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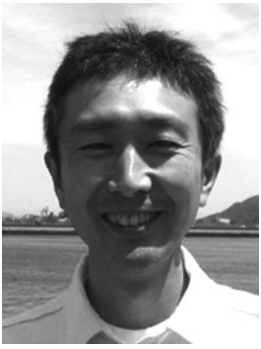

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J. Seckbach and J.P. Kociolek (eds.), *The Diatom World*,
Cellular Origin, Life in Extreme Habitats and Astrobiology 19, 211–225
DOI 10.1007/978-94-007-1327-7_9, © Springer Science+Business Media B.V. 2011

DIATOM VIRUSES

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1. Viruses in Marine Environments

Since the first reports of large numbers of virus-like particles (VLPs) in natural seawater, the aquatic viruses have been intensively studied (Bergh et al., 1989; Wommack and Colwell, 2000). Currently, the viruses are regarded as one of the major biological factors that regulate carbon cycling, microbial biomass, and the genetic diversity of protists and algae (Fuhrman, 1999; Brussaard, 2004; Suttle, 2005; Brussaard et al., 2008). The number of viruses in natural waters is estimated to be *ca.* 10^6 particles ml^{-1} in oligotrophic waters and *ca.* 10^8 particles ml^{-1} in higher productive areas; however, this may be underestimated due to insufficiencies in the detection methods and instruments (Suttle, 2007; Tomaru and Nagasaki, 2007).

Most of the virus particles in aquatic environments are considered to be bacteriophages because of the high abundance of their hosts in natural waters. And the viruses infecting eukaryotic phytoplankton may rank second in abundance. At least 29 viruses infecting eukaryotic microalgae have been identified and reported (Suttle, 2007; Nagasaki, 2008). Many of these viruses harbor a large double-stranded DNA (dsDNA) genome and thus are classified into the family Phycodnaviridae based on the deduced amino acid sequences of the DNA polymerase domain (Wilson et al., 2005). Other than phycodnaviruses, recent studies show diverse microalgal virus species harboring single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA) genomes (Brussaard and Martinez, 2008). Although the basic biological characters of these viruses have been intensively studied, their taxonomic position is not sufficiently understood due to the few marine viruses in the databases. This indicates the study of marine viruses is still one of the frontier fields within the aquatic sciences.

Several microalgal host–virus systems in natural environments have been studied, e.g., *Emiliania huxleyi* (Prymnesiophyceae) (Schroeder et al., 2003; Allen et al., 2007), *Phaeocystis globosa* (Prymnesiophyceae) (Baudoux and Brussaard, 2005), *Micromonas pusilla* (Prasinophyceae) (Zingone et al., 1999, 2006), *Heterosigma akashiwo* (Raphidophyceae) (Nagasaki and Yamaguchi, 1997; Tomaru et al., 2008a), and *Heterocapsa circularisquama* (Dinophyceae) (Nagasaki et al., 2004b; Tomaru and Nagasaki, 2004) and their respective viruses. In these

Table 1. Viruses infecting diatoms.

Virus	Host	Size (nm)	Genome	Reference
RsetRNAV	*Rhizosolenia setigera*	32	ssRNA	Nagasaki et al. (2004a)
CtenRNAV	*Chaetoceros tenuissimus*	31	ssRNA	Shirai et al. (2008)
CsfrRNAV	*Chaetoceros socialis* f. *radians*	22	ssRNA	Tomaru et al. (2009)
CsalDNAV	*Chaetoceros salsugineum*	38	ssDNA	Nagasaki et al. (2005b)
CdebDNAV	*Chaetoceros debilis*	32	ssDNA	Tomaru et al. (2008b)
CspNIV	*Chaetoceros* cf. *gracilis*	25	nd	Bettarel et al. (2005)

relationships, the viruses contribute to the disintegration of the host blooms and the succession of host clonal composition (Nagasaki et al., 2004b; Tomaru et al., 2004b). Therefore, the roles of viruses in natural environments are important from the viewpoint of the ecological dynamics of microalgal host populations.

Although the importance of diatoms as key players in the oceanic carbon cycle has been recognized (Smetacek, 1999), the existence of diatom viruses has been scarcely known until recently. Transmission electron microscopy showed VLPs were occasionally found in unidentified diatom cells that were involved in phytoplankton aggregations in sediment trap samples collected from the north-eastern Pacific Ocean (Proctor and Fuhrman, 1991); however, until recently, no isolation of diatom viruses has been reported. The first diatom virus was reported in 2004, an ssRNA virus infecting *Rhizosolenia setigera* (Table 1) (Nagasaki et al., 2004a). After the initial discovery, several *Chaetoceros* viruses have been successfully isolated and characterized. These discoveries are very important to further understand diatom ecology, the carbon cycle related to diatom production and evolution of diatoms. In the following sections, we summarize the basic ecology, physiology, and genetic features of diatom viruses isolated thus far.

2. Diatom Viruses

2.1. SINGLE-STRANDED RNA DIATOM VIRUSES

2.1.1. Rhizosolenia setigera RNA Virus

Rhizosolenia setigera RNA Virus (RsetRNAV) is an icosahedral virus (32 nm in diameter) lacking a tail (reported as RsRNAV in Nagasaki et al., 2004a). Virus particles accumulate in the host cytoplasm. This virus was first isolated from water samples of Ariake Sound in western Japan in April 2002. The latent period and burst size of RsetRNAV are 48 h and 1,100–3,000 infectious units per host cell, respectively. The infection specificity of this virus is strain specific rather than species specific (see Sect. 3.1). The major structural proteins of RsetRNAV are 41.5, 41.0, and 29.5 kDa. The RsetRNAV genome is an ssRNA which is 8,877 nt long, polyadenylated, lacking a cap structure, and has two major open reading frames (ORFs): ORF-1 (4,818 nt) and ORF-2 (2,883 nt) (Shirai et al., 2006).

2.1.2. Chaetoceros tenuissimus RNA Virus

Chaetoceros tenuissimus RNA Virus (CtenRNAV) causes the lysis of the bloom forming marine diatom *Chaetoceros tenuissimus* Meunier (Shirai et al., 2008). CtenRNAV was first isolated from water samples of Ariake Sound in western Japan during June 2004. This virus is an icosahedral virus (31 nm in diameter) and lacks a tail (Fig. 1).

Virus particles accumulate in the host cytoplasm in a crystalline array formations (Fig. 2). The latent period and burst size of CtenRNAV are <24 h

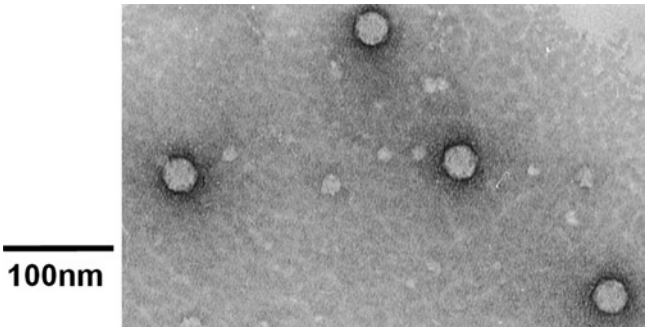

Figure 1. Negatively stained CtenRNAV particles.

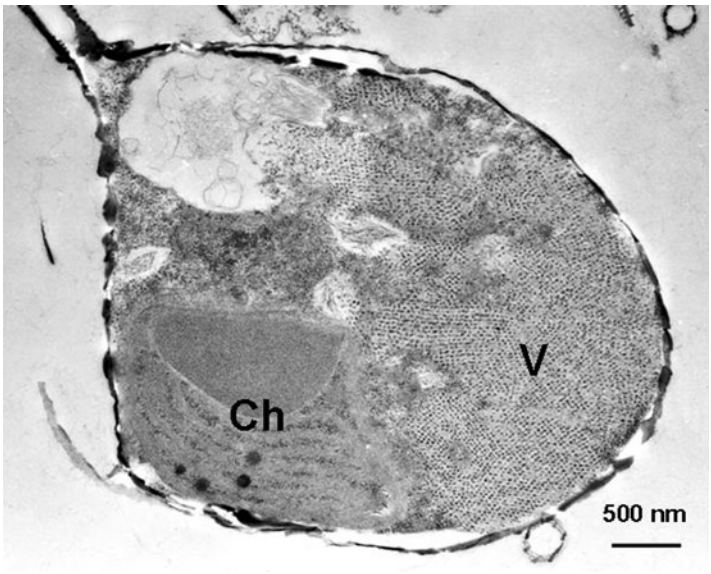

Figure 2. Transmission electron micrograph of a thin section of a CtenRNAV-infected *Chaetoceros tenuissimus* cell at 48 h post-virus inoculation. Virus-like particles (VLPs) accumulate in the host cytoplasm. Ch and V indicate chloroplast and VLP, respectively.

and ~10^4 infectious units per host cell, respectively. One of the unique features of this virus is its exceptionally high yields at ~10^{10} infectious units ml^{-1}; this is much higher than those for any other microalgal viruses previously characterized. This is advantageous for investigators to conduct future characterizations where large numbers of virions are necessary. Another noted character is the virus sensitivity of the host culture is different from other host–virus systems. Microalgal cultures are generally more sensitive to virus infections in logarithmic growth phase than stationary growth phase, whereas *C. tenuissimus* cultures show opposite responses to CtenRNAV, i.e., cultures in stationary growth phase show faster lysis than in logarithmic phase cultures. This phenomenon may be a key to understanding the host–virus relationship in natural marine environments; however, the reason has not been determined.

CtenRNAV harbors a ssRNA genome that is 9,431 nt (excluding a poly-A tail region) that includes two ORFs: ORF-1 (5,211 nt) and ORF-2 (2,646 nt). The major structural proteins are 33.5, 31.5, and 30.0 kDa.

2.1.3. Chaetoceros socialis f. radians RNA Virus

Chaetoceros socialis f. *radians* RNA Virus (CsfrRNAV) causes lysis of the bloom-forming diatom species, *Chaetoceros socialis* Lauder f. *radians* (Schütt) Proschkina-Lavrenko (Tomaru et al., 2009). CsfrRNAV was first isolated from water samples of Hiroshima Bay in western Japan in April 2005. CsfrRNAV is a very small polyhedral virus (22 nm in diameter) that lacks a tail. The virus particles accumulate in the host cytoplasm. The latent period and burst size of CsfrRNAV are <48 h and 66 infectious units per host cell, respectively. CsfrRNAV harbors an ssRNA genome that encodes at least three polypeptides of 32.0, 28.5, and 25.0 kDa. Using a RNA sequencing analysis, the genome was shown to be 9,467 nt (excluding a poly-A tail) that has two ORFs: ORF-1 (5,070 nt) and ORF-2 (2,688 nt).

2.1.4. Bacillarnavirus

Five different ssRNA viruses infecting marine stramenopiles are recognized: HaRNAV infecting a bloom-forming raphidophyte *Heterosigma akashiwo* (Tai et al., 2003; Lang et al., 2004), SssRNAV infecting a fungoid protist *Aurantiochytrium* sp. (Takao et al., 2005, 2006) and the three ssRNA diatom viruses described above. Their phylogenetic analysis was conducted where molecular biological features were compared and they were phylogenetically analyzed by Tomaru et al. (2009). The AU ratios of the three ssRNA diatom viruses were from 60.4% to 63.7%, while the HaRNAV and SssRNAV were much lower at 53.1% and 50.2%, respectively. The ssRNA diatom viruses, RsetRNAV, CtenRNAV, and CsfrRNAV, harbor an ssRNA genome with two ORFs (Fig. 3) that encode putative replication-related proteins and capsid proteins. In contrast, HaRNAV and SssRNAV genomes include one and three ORFs, respectively. A BLASTP analysis showed the amino acid sequences of ssRNA diatom viruses are highly similar to each other (E value = 0 ~ $2E-108$), while they were less similar to the HaRNAV and SssRNAV (E value = $3E-72$ ~ $5E-22$) (Tomaru et al., 2009). The basic genome structures of the ssRNA diatom viruses, therefore,

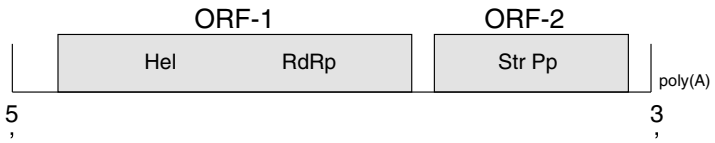

Figure 3. Schematic genome structure of single-stranded RNA diatom viruses, RsetRNAV, CtenRNAV, and CsfrRNAV. The genome size is *ca*. 9 kb, excluding a poly-A tail region and includes two open reading frames (ORFs). ORF-1, *ca*. 5k nt, encodes a putative RNA helicase (Hel) and a RNA-dependent RNA polymerase (RdRp) and ORF-2, *ca.* 2.6k nt, encodes a structural polyprotein (Str Pp) (Shirai et al., 2006, 2008; Tomaru et al., 2009).

are considered to be different from the two other stramenopile-infecting viruses. Further, the phylogenetic relationships based on the deduced amino acid sequence of the RNA-dependent RNA polymerase (RdRp) domains among positive-sense ssRNA viruses were analyzed. The result strongly supported the monophyly of RsetRNAV, CtenRNAV, and CsfrRNAV with a bootstrap value of 100% using both the neighbor-joining method and maximum likelihood method. Based on this data, this virus group is approved by the International Committee on Taxonomy of Viruses (ICTV) as a new genus, *Bacillarnavirus*, in 2010.

2.2. SINGLE-STRANDED DNA DIATOM VIRUSES

2.2.1. Chaetoceros salsugineum DNA Virus

Chaetoceros salsugineum DNA Virus (CsalDNAV) is a 38-nm icosahedral virus accumulating in the nucleus of *C. salsugineum* (reported as CsNIV in Nagasaki et al., 2005b). CsalDNAV was first isolated from water samples of Ariake Sound in western Japan in April 2003. The latent period and burst size are <24 h and ~300 infectious units per host cell, respectively. The CsalDNAV genome structure is unique among those of previously reported viruses. It consists of a single molecule of covalently closed, circular single-stranded DNA (ssDNA; 6,000 nt) as well as a segment of linear ssDNA (997 nt) (Fig. 4). The linear segment is complementary to a portion of the closed circle creating a partial double-stranded region. Six ORFs are found in the genome.

2.2.2. Chaetoceros debilis DNA Virus

Chaetoceros debilis DNA Virus (CdebDNAV) is a polyhedral virus (30 nm in diameter) lacking a tail and infects the cosmopolitan marine diatom *Chaetoceros debilis* Cleve (Tomaru et al., 2008b). This virus was first isolated from water samples of Ariake Sound in western Japan in March 2003. The virus particles accumulate primarily in the cytoplasm of *C. debilis* (Fig. 5); however, accumulations are also observed in the host's nucleus.

Host specificity of CdebDNAV is strain specific as shown with RsetRNAV. The latent period is <24 h. Agarose gel electrophoresis of the extracted CdebDNAV genome shows four bands at *ca.* 7, 5, 1.4, and 0.8 kb; they are

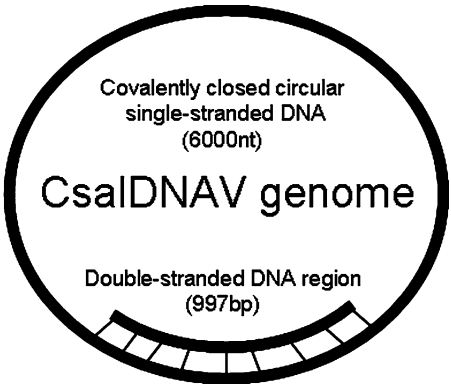

Figure 4. Schematic genome structure of CsalDNAV (Nagasaki et al., 2005b).

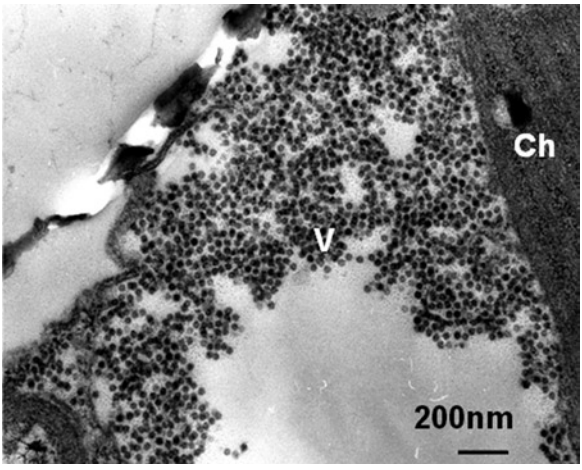

Figure 5. Transmission electron micrograph of a thin section of a CdebDNAV-infected *Chaetoceros debilis* cell at 48 h post-virus inoculation. Virus-like particles (VLPs) accumulate in the host cytoplasm. Ch and V indicate chloroplast and VLP, respectively.

completely digested using a S1 nuclease. This indicates the genome type of CdebDNAV is ssDNA. Its structure, however, is not understood. Sequence analysis shows the existence of at least one large contiguous segment at *ca*. 7 kb (unpublished data) that includes several ORFs.

The burst size of CdebDNAV was estimated to be ~50 infectious units per host cell based on a growth test where we calculated the decrease in host cell number and increase in virus number. This value, however, may be underestimated by comparing this data and the particle numbers found in a thin section view of the infected cells, e.g., Fig. 5. Such a discrepancy between the burst size data and the thin section view has been observed with CsfrRNAV and

CsalDNAV. Possible explanations for the small burst size may be aggregation of virus particles that causes an underestimation of the most probable number measuring the infectious virus units, difficulties in distinguishing dead cells and living cells using optical microscopy, or a dominance of defective particles lacking infectivity.

2.2.3. Bacilladnavirus

Two different ssDNA viruses infecting *Chaetoceros* species, CsalDNAV and CdebDNAV, have been isolated. The deduced amino acid sequences of their putative replicase gene are highly similar (*E* value = 2*E* – 69), and also they have a low similarity (*E* value > 2E − 4) to bird-infecting circoviruses which harbor a similar single-stranded circular genomic DNA (Tomaru et al., 2008b). These two viruses are considered to be similar based on the data, however, the genome structure of CdebDNAV is not enough understood. The phylogenetic relationship and taxonomic position of the ssDNA diatom viruses have not been fully understood due to the insufficient number of ssDNA viruses in the data bases. Therefore, only CsalDNAV have been approved as a sole member of a new genus, *Bacilladnavirus*, by ICTV in 2010. Characterizations of the new ssDNA diatom viruses, are in progress, e.g., morphologic, physiologic, and genomic characters of ssDNA viruses infecting several *Chaetoceros* sp. strains are highly similar to that of the previously reported ssDNA diatom viruses (unpublished data). Future studies may show further characteristics of the new genus "*Bacilladnavirus*."

2.3. OTHER DIATOM VIRUSES

2.3.1. Chaetoceros Nuclear Inclusion Virus

Chaetoceros Nuclear Inclusion Virus (CspNIV) is a lytic virus infecting *Chaetoceros* cf. *gracilis* isolated from Chesapeake Bay, USA, in April 2003 (Bettarel et al., 2005). The virus particles are *ca.* 25 nm in diameter and accumulate in the nucleus of *C.* cf. *gracilis* forming paracrystalline arrays. The latent period of CspNIV is <24 h. The genome structure of CspNIV has not been reported. Another small virus (*ca.* 30 nm in diameter) infecting *C.* cf. *wighamii* and accumulating in the host nucleus is also reported (Eissler et al., 2009) its genome characters are also unrevealed. Further study is expected to reveal genome structure for these viruses.

3. Ecology of Diatom Viruses

Ecological relationships between microalgal hosts and viruses in natural environments have been extensively studied during the last two decades (Brussaard and Martinez, 2008; Nagasaki, 2008). The effects of microalgal viruses on its host populations are roughly divided into two aspects: (1) quantitative effects that

cause a rapid decrease or restriction of the population abundance and (2) qualitative effects that change the clonal composition of the host populations that have a variety of virus sensitivities. The relationships between diatoms and their viruses *in situ* are relatively unknown; however, some authors have indicated their ecological interactions in nature based on field surveys and physiological studies (Shirai et al., 2008; Tomaru et al., 2008b).

3.1. INFECTION SPECIFICITY OF DIATOM VIRUSES

The infection of RsetRNAV and CdebDNAV is strain specific rather than species specific, and further, the specificity is diverse among virus clones. This indicates the virus sensitivities of diatom host clones are diverse among host clones (e.g., schematic diagram Fig. 6).

Tomaru et al. (2008b) conducted a cross reactivity test between 19 *C. debilis* strains and 29 virus clones infecting *C. debilis* where both were isolated from Ariake Sound in western Japan during blooms in 2005. The results show the intraspecies host specificity is highly diverse among the virus clones tested; therefore, they concluded that a natural *C. debilis* population is composed of highly diverse host clones that differ in virus sensitivity spectra.

The complex relationships between microalgal host–virus systems shown in the schematic relationship of Fig. 6 are generally observed in other microalgal host–virus relationships, e.g., *M. pusilla* and MpV (Sahlsten, 1998), *H. akashiwo* and HaNIV or HaV (Lawrence et al., 2001; Tomaru et al., 2004b), and *H. circularisquama* and HcRNAV (Tomaru et al., 2004a; Nagasaki et al., 2005a; Mizumoto et al., 2007); this is considered to be significant in preventing the complete extinction of microalgal host species due to viral infection.

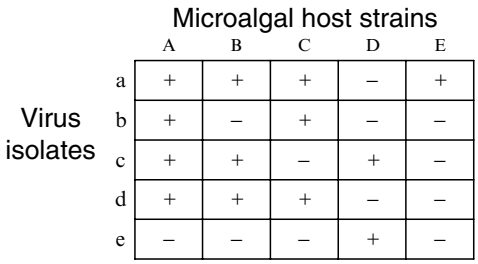

Figure 6. Schematic relationship between microalgal host clones ("A" to "E") and virus clones ("a" to "e") having different virus sensitivity and host specificity, respectively. "+" and "−" indicate lytic and not infectious, respectively.

3.2. INFECTION MECHANISMS

The infection mechanism of diatom viruses is unknown. The diatom's silica wall may restrict access of viruses to the cell membrane; however, most diatoms have a number of pores in the frustule. The particle sizes of RsetRNAV (32 nm) and CsalDNAV (38 nm) are smaller than that of *R. setigera* frustule pores (ca. 80 nm in diameter; Nagasaki et al., 2004a) and *C. salsugineum* setae pores (Nagasaki et al., 2005b), respectively; this may be the possible route of viral infection.

3.3. QUANTITATIVE EFFECTS ON DIATOM POPULATIONS

Bettarel et al. (2005) reported the most widespread occurrence of viruses infecting *Chaetoceros* cf. *gracilis* in Chesapeake Bay was recorded in April 2003 *ca.* 1 month after the winter-spring *Chaetoceros* bloom. The results suggest the importance of diatom viruses in the crash of their host *Chaetoceros* blooms (i.e., quantitative effects of virus infection) because the increase of virus numbers in the water column are considered likely to be the results of its host cells' death due to viral infection.

3.4. STABILITY OF INFECTIVITY

The results given by Bettarel et al. (2005) indicate that the viruses infecting *C.* cf. *gracilis* remain infectious in water columns of the bay at least 1 month after the disappearance of its host diatom. The high stability seems to be a general character of the diatom viruses isolated, e.g., infectious titers of CsfrRNAV suspension after 50 days of storage at 20°C, 10°C, and 4°C in the dark were 25%, 66%, and 145% of the initial titer, respectively (Tomaru et al., 2009); similar results were reported for RsetRNAV, CsalDNAV, CtenRNAV, and CdebDNAV. This feature may support the persistence of diatom viruses in natural environments.

The decay or elimination rate of diatom viruses from natural waters should be far higher due to exposure to irradiation with ultraviolet light, adsorption to various particles, external enzymes from bacteria, ingestion by heterotrophic microorganisms, and other unknown factors (Bitton and Mitchell, 1974; Kapuscinski and Mitchell, 1980; Suttle and Chen, 1992; Noble and Fuhrman, 1997). Some percentage of the viruses which successfully propagate during the host bloom, however, may be preserved in natural environments, enabling their revival in successive host blooms. One of the possible reservoirs of microalgal viruses is likely to be in sediments (Lawrence et al., 2002; Tomaru et al., 2005, 2007) where viruses infecting diatoms have been isolated frequently from bottom sediment samples (Nagasaki et al., 2005b; Tomaru et al., 2008b).

3.5. RESTING SPORES AND VIRUSES

Formations of resting spores by diatoms are essential in their survival strategies in various environments, and they are buried in sediments in many cases. Several studies predicted the possibilities of the diatom resting spores escaping from viral infections. Tomaru et al. (2009) found resting spores of *C. socialis* f. *radians* remaining in CsfrRNAV-inoculated cultures after 23 days postinoculation. The resting spores had chlorophyll fluorescence indicating viability in spite of the existence of numerous ambient virus particles; it is unknown whether formation of resting spores is enhanced by viral inoculation. Similar results were reported in the relationship between *C.* cf. *gracilis* and CspNIV (Bettarel et al., 2005) and between *C. debilis* and CdebDNAV (Tomaru et al., 2008b).

Possibly resting spores are significant for protecting diatom populations against viral attack. The lytic viruses infecting diatoms are frequently isolated from sediments (see above); therefore, the hatching of the resting spores from the sediments without viral infection may be essential for bloom formation. The relationships between the resting spores and the viral infection should be the focus of future studies to reveal the survival strategies of diatoms in natural environments.

3.6. DYNAMICS OF HOST AND VIRUS

Previous studies indicated the significant effects of viruses on their host population dynamics (Brussaard, 2004). Whereas, field research on diatom viruses have been scarcely reported. One of the principal reasons is the difficulty in identifying and counting diatom cells at the species level using light microscopy. The genus *Chaetoceros* includes more than 400 species (Rines and Hargraves, 1988), and differential identification of these species in natural water samples is almost impossible, especially for the small cells (e.g., *C. tenuissimus*) using the light microscope. Therefore, to estimate the impact of diatom viruses on their hosts' dynamics, the development of quantitative detection methods for diatoms is needed. This distinction at the species level is possible using real-time PCR.

4. Proposal for Diatom Virus Nomenclature

There have been no universal codes of algal virus nomenclature. The nomenclature used previously (e.g., *Heterosigma akashiwo* virus = HaV, *Emiliania huxleyi* virus = EhV) is no longer available due to the recent increase of algal viruses in culture. One remedy is to include more host information in the virus abbreviation name, e.g., using four letters out of the host scientific name composed of the initial letter from the host genus name and three letters from the species name, *i.e., Chaetoceros tenuissimus* is represented by "Cten" and *Chaetoceros socialis* f. *radians* is "Csfr." And, including the genome type (DNA or RNA) is meaningful

as we already know one host alga can be infected by various genome types of virus (ex. Brussaard and Martinez, 2008; Nagasaki, 2008; Tomaru et al., 2008a). Here we do not recommend using "NI (nuclear inclusion)" representing the virus replication site when the virus genome type is clear as in the case of CsNIV (Nagasaki et al., 2005b). Hence, following the above rule, RsRNAV (Nagasaki et al. 2004) and CsNIV (Nagasaki et al., 2005b) should be respectively, renamed as RsetRNAV and CsalDNAV, and this has been approved by ICTV in 2010. Of course, this rule may be changed appropriately when there is an increase in the variety of cultured algal viruses.

5. Conclusions

The discovery and successful isolation of diatom viruses imply their potential importance for controlling the quantity (biomass) and quality (clonal composition) of diatom populations in natural environments. Studies about diatom viruses have just began. There are numerous questions concerning diatom viruses, e.g., the effects of biogeochemical cycles in controlling diatom populations, unfound diatom viruses distinct from ssRNA and ssDNA viruses, coevolutions between diatoms and their viruses, and viruses infecting pennate diatoms and freshwater species. Further studies on various diatom host–virus systems should provide answers to these and other questions.

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