Article

Novel Protocol for Estimating Viruses Specifically Infecting the Marine Planktonic Diatoms

Yuji Tomaru ^{1,*} and Kei Kimura ²

¹ National Research Institute of Fisheries and Environment of the Inland Sea, Japan Fisheries Research and Education Agency, Hiroshima 739-0452, Japan

² Faculty of Agriculture, Saga University, Saga 840-8502, Japan; kimurak@cc.saga-u.ac.jp

* Correspondence: tomaruy@affrc.go.jp; Tel.: +81-829-55-0666; Fax: +81-829-54-1216

Received: 9 April 2020; Accepted: 2 June 2020; Published: 4 June 2020

Abstract: Since their discovery, at least 15 diatom viruses have been isolated and characterised using a culture method with two cycles of extinction dilution. However, the method is time consuming and laborious, and it isolates only the most dominant virus in a water sample. Recent studies have suggested inter-species host specificity of diatom viruses. Here, we describe a new protocol to estimate previously unrecognised host-virus relationships. Host cell cultures after inoculation of natural sediment pore water samples were obtained before complete lysis. The proliferated viral genomes in the host cells were amplified using degenerate primer pairs targeting protein replication regions of single-stranded RNA (ssRNA) and single-stranded DNA (ssDNA) viruses, and then sequenced. Diverse ssRNA virus types within known diatom virus group were detected from inoculated *Chaetoceros tenuissimus* and *C. setoensis* cells. A previously unknown ssDNA virus type was detected in inoculated *C. tenuissimus* cells, but not in *C. setoensis* cells. Despite the possible protocol biases, for example non-specific adsorptions of virions onto the host cells, the present method helps to estimate the viruses infectious to a single host species. Further improvements to this protocol targeting the proliferated viral genomes might reveal unexpected diatom–virus ecological relationships.

Keywords: marine diatom; *Chaetoceros tenuissimus*; *Chaetoceros setoensis*; host-virus specificity; RdRp; ssRNA virus; ssDNA virus; viral genome

1. Introduction

Diatoms (Bacillariophyta) are unicellular, photosynthetic, eukaryotic algae found in aquatic environments worldwide. Their contribution to global biogeochemical cycling is significant [1], and they account for approximately 40% of marine primary production [2]. Complete diatom genome sequences reported to date [3,4] have revealed many aspects of diatom evolutionary history, ecology, metabolism, and influence on biogeochemical cycling [1,5–7].

Population dynamics of diatoms are affected by both abiotic factors [8] and biotic factors, and examples of the latter include viruses [9], zooplankton grazing, and bacterial and fungal parasites [10]. To date, at least 15 different diatom viruses have been reported [9]. Furthermore, recent metagenomic studies have predicted a high sequence diversity of diatom viruses in aquatic environments [11–14]. Metagenomic studies continue to provide diatom virus-like sequences owing to methodological progress. One of the problems in these studies is that the implemented methodologies can detect the virus-like sequences in the environment but not their host organisms. To understand the viral effects on the diatom dynamics, it is important to reveal the host-virus relationships. Therefore, many previous studies have reported isolations of viruses using specific diatom hosts [9].

The isolation of diatom viruses is essential to understand their basic biological features. The adopted method for virus isolation that employs two cycles of extinction dilution is time

consuming and labour intensive. For example, establishment of an axenic diatom virus isolate using two cycles of extinction-dilution method generally required 2–3 months in our previous studies [9]. In the first step of the isolation procedure, samples of filtered water or sediment pore water are added to diatom cultures. After the lysis of cells, the viruses are cloned through two cycles of extinction dilution, which isolates only the most dominant virus but not the minor viruses. A recent study showed that infections with a single-stranded RNA (ssRNA) diatom virus CtenRNAV type II are not species specific, but the virus infects several species [15]. To understand the diverse relationship between the host and virus, efficient methods for identifying viruses other than the dominant ones are necessary.

To this end, we developed and tested a new protocol to estimate host-virus relationships and detected previously unrecognised viruses that specifically infect *Chaetoceros* species. In general, viral metagenomic studies directly analyse viral genomes in water. The new protocol we tested and present here aims to detect specific viral genomes amplified in the host algae. The host cell cultures after inoculation with natural sediment pore water samples were obtained before complete lysis. The proliferated viral genomes in the host cells were amplified using degenerate primer pairs, and then sequenced. We used two different primer pairs to amplify replication protein regions of single-stranded DNA (ssDNA) and ssRNA viruses; they include both conserved and highly diverse regions in general and are used for virus taxonomy [16,17]. One primer pair, *Mplsc2*, amplifies RNA-dependent RNA polymerase (RdRp) region of picornaviruses [11]. We designed the second primer pair that targeted the ssDNA diatom virus replication protein (Rep) region, based on several ssDNA diatom virus sequences. The goal of this method was not to isolate viruses, but to estimate potential relationships between host diatoms and their infectious viruses within the limit of the primer pair performance.

2. Materials and Methods

Sediment samples were collected in Hiroshima Bay using an Ekman–Birge type bottom sampler (0–1 cm depth) and preserved at $-20\text{ }^{\circ}\text{C}$ until analysis. Three grams of each sediment sample was shaken with 3 mL of modified SWM3 medium [18] at 400 rpm for 30 min, and centrifuged at $716\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. The supernatant was filtered through a $0.2\text{-}\mu\text{m}$ pore-sized Dismic-25cs filter (Advantec, Tokyo, Japan) to remove bacteria. Aliquots (500 μL) of the filtered supernatants were added to 20 mL of exponentially growing *C. setoensis* (NIES-3712) and *C. tenuissimus* (NIES-3714 and NIES-3715) cultures. The cultures were incubated at $20\text{ }^{\circ}\text{C}$ under a 12 h light and 12 h dark cycle and light intensity of $130\text{ to }150\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$, which was produced by cool white fluorescent illumination. Two days post-inoculation, 2 mL of aliquots was centrifuged at $17,400\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. These inoculation and culture procedures are necessary for the viruses to infect and amplify themselves in the specific microalgal host strain cells. The supernatants were removed and the host cell pellets were preserved at $-80\text{ }^{\circ}\text{C}$ until analysis.

DNA and RNA of both host cell pellets with no inoculum and 500 μL of natural sediment pore water samples were isolated following the same method.

2.1. RNA Virus Detection by PCR

RNA was extracted from the cell pellets using the RNeasyPlus Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's recommendation. The extracted RNA was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System with random hexamers (Thermo Fisher Scientific, Waltham, MA, USA). The partial RdRp region of ssRNA viruses was amplified using a degenerate primer pair, *Mplsc2F*: 3'-ITWGCIGGIGATTWCA-5' and *Mplsc2R*: 3'-CKYTTCARRAAWTCAGCATC-5' [11]. PCR amplification was conducted using 20 μL of mixtures containing 5 μL of cDNA, $1\times$ BlendTaq buffer (Toyobo, Tokyo, Japan), 200 nM of dNTPs, 0.2 μM of each primer, and 1 U of BlendTaq DNA polymerase. The amplification was performed using GeneAmp PCR system 9700 (Thermo Fisher Scientific, USA), according to the following cycle parameters: 40 cycles of denaturation at $94\text{ }^{\circ}\text{C}$ (30 s), annealing at $43\text{ }^{\circ}\text{C}$ (30 s), and extension at $72\text{ }^{\circ}\text{C}$ (30 s).

2.2. DNA Virus Detection by PCR

DNA was extracted from the cell pellets using the DNeasy Plant Mini Kit (Qiagen) according to the manufacture's recommendation. The partial Rep region of diatom ssDNA viruses was amplified using a degenerate primer pair, *BDVRepF1*: 5'-GWCDTGGGAHCAATHSTTC-3' and *BDVRepR1*: 5'-YKSMAHTCNTCVGTYTGRTC-3', designed in this study (Figure S1). PCR amplification was conducted with 20 µL of reaction mixtures, which contained 5 µL of DNA, 1× BlendTaq buffer (Toyobo, Japan), 200 nM of dNTPs, 0.2 µM of each primer, and 1 U of BlendTaq DNA polymerase, using GeneAmp PCR system 9700 and the following cycle parameters: 40 cycles of denaturation at 95 °C (30 s), annealing at 55 °C or 50 °C (30 s), and extension at 72 °C (30 s). The annealing temperature of 55 °C was used for sediment pore water samples collected in July 2011 and August 2013 and inoculated to the two *C. tenuissimus* strains; 50 °C was used for all other samples.

2.3. Sequencing of PCR Products

The PCR products were then electrophoresed on 1% (w/v) agarose ME gels (WAKO Pure Chemical Industries, Osaka, Japan), and the nucleic acids were visualised using Midori green nucleic acid stain (NIPPON Genetics, Tokyo, Japan). PCR amplicons of approximately 0.7 kb were excised, and their nucleic acids were extracted (NucleoSpin® Gel and PCR Clean-up; Macherey-Nagel GmbH and Co., KG, Düren, Germany). The PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α-competent cells (Toyobo, Japan). Sequencing was conducted using the dideoxy method with ABI PRISM 3100 Genetic Analyser (Thermo Fisher Scientific). The resultant sequences were treated with GENETYX v12 (GENETYX, Tokyo, Japan) and their homology to viral replication proteins was verified using BLASTP [19].

2.4. Phylogenetic Analysis

The sequenced PCR fragments of the partial *RdRp* gene in ssRNA viruses and the partial *Rep* gene in ssDNA viruses were translated to amino acid (AA) sequences. The obtained *RdRp* AA sequences of ssRNA viruses were compared with the diatom ssRNA virus *RdRp* sequence dataset. The merged ssRNA virus *RdRp* dataset was aligned using Clustal W [20], and then manually adjusted. Similarly, the AA sequences of the partial *Rep* were compared with those of the diatom ssDNA virus sequence dataset, aligned using Clustal W [20], and then manually adjusted. Phylogenetic trees based on ssRNA virus *RdRp* and ssDNA virus *Rep* were constructed using the maximum likelihood (ML) method with the Jones–Taylor–Thornton matrix (JTT model) package in MEGA X [21]. Bootstrap consensus trees were constructed using 1000× bootstrap replications in each tree. Accession numbers of the sequences used in this study are listed in Table A1.

2.5. Pairwise Sequence Identity Comparisons

The aligned AA database was rearranged according to an ML tree. Pairwise sequence identities for the AA sequences were calculated using the Sequence Identity Matrix implemented in the BioEdit Sequence Alignment Editor [22]. The resulting identity matrix was edited using Microsoft Excel.

3. Results

No virus-like sequence was detected in host diatom cultures with no inoculum. This suggested that the host genomes were harbouring no virus-like sequence targeted in this study, and therefore, did not affect the results.

The detected *RdRp* sequences were divided into 19 types (A–S) with pairwise identities of >0.9 (Figure 1, Figures S2 and S3). *RdRp* sequences were detected in 21 of the 28 samples of sediment pore water; all 19 types of *RdRp* sequences, except for types F, H, and P, were confirmed in these samples. Types F, H, and P were detected only in inoculated diatom cell pellets. The *RdRp* sequences detected in *C. setoensis* NIES-3712 belonged to types A, B, C, D, E, F, H, O, and P (Figure 1; Table 1). The *RdRp*

sequences of *C. tenuissimus* NIES-3714 were detected in seven samples, those collected in July 2011 (type E), November 2011 (type D), July 2013 (type E), January 2014 (type N), April 2014 (type A), May 2014 (type A), and June 2014 (type C) (Table 1). RdRp types G, I, J, K, L, M, R, and S were detected only in sediment pore water samples, but not in inoculated diatom cells.

Figure 1. Phylogenetic analysis of ssRNA viruses. Maximum likelihood (ML) trees of ssRNA viruses on the basis of RNA-dependent RNA polymerase sequences. Bootstrap values (percent) from 1000 replicates are shown at the nodes. ML distance scale is shown under the ML tree.

Table 1. Viral RdRp and Rep types detected from sediment pore water in Hiroshima Bay, Japan, using diatom strains *Chaetoceros setoensis* and *Chaetoceros tenuissimus* during the study period. RdRp (A–S) and Rep (T–V) types were determined based on the phylogenetic trees in Figures 1 and 2, respectively. *Mplsc2F/R** and *BDVrepF1/R1*** are primer pairs used for the amplification of the RdRp and Rep regions, respectively. ‘-’ indicates not detected.

Date		ssRNA Virus, <i>Mplsc2F/R*</i>				ssDNA Virus, <i>BDVrepF1/R1**</i>			
		Sediment Pore Water	<i>C. setoensis</i>	<i>C. tenuissimus</i>		Sediment Pore Water	<i>C. setoensis</i>	<i>C. tenuissimus</i>	
			NIES-3712	NIES-3714	NIES-3715		NIES-3712	NIES-3714	NIES-3715
2011	Jul 23	A, E, I, K	E	E	E	-	-	T	-
2011	Aug 10	A, I, J, R	-	-	E	-	-	T	T
2011	Sep 7	C, N	-	-	E	-	-	T	T
2011	Oct 3	A, C, J	-	-	E	-	-	-	-
2011	Nov 4	C, D	D	D	E	-	-	-	-
2011	Dec 12	A, C, D, E, Q	C, D	-	E	-	-	T	-
2013	May 21	A, O	A	-	-	-	-	T	T
2013	Jun 28	J, R	-	-	E	-	-	T	-
2013	Jul 10	A	-	E	E	-	-	-	-
2013	Aug 7	J	-	-	E	-	-	-	-
2013	Sep 5	-	-	-	E	-	-	V	-
2013	Oct 18	S	-	-	E	-	-	V	-
2013	Nov 20	A, B, G, J	-	-	-	-	-	V	T
2013	Dec 19	A, B, C, L, N, Q	-	-	-	-	-	-	T
2014	Jan 22	A, C,	-	N	Q	U	-	-	-
2014	Feb 19	A, C, G, J, L, N, Q, S	F, H	-	-	U	-	T, V	T
2014	Mar 12	-	-	-	-	-	-	T	T
2014	Apr 17	A	C	A	-	-	-	-	T
2014	May 22	A, R	-	A	-	U	-	-	-
2014	Jun 16	A	A	C	A	-	-	T	T
2014	Jul 14	-	A	-	E	-	-	-	-
2014	Aug 11	-	-	-	E	-	-	T	-
2014	Sep 17	-	B	-	E	-	-	T	-
2014	Oct 15	-	C	-	E	-	-	T	T
2014	Nov 12	A, C, G, K	C	-	E	-	-	T	T
2015	Jan 21	C	C	-	-	-	-	T	T
2015	Feb 24	-	C, P	-	-	-	-	T	-
2015	Mar 25	M, N	C, O	-	-	-	-	-	T

Figure 2. Phylogenetic analysis of ssDNA viruses. Maximum likelihood (ML) trees of ssDNA viruses based on replication protein sequences. Bootstrap values (percent) from 1000 replicates are shown at the nodes. ML distance scale is shown under the ML tree.

The frequency of RdRp type E was only 3.4% of the sediment pore water samples (2/58, both samples collected in 2011), but it was dominant in >89% of *C. tenuissimus* NIES-3715 samples and was detected even when its presence was not confirmed in sediment pore water samples (Table 1). RdRp type E sequences of *C. tenuissimus* NIES-3714 or *C. setoensis* NIES-3712 were detected only in samples collected in July 2011 and July 2013.

The ssDNA virus Rep sequences detected in this study were grouped into three types (T, U, and V) at pairwise identities of >0.9 (Figure 2, Figures S4 and S5; Table 1). Most of the detected sequences were grouped into Rep type T, including CtenDNAV types I and II, which were detected only from the inoculated *C. tenuissimus* cells. Type U was detected only in sediment pore water samples collected in January 2014, February 2014, and May 2014. Type V was detected in inoculated *C. tenuissimus* NIES-3714, but not in NIES-3715 (Table 1). No Rep sequences were detected in *C. setoensis*.

4. Discussion

The results of the present study based on the new protocol suggested that the diverse RdRp type viruses have relationships with the specific diatom host strains. These results provide new insights into the diatom-virus ecology and evolution. For instance, no RNA virus infectious to *C. setoensis* NIES-3712 and *C. tenuissimus* NIES-3714 has been isolated to date (unpublished data), but RdRp sequences were detected in the inoculated cells of these two strains (Table 1). The diversity of RdRp types in the two diatom strains was higher than that of *C. tenuissimus* NIES-3715—nine and five types were detected using *C. setoensis* NIES-3712 and *C. tenuissimus* NIES-3714, respectively, vs. three in *C. tenuissimus* NIES-3715 (Table 1).

The major step in determining successful viral infection is dependent on the specific attachment of viruses to susceptible host cells; that is, viral tropism can be determined by cellular receptor-viral ligand interactions. One of the well-studied examples is poliovirus in the family Picornaviridae. The immunoglobulin superfamily molecule CD155 is regarded as the sole human cellular receptor for poliovirus [23]. Although rodents generally resist poliovirus infection because of the absence of CD155, transgenic mice expressing CD155 become susceptible to poliovirus infection [24]. Intra-species host specificity of HcRNAV infectious to the bloom-forming photosynthetic dinoflagellate *Heterocapsa circularisquama* is considered to be primarily determined in its upstream event—specific binding of

a virus particle to its corresponding host cell surface receptor—as demonstrated in a transfection experiment [25]. Most of the substituted amino acid residues of the capsid proteins between two distinct HcRNAV isolates, which have different host specificities, are located on the exterior surface of the virion [26].

In the present study, RdRp type E was observed in all diatom strains. The currently isolated genomes of diatom RNA viruses are composed of two open reading frames, replication-related proteins including RdRp, and structural proteins [27]. Therefore, the RdRp type E viruses would share the same RdRp sequence but have diverse capsid proteins, for example, amino acid sequence and protein modification patterns, that determine viral infection specificities to these diatom species and/or strains. Similarly, type A was detected in all strains, whereas type C was detected in *C. setoensis* NIES-3712 and *C. tenuissimus* NIES-3714, but not in NIES-3715 (Table 1). These results suggest that diatom RNA virus genomes are diverse in terms of combinations of RdRp types and capsid proteins. The phylogenetic analysis of ssDNA virus Rep protein sequences placed CtenDNAV types I and II in the same group (Figure 2), although their structural protein similarity is only 27.6% [15]. Similar to ssRNA viruses, the combinations of Rep types and capsid proteins for diatom ssDNA viruses is expected to be diverse.

Another determinant of tropism is virus replication. Poxvirus tropism is regulated by intracellular events downstream of the virus binding and entry sites, rather than at the level of specific host receptors [28]. As for microalgal RNA viruses, the HcRNAV genome was successfully proliferated in cells of several *H. circularisquama* strains using transfection methods, but not in another species within the same genus, the marine dinoflagellate *Heterocapsa triquetra* [25]. Although both species are from the genus *Heterocapsa*, the intracellular condition of *H. triquetra* is not suitable for the replication of HcRNAV. The infections of CtenRNAV type II are inter-specific [15]. In addition to the original host strain *C. tenuissimus* NIES-3715, the virus infects and lyses at least four different *Chaetoceros* species including *C. setoensis*. However, the genome replication of CtenRNAV type II in the different host species seemed lower than that in the original host cells. These observations suggest that the cells permitting virus production may have different levels of permissiveness; that is, some isolates may produce large amounts of virus, whereas others may inhibit or permit only a low level of viral replication. The mechanisms determining the genome replication of microalgal RNA viruses are thus considered to be different at the intra-species and inter-species levels. The high frequency of RdRp type E in *C. tenuissimus* NIES-3715 reflects its higher replication efficiency in the cells. CtenRNAV type II was originally isolated in *C. tenuissimus* NIES-3715 [15] and was phylogenetically grouped within RdRp type E in this study. Considering the above discussion, the RdRp type E viruses and *C. tenuissimus* NIES-3715 are highly compatible and have successfully co-evolved.

Six different RdRp type viruses infectious to *C. tenuissimus* were present in the coastal sediments (Table 1). Of those, four types were also detected in the inoculated *C. setoensis* cells. The diatom virus densities are specifically increased during its host bloom periods as observed in other microalgae and virus systems [29]. In general, diatom blooms are usually composed of diverse species. Viruses produced by some diatom host species might not only affect host population dynamics, but also the dynamics of other species in diatom communities during bloom periods.

The protocol used in this study enables estimation of the host diatom and its infectious virus-type relationships, and is easier and quicker than the extinction dilution method. However, these results are affected by several technical problems and limitations. The detection efficiency of the target sequences, that is, viral replication proteins in this study, may be affected by higher concentrations of non-targeted nucleic acids derived from host organisms and bacteria, such as rRNA, during PCR amplifications. Further optimisation of the PCR protocols, for example, improving nucleic acid extraction and purification and PCR temperature settings, might eliminate these biases. The insufficient specificity of degenerate primer pairs for detecting diatom ssDNA viruses would also limit detection. Our preliminary tests showed that the degenerate primer pair for amplifying the ssDNA virus replication protein region failed to detect the previously known CtenDNAV type II. Hence, further improvements

to the primers and protocols would be necessary to elucidate the host-virus systems, especially for ssDNA viruses.

We could not completely exclude the possibility of non-specific adsorptions of virions onto the host cells, which will overestimate the diversity of infectious viruses. However, the present results demonstrated that the viral RdRp types detected in cell pellets were often not detected in sediment pore water samples used for inoculation, and vice versa. For example, no RdRp sequence was detected in sediment pore water collected in September 2013, but type E was detected in the inoculated *C. tenuissimus* NIES-3715 cells. Furthermore, six different RdRp types were detected in the sediment pore water sampled in December 2013, but not in inoculated cells. The direct detection of ssDNA virus Rep proteins from sediment pore water samples was mostly unsuccessful, and only Rep type U was detected three times in water samples but never confirmed in the inoculated *C. tenuissimus* cells (Table 1). Rep types T and V were detected at higher frequencies in inoculated *C. tenuissimus* cells (Table 1). Therefore, the present method may not have enough resolution to detect non-specific virions adsorbed onto the host cell surface. For an improved understanding of the host-virus system, development of efficient protocols is necessary to detect the proliferated target sequences in the host cell. For example, detection of negative-stranded genome RNA for ssRNA viruses and mRNA sequences for ssDNA viruses might be more effective in determining the host organisms.

The lytic effects of microalgal viruses are affected by abiotic environmental factors and their effect on host cells, especially water temperature [30,31]. Viral proliferation in *C. tenuissimus* cells changes with respect to water temperature [27,32]. In this study, water temperature was set at 20 °C, but different experimental conditions may affect the results. In addition to water temperature, salinity and nutrient conditions potentially affect viral infection [33].

Nevertheless, although the present protocol has several technical problems, its methodology can be utilised to understand diatom and virus relationships. For example, our results indicated that a diatom host permits the proliferation of various RdRp type viruses. Furthermore, an efficient proliferation of the viral genome is determined by the compatibility of the RdRp types to the host intracellular machinery. Our study lays the foundation for further studies on virus–diatom interactions.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/6/225/s1>, Figure S1: A primer pair, BDVRepF1/R1, used for the amplification of the partial replication protein (Rep) region of diatom ssDNA viruses. The primers were designed based on the alignment result of diatom ssDNA virus Rep sequences.; Figure S2: Full data set of Figure 1; Figure S3: Pairwise sequence identity comparisons (%) of the RNA-dependent RNA polymerase (RdRp) region among all ssRNA virus samples; Figure S4: Full data set of Figure 2; Figure S5: Pairwise sequence identity comparisons (%) of the replication protein (Rep) region among all ssDNA virus samples.

Author Contributions: Conceptualisation, Y.T.; methodology, Y.T.; validation, Y.T. and K.K.; formal analysis, K.K.; resources, Y.T. and K.K.; writing—original draft preparation, Y.T.; writing—review and editing, Y.T. and K.K.; visualisation, K.K.; supervision, Y.T. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by KAKENHI (19H00956) and grants for Scientific Research on Innovative Areas (16H06429, 16K21723, and 16H06437) from the Ministry of Education, Culture, Science, Sports and Technology (MEXT) of Japan.

Acknowledgments: We are grateful to T.M. for technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. List of accession numbers of the sequences determined in this study.

Accession Number	Sequence	Accession Number	Sequence
LC534420	2011_Aug_Sd-a	LC534488	2013_Jul_3715
LC534421	2011_Aug_Sd-b	LC534489	2013_Aug_3715
LC534422	2011_Aug_Sd-c	LC534490	2013_Sep_3715
LC534423	2011_Aug_Sd-d	LC534491	2013_Oct_3715
LC534424	2011_Dec_Sd-a	LC534492	2014_Jan_3714
LC534425	2011_Dec_Sd-b	LC534493	2014_Jan_3715
LC534426	2011_Dec_Sd-c	LC534494	2014_Apr_3714
LC534427	2011_Dec_Sd-d	LC534495	2014_May_3714
LC534428	2011_Dec_Sd-e	LC534496	2014_Jun_3714
LC534429	2011_Jul_Sd-a	LC534497	2014_Jun_3715
LC534430	2011_Jul_Sd-d	LC534498	2014_Jul_3715
LC534431	2011_Jul_Sd-e	LC534499	2014_Aug_3715
LC534432	2011_Jul_Sd-f	LC534500	2014_Sep_3715
LC534433	2011_Nov_Sd-a	LC534501	2014_Oct_3715
LC534434	2011_Nov_Sd-b	LC534502	2014_Nov_3715
LC534435	2011_Oct_Sd-a	LC534503	2011_Jul_3712
LC534436	2011_Oct_Sd-b	LC534504	2011_Nov_3712
LC534437	2011_Oct_Sd-c	LC534505	2011_Dec_3712-A
LC534438	2011_Sep_Sd-a	LC534506	2011_Dec_3712-B
LC534439	2011_Sep_Sd-b	LC534507	2013_May_3712
LC534440	2013_Aug_Sd	LC534508	2014_Feb_3712-A
LC534441	2013_Dec_Sd-a	LC534509	2014_Feb_3712-B
LC534442	2013_Dec_Sd-b	LC534510	2014_Apr_3712
LC534443	2013_Dec_Sd-c	LC534511	2014_Jun_3712
LC534444	2013_Dec_Sd-d	LC534512	2014_Jul_3712
LC534445	2013_Dec_Sd-e	LC534513	2014_Sep_3712
LC534446	2013_Dec_Sd-f	LC534514	2014_Oct_3712
LC534447	2013_Jul_Sd	LC534515	2014_Nov_3712
LC534448	2013_Jun_Sd-a	LC534516	2015_Jan_3712
LC534449	2013_Jun_Sd-b	LC534517	2015_Feb_3712-A
LC534450	2013_May_Sd-a	LC534518	2015_Feb_3712-B
LC534451	2013_May_Sd-b	LC534519	2015_Mar_3712-A
LC534452	2013_Nov_Sd-a	LC534520	2015_Mar_3712-B
LC534453	2013_Nov_Sd-b	LC534521	2014_Jan_Sd
LC534454	2013_Nov_Sd-c	LC534522	2014_Feb_Sd
LC534455	2013_Nov_Sd-d	LC534523	2014_May_Sd
LC534456	2013_Oct_Sd	LC534524	2011_Sep_3715
LC534457	2014_Apr_Sd	LC534525	2011_Sep_3714
LC534458	2014_Feb_Sd-a	LC534526	2011_Aug_3714
LC534459	2014_Feb_Sd-b	LC534527	2011_Jul_3714
LC534460	2014_Feb_Sd-d	LC534528	2015_Mar_3715
LC534461	2014_Feb_Sd-e	LC534529	2015_Feb_3714
LC534462	2014_Feb_Sd-g	LC534530	2014_Mar_3714
LC534463	2014_Feb_Sd-h	LC534531	2014_Mar_3715
LC534464	2014_Feb_Sd-i	LC534532	2014_Feb_3714-a
LC534465	2014_Feb_Sd-j	LC534533	2014_Feb_3714-b
LC534466	2014_Jan_Sd-a	LC534534	2014_Feb_3715
LC534467	2014_Jan_Sd-b	LC534535	2011_Dec_3714
LC534468	2014_Jun_Sd	LC534536	2013_May_3714
LC534469	2014_May_Sd-a	LC534537	2013_May_3715
LC534470	2014_May_Sd-b	LC534538	2013_Jun_3714
LC534471	2014_Nov_Sd-a	LC534539	2011_Aug_3715
LC534472	2014_Nov_Sd-c	LC534540	2013_Sep_3714
LC534473	2014_Nov_Sd-d	LC534541	2013_Oct_3714
LC534474	2014_Nov_Sd-e	LC534542	2013_Nov_3715
LC534475	2015_Jan_Sd	LC534543	2013_Dec_3715
LC534476	2015_Mar_Sd-a	LC534544	2013_Nov_3714
LC534477	2015_Mar_Sd-b	LC534545	2014_Apr_3715
LC534478	2011_Jul_3714	LC534546	2014_Jun_3714
LC534479	2011_Jul_3715	LC534547	2014_Jun_3715
LC534480	2011_Aug_3715	LC534548	2014_Aug_3714
LC534481	2011_Sep_3715	LC534549	2014_Nov_3714
LC534482	2011_Oct_3715	LC534550	2014_Oct_3715
LC534483	2011_Nov_3714	LC534551	2014_Oct_3714
LC534484	2011_Nov_3715	LC534552	2014_Sep_3714
LC534485	2011_Dec_3715	LC534553	2014_Nov_3715
LC534486	2013_Jun_3715	LC534554	2015_Jan_3714
LC534487	2013_Jul_3714	LC534555	2015_Jan_3715

References

- Tréguer, P.; Bowler, C.; Moriceau, B.; Dutkiewicz, S.; Gehlen, M.; Aumont, O.; Bittner, L.; Dugdale, R.; Finkel, Z.; Iudicone, D.; et al. Influence of diatom diversity on the ocean biological carbon pump. *Nat. Geosci.* **2018**, *11*, 27–37.
- Field, C.B.; Behrenfeld, M.J.; Randerson, J.T.; Falkowski, P.G. Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science* **1998**, *281*, 237–240. [[CrossRef](#)]
- Armbrust, E.V.; Berges, J.A.; Bowler, C.; Green, B.R.; Martinez, D.; Putnam, N.H.; Zhou, S.; Allen, A.E.; Apt, K.E.; Bechner, M.; et al. The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism. *Science* **2004**, *306*, 79–86. [[CrossRef](#)]
- Bowler, C.; Allen, A.E.; Badger, J.H.; Grimwood, J.; Jabbari, K.; Kuo, A.; Maheswari, U.; Martens, C.; Maumus, F.; Ottillar, R.P.; et al. The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* **2008**, *456*, 239–244. [[CrossRef](#)]
- Armbrust, E.V. The life of diatoms in the world's oceans. *Nature* **2009**, *459*, 185–192. [[CrossRef](#)]
- Bowler, C.; Vardi, A.; Allen, A.E. Oceanographic and biogeochemical insights from diatom genomes. *Annu. Rev. Mar. Sci.* **2010**, *2*, 333–365. [[CrossRef](#)]
- Benoiston, A.-S.; Ibarbalz, F.M.; Bittner, L.; Guidi, L.; Jahn, O.; Dutkiewicz, S.; Bowler, C. The evolution of diatoms and their biogeochemical functions. *Philos. Trans. R. Soc. Lond., B Biol. Sci.* **2017**, *372*, 20160397. [[CrossRef](#)]
- Sarthou, G.; Timmermans, K.R.; Blain, S.; Tréguer, P. Growth physiology and fate of diatoms in the ocean: A review. *J. Sea Res.* **2005**, *53*, 25–42. [[CrossRef](#)]
- Tomaru, Y.; Toyoda, K.; Kimura, K. Marine diatom viruses and their hosts: Resistance mechanisms and population dynamics. *Perspect. Phycol.* **2015**, *2*, 69–81. [[CrossRef](#)]
- Raven, J.A.; Waite, A.M. The evolution of silicification in diatoms: Inescapable sinking and sinking as escape? *New Phytol.* **2004**, *162*, 45–61. [[CrossRef](#)]
- Culley, A.I.; Mueller, J.A.; Belcaid, M.; Wood-Charlson, E.M.; Poisson, G.; Steward, G.F. The characterization of RNA viruses in tropical seawater using targeted PCR and metagenomics. *mBio* **2014**, *5*, e01210–01214. [[CrossRef](#)]
- McDaniel, L.D.; Rosario, K.; Breitbart, M.; Paul, J.H. Comparative metagenomics: Natural populations of induced prophages demonstrate highly unique, lower diversity viral sequences. *Environ. Microbiol.* **2014**, *16*, 570–585. [[CrossRef](#)]
- Dayaram, A.; Goldstien, S.; Argüello-Astorga, G.R.; Zawar-Reza, P.; Gomez, C.; Harding, J.S.; Varsani, A. Diverse small circular DNA viruses circulating amongst estuarine molluscs. *Infect. Genet. Evol.* **2015**, *31*, 284–295. [[CrossRef](#)]
- Miranda, J.A.; Culley, A.I.; Schvarcz, C.R.; Steward, G.F. RNA viruses as major contributors to Antarctic viroplankton. *Environ. Microbiol.* **2016**, *18*, 3714–3727. [[CrossRef](#)]
- Kimura, K.; Tomaru, Y. Discovery of two novel viruses expands the diversity of ssDNA and ssRNA viruses infecting a cosmopolitan marine diatom. *Appl. Environ. Microbiol.* **2015**, *81*, 1120–1131. [[CrossRef](#)]
- Varsani, A.; Krupovic, M. Sequence-based taxonomic framework for the classification of uncultured single-stranded DNA viruses of the family Genomoviridae. *Virus Evol.* **2017**, *3*, vew037. [[CrossRef](#)]
- Vlok, M.; Lang, A.S.; Suttle, C.A. Application of a sequence-based taxonomic classification method to uncultured and unclassified marine single-stranded RNA viruses in the order Picornavirales. *Virus Evol.* **2019**, *5*, vez056. [[CrossRef](#)]
- Imai, I.; Itakura, S.; Matsuyama, Y.; Yamaguchi, M. Selenium requirement for growth of a novel red tide flagellate *Chattonella verruculosa* (Raphidophyceae) in culture. *Fish. Sci.* **1996**, *62*, 834–835. [[CrossRef](#)]
- Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [[CrossRef](#)]
- Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; McGettigan, P.A.; McWilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R.; et al. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–2948. [[CrossRef](#)]
- Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [[CrossRef](#)]

22. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
23. Mendelsohn, C.L.; Wimmer, E.; Racaniello, V.R. Cellular receptor for poliovirus: Molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* **1989**, *56*, 855–865. [[CrossRef](#)]
24. Ren, R.; Costantini, F.; Gorgacz, E.J.; Lee, J.J.; Racaniello, V.R. Transgenic mice expressing a human poliovirus receptor: A new model for poliomyelitis. *Cell* **1990**, *63*, 353–362. [[CrossRef](#)]
25. Mizumoto, H.; Tomaru, Y.; Takao, Y.; Shirai, Y.; Nagasaki, K. Intraspecies host specificity of a single-stranded RNA virus infecting a marine photosynthetic protist is determined at the early steps of infection. *J. Virol.* **2007**, *81*, 1372–1378. [[CrossRef](#)]
26. Nagasaki, K.; Shirai, Y.; Takao, Y.; Mizumoto, H.; Nishida, K.; Tomaru, Y. Comparison of genome sequences of single-stranded RNA virus infecting the bivalve-killing dinoflagellate *Heterocapsa circularisquama*. *Appl. Environ. Microbiol.* **2005**, *71*, 8888–8894. [[CrossRef](#)]
27. Tomaru, Y.; Kimura, K.; Yamaguchi, H. Temperature alters algicidal activity of DNA and RNA viruses infecting *Chaetoceros tenuissimus*. *Aquat. Microb. Ecol.* **2014**, *73*, 171–183. [[CrossRef](#)]
28. McFadden, G. Poxvirus tropism. *Nat. Rev. Microbiol.* **2005**, *3*, 201–213. [[CrossRef](#)]
29. Tomaru, Y.; Fujii, N.; Oda, S.; Toyoda, K.; Nagasaki, K. Dynamics of diatom viruses on the western coast of Japan. *Aquat. Microb. Ecol.* **2011**, *63*, 223–230. [[CrossRef](#)]
30. Demory, D.; Arsenieff, L.; Simon, N.; Six, C.; Rigaut-Jalabert, F.; Marie, D.; Ge, P.; Bigeard, E.; Jacquet, S.; Sciandra, A.; et al. Temperature is a key factor in *Micromonas*–virus interactions. *ISME J.* **2017**, *11*, 601–612. [[CrossRef](#)]
31. Maat, D.S.; Biggs, T.; Evans, C.; Van Bleijswijk, J.D.; Van Der Wel, N.N.; Dutilh, B.E.; Brussaard, C.P. Characterization and temperature dependence of arctic *Micromonas polaris* viruses. *Viruses* **2017**, *9*, 134. [[CrossRef](#)] [[PubMed](#)]
32. Kimura, K.; Tomaru, Y. Effects of temperature and salinity on diatom cell lysis by DNA and RNA viruses. *Aquat. Microb. Ecol.* **2017**, *79*, 79–83. [[CrossRef](#)]
33. Mojica, K.D.; Brussaard, C.P. Factors affecting virus dynamics and microbial host-virus interactions in marine environments. *FEMS Microbiol. Ecol.* **2014**, *89*, 495–515. [[CrossRef](#)] [[PubMed](#)]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).