary Fig. 1). Environmental factors affecting infectivity should be considered as well, as they are known to influence infectivity (Mojica and Brussaard, 2014; Maat et al., 2016). The isolation and characterization of *P. galaxiae* viruses is however of importance as it will allow us to further study these coevolutionary processes.

Viruses infecting *Pseudo-nitzschia* have been predicted previously (Carlson et al., 2016), but they have not been adequately characterized or brought into culture. Given the extent of previous work on the diatom genus *Pseudo-nitzschia*, the description of its first virus is of great interest as it provides the possibility to test ecologically relevant questions related to bloom dynamics (Brussaard et al., 2004; Pelusi et al., 2021; Diaz et al., 2023) and the fate of the lysed diatoms in terms of the carbon pump (Bidle, 2016; Laber et al., 2018; Nissimov et al., 2018). While the *P. galaxiae* strains infected by the described viruses did not produce DA (data not shown), obtaining a toxic *P. galaxiae* strain (or any other toxigenic *Pseudo-nitzschia*) with an infectious virus will also enable to probe the potential impact infection might have on toxin production. Finally, using *Pseudo-nitzschia* and its viruses as a model system will also allow us to evaluate the impact that different environmental factors have on virus-host interactions and the mentioned processes.

3.3. *Chaetoceros tenuissimus virus ChTenDNAV type-II*

3.3.1. *Viral culture*

A potential virus causing partial lysis of *Chaetoceros tenuissimus* strain ChTen-GoT was propagated from Conc-Aug23 concentrate (LysChV). The virus was transmissible between fresh cultures after filtration through 0.22 μm sterile syringe filters. Monitoring of Chl-a auto-fluorescence showed that the virus caused lysis of the diatoms but did not lead to a complete collapse of the culture (at least not within the relevant time window where external factors can still be excluded). No lysis was observed after 0.02 μm filtration and UV sterilization of the lysate. Negative contrast staining and TEM examination of the filtered lysate revealed moderate amounts of virus particles (Fig. 6). All particles lacked an outer membrane. The width was measured to be 37 ± 3 nm (n = 9), like the previously described ChTenDNAV type-II virus (Kimura and Tomaru, 2015). The genome analysis presented below confirmed this and showed that the virus was indeed the known ssDNA virus ChTenDNAV type-II from the *Bacilladnaviridae* family. Lysates stored at 4 °C in native media in the dark were still infectious after almost a year of storage, again highlighting the potential persistence of these viruses in the environment.

The ability of the lysate LysChV containing this virus to lyse other *Chaetoceros* species was tested against a strain of *C. socialis* H.S. Lauder. Interestingly, complete lysis of this culture was observed (all host cells lysed), in contrast to the partial lysis of the *C. tenuissimus* culture. ChTenDNAV type-II from the original description did not lyse *C. socialis* (Kimura and Tomaru, 2015). Despite the similarity of the replicase protein, this virus appears to have a broader host range compared to ChTenDNAV type-II from the original description. This is the first report of an ssDNA diatom virus infecting multiple species. However, we cannot exclude the possibility that another virus was present in the filtered lysate used for host specificity testing that we subsequently failed to detect metagenomically. It has previously been shown that even the serial extinction dilution method for virus isolation can miss certain viruses, as the most abundant ones are selectively amplified (Kimura and Tomaru, 2015; Tomaru et al., 2009; Tomaru and Kimura, 2020).

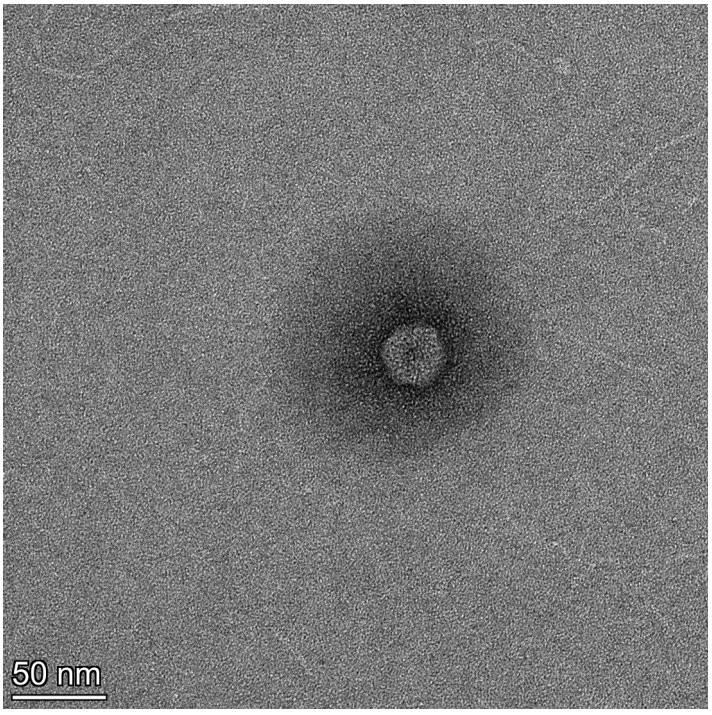

Fig. 6. Intact viral particle in LysChV. There were not many virus particles visible in this lysate, but the ones that were present resembled those of the described ChTenDNAV with a size of roughly 37 nm and icosahedral shape.

3.3.2. Genomic characterization

Genomic characterization began with the assembly of several large contigs (see Supplementary Table 3) that showed similarity to other *C. tenuissimus* viruses based on the DIAMOND blastX search. The largest contig (NODE_2446_length_4849_cov_4617.537130) showed similarity to the previously described Chaetoceros tenuissimus DNA virus type-II. The predicted replication-associated protein had 95 % identity with blastX, while the other most likely capsid-associated proteins showed 78 % identity to the previously described viral proteins. The viral genome was a circular ssDNA genome approximately 5.5 kb long, an organization typical for members of the *Bacilladnaviridae* family. The original assembly of the genome revealed a single large contig (4.6 kb) and two shorter contigs of about 1 kb that were homologous but had different sequences (NODE_10,062_length_977_cov_1726.121475 and NODE_10,097_length_971_cov_942.750000). These contigs were not artifacts or chimeras, as they had a very high coverage of reads (Supplementary Table 3). The 5′ and 3′ ends of two of these contigs matched the 5′ and 3′ ends of the large contig, effectively closing the circular genome. A portion of the sequence on contigs NODE_10,062 and NODE_10,097 encoded the replication protein. Therefore, to close the genome, we performed PCR on the unclear region and sequenced the products, resulting in a sequence that matched the NODE_10,097 contig (Supplementary Fig. 5., alignment in Supplementary Material - ChV_ambiguousregion_sequencing.fasta). This was then used to effectively close the genome. The nature of these two contigs was unclear. One possible explanation for the origin of the other contig could be that it is part of a dsDNA region, as all viruses from *Bacilladnaviridae* described so far have circular ssDNA genomes with a small dsDNA region (Kazlauskas et al., 2017). Another possibility is that the alternative contig could be the result of a form of alternative splicing of the replication protein. Alternative splicing of mRNAs has been described in diatoms (Kinoshita et al., 2001; Irimia et al., 2007) and is well documented in several human viruses, including HIV, hepatitis B virus and human papillomavirus (Stoltzfus, 2009; Graham and Faizo, 2017; Kremsdorf et al., 2021).

DNA polymerase phylogeny of the isolated virus showed that it clustered with a relatively high bootstrap support with Chaetoceros tenuissimus DNA virus type-2 and formed a sister clade to Chaetoceros tenuissimus DNA virus type-I (Fig. 7). Based on the taxonomy proposed by Varsani & Krupovic (ICTV, 2022) with a threshold of 75 % amino acid identity for the most conserved replication initiation protein (Rep) as a delineation criterion, the discovered sequence does not represent a new virus species, as determined by pairwise alignments with known members of *Bacilladnaviridae* (Supplementary Fig. 6), but represents a new variant of Chaetoceros tenuissimus DNA virus type-II within the genus *Protobacilladnavirus*, among which several other viruses infecting

Fig. 7. Phylogenetic tree of members of *Bacilladnaviridae* as proposed in ICTV (Varsani and Krupovic, 2022) including new sequence of Chaetocerus tenuissimus DNA virus type-II. Phylogeny is inferred from DNA polymerase amino acid sequence alignment (G-INS-1 + TrimAI) and generated with RaxML (DAYHOF + G4 + *I*, 1000 bootstraps). Branch length represents the average number of amino acid substitutions per site. Chaetoceros tenuissimus DNA virus type-II from the GoT is shown in red. The outgroup is a set of sequences from members of the *Circoviridae*.

Chaetoceros species can be found. It is worth noting that despite the high similarity of the replicase protein, the capsid proteins and the genome are quite different. This could possibly also explain the observed difference in host specificity. The capsid proteins of *Bacilladnaviridae* are generally divergent and are therefore thought to be the main drivers of diversification and specificity of these viruses (Tisza et al., 2020; Tomaru and Kimura, 2020). Nevertheless, the isolation of a virus in the GoT belonging to the same viral species suggests that these viruses are widespread, as are their hosts. Studies have shown that a certain type of DNA virus has been endogenized in the genome of *C. tenuissimus*, forming a so-called endogenous virus-like fragment – EVLF (Hongo et al., 2021). These EVLPs are found in strains of *C. tenuissimus* from a variety of world regions and are transcribed and show high similarity to the ChTenDNAV type I replication protein, suggesting that endogenization occurred relatively recently in evolutionary time (Hongo et al., 2021). This suggests that the evolutionary history of diatoms and their viruses is intertwined and manifests in various ways, from ecological and physiological adaptations to speciation. Although several viruses infecting *C. tenuissimus* have been cultured and characterized to date (Shirai et al., 2008; Tomaru et al., 2011a; Kimura and Tomaru, 2015), all were isolated in Japanese waters. Our work is the first isolation from a different oceanographic region.

4. Conclusions

We have characterized a novel ssRNA *Picornavirales* virus, PnGalRNAV, that infects *Pseudo-nitzschia galaxiae*, a species that has been shown to be toxic and is highly abundant in the study area. Two strains of this virus were found, designated as type-I and type-II, both with unique host preferences. This virus exhibits typical features of other *Picornavirales*, and resembles other described ssRNA diatom viruses. Sequences nearly identical to the RdRP of PnGalRNAV have been found in published metatranscriptomic datasets, although the prevalence of these data is not high. These data suggest these viruses are globally spread. Another virus infecting the potentially toxic species *Pseudo-nitzschia calliantha* was propagated, albeit lost. Our study is an important addition to the extensive knowledge on the harmful diatom genus *Pseudo-nitzschia* and will allow further research on the role of viruses in the regulation of blooms, carbon turnover and possibly even toxin production. In addition, our study extends the knowledge of one of the best-studied diatom virus systems, that of the bloom-forming *Chaetoceros tenuissimus*, by isolating an ssDNA virus from a location other than Japan. The virus is closely related to these viruses from the *Bacilladnaviridae* family, indicating that its genome is relatively conserved.

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Data availability

Raw reads deposited were deposited in the Sequence Read Archive (SRA, Accession number: PRJNA961029). List of assembled contigs, alignments and MEGAN files were deposited in Mendeley Data (DOI: 10.17632/489c8tjjxv.1). Virus sequences associated with *Chaetoceros* species were deposited in GenBank (Accessions OR283035-OR283038). Virus genomes associated with *Pseudo-nitzschia* species were deposited in GenBank (Accessions OR271362.1 and PP728770).

CRediT authorship contribution statement

Timotej Turk Dermastia: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Denis Kutnjak:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology. **Ion Gutierrez-Aguirre:** Writing – review & editing, Supervision, Resources, Methodology. **Corina P.D. Brussaard:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Katarina Bačnik:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102737.

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