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Discovery of Two Novel Viruses Expands the Diversity of Single-Stranded DNA and Single-Stranded RNA Viruses Infecting a Cosmopolitan Marine Diatom

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Recent studies have suggested that diatom viruses are an important factor affecting diatom population dynamics, which in turn are important in considering marine primary productivity. The marine planktonic diatom *Chaetoceros tenuissimus* Meunier is a cosmopolitan species and often causes blooms off the western coast of Japan. To date, two viruses, *C. tenuissimus* DNA virus (CtenDNAV) type I and CtenRNAV type I, have been identified that potentially affect *C. tenuissimus* population dynamics in the natural environment. In this study, we successfully isolated and characterized two additional novel viruses (CtenDNAV type II and CtenRNAV type II). This paper reports the basic characteristics of these new viruses isolated from surface water or sediment from the Hiroshima Bay, Japan. The physiological and morphological characteristics of the two new viruses were similar to those of the previously isolated viruses. However, the amino acid sequences of the structural proteins of CtenDNAV type II and CtenRNAV type II were clearly distinct from those of both type I viruses, with identity scores of 38.3% and 27.6%, respectively. Our results suggest that at least four genetically distinct viruses sharing the same diatom host are present in western Japan and affect the population dynamics of *C. tenuissimus*. Moreover, the result that CtenRNAV type II lysed multiple diatom species indicates that RNA viruses may affect various diatom populations in the natural environment.

Following the first reports of large numbers of virus-like particles (VLP) in seawater, aquatic viruses have been intensively studied (1, 2). Despite their small size, viruses represent a large proportion of the biomass of the oceans (3) and are regarded as an important component of aquatic ecosystems; they are known to regulate carbon cycling, microbial biomass, and the genetic diversity of their host organisms (3, 4, 5, 6). Microalgae sustain a large portion of aquatic primary production, and viruses that infect them are considered to significantly affect their population dynamics (5). To improve our understanding of aquatic ecology, microalgal viruses and their hosts should be investigated in greater detail.

Marine diatoms generate as much organic carbon via photosynthesis as the globe's rainforests each year (7, 8, 9). Recently, several reports have suggested that diatom population dynamics are potentially affected by viruses as well as by diverse physical, chemical, and biological factors (10, 11). The cosmopolitan marine planktonic diatom *Chaetoceros tenuissimus* Meunier often causes blooms off the coast of western Japan. To date, two distinct viruses that infect it have been isolated and characterized: *C. tenuissimus* DNA virus (defined as CtenDNAV type I in this study) (12) and *C. tenuissimus* RNA virus (defined as CtenRNAV type I) (13). The two viruses are morphologically similar; both are icodahedral and 31 to 37 μm in diameter. However, their genome structures and infectious features differ. The burst sizes (calculated on the basis of the increase in the infectious titer per decreased host cell number in a given period) of CtenDNAV type I and CtenRNAV type I are approximately 10^2 and 10^4 infectious units · $cell^{-1}$, respectively. The two viruses also differ genetically. The genome of CtenDNAV type I is a covalently closed circular single-stranded DNA (ssDNA) of 5,639 nucleotides (nt) that includes a partially double-stranded region (875 bp) (12), and that of CtenRNAV type I is a linear single-stranded RNA (ssRNA) of 9,431 nt (13). A recent study has also suggested that their lytic effects on the host cells, along with the effects of variations in water temperature, are clearly dissimilar (14). These two distinctive viruses are therefore considered to share a host population and coexist in the natural environments.

In the present study, we isolated and characterized two novel ssDNA and ssRNA viruses that infect *C. tenuissimus* from the Hiroshima Bay in western Japan. Here we report their basic morphological, genetic, and infection characteristics. The two novel viruses differ markedly from the previously isolated *C. tenuissimus* viruses in their infection characteristics and genetic features.

MATERIALS AND METHODS

Algal cultures and growth conditions. The axenic clonal diatom strain *C. tenuissimus* 2-10 (13) (Fig. 1) was maintained at 15°C in modified SWM3 medium enriched with 2 nM Na_2SeO_3 (15) under a 12-h-light/12-h-dark cycle of ca. 110 to 150 μmol of photons $m^{-2} \cdot s^{-1}$ with cool white fluorescent illumination. Using the host strain for following experiments, the

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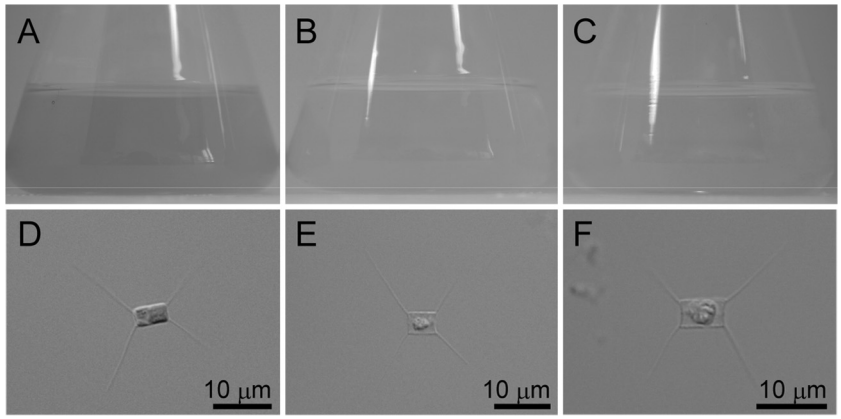

FIG 1 Cultures and micrographs of *Chaetoceros tenuissimus* strain 2-10. (A) Cultures without viral inoculation. (B) Cultures inoculated with CtenDNAV type II at 7 days postinoculation (dpi). (C) Cultures inoculated with CtenRNAV type II at 7 dpi. (D) Optical micrograph of an intact cell. (E) Optical micrograph of a CtenDNAV type II-inoculated cell. (F) Optical micrograph of a CtenRNAV type II-inoculated cell.

host culture was naturalized at 20°C under the light conditions described above for 1 week.

Virus isolation. Sediment samples (depth, 0 to 1 cm) were collected from a jetty of the National Research Institute of Fisheries and Environment of Inland Sea (FEIS) (34°27.525′N, 132°26.653′E) in the Hiroshima Bay, Japan, on 1 July 2010 and on 20 July 2010 using an Ekman-Birge bottom sampler equipped with a 36-mm-inner-diameter corer tube (16). A volume of SWM3 medium equal to each sample's weight was added to each sediment sample, and the samples were shaken at 400 rpm and 20°C for 30 min. Samples were centrifuged at 860 × *g* and 4°C for 10 min. Each supernatant was filtered using a 0.2-μm-pore-size Dismic-25cs syringe filter (Advantec Toyo, Tokyo, Japan). The filtrate samples were diluted in modified SWM3 medium over a series of 10-fold dilution steps. To isolate the viruses, 2 extinction-dilution cycles (17, 18) were then conducted using *C. tenuissimus* 2-10, which was naturalized at 20°C. Aliquots (100 μl) of filtrate from each dilution step were added to 8 wells of a 96-well flat-bottom plate (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) that contained 150 μl of an exponentially growing host culture, and cell suspensions were then incubated at 20°C under the lighting conditions described above. Cell conditions were observed using an inverted optical microscope (Ti-U; Nikon, Tokyo, Japan). Subsequently, the algal lysate produced in the vessels with the highest dilution from the first assay was carried over to the second extinction-dilution cycle. The resultant lysate from the final endpoint dilution was used as a clonal lysate, for which the probability of the presence of 2 or more viruses (i.e., failure in cloning) was estimated to be <0.0106 (17). Contaminating bacteria were removed from the clonal lysate by filtration through a 0.1-μm-pore-size Millex syringe filter (Merck, Billerica, MA), after which the lysate was transferred to an exponentially growing host culture. Each lysate was examined for bacterial contamination using epifluorescence microscopy after staining with SYBR-gold (Life Technologies, Carlsbad, CA, USA). Briefly, the lysate was fixed with 1% glutaraldehyde and a 1.0×10^{-4} dilution of commercial SYBR-gold stock was added to each fixed sample. The stained samples were filtered through a polycarbonate membrane filter (Nuclepore; Whatman, Kent, United Kingdom) (pore size, 0.2 μm). Subsequently, the filters were mounted on glass slides with a drop of low-fluorescence immersion oil and then covered with another drop of immersion oil and a coverslip. The slides were viewed at a magnification of ×1,000 with an Olympus BX50 epifluorescence microscope (excitation, 470 to 490 nm; emission, 510 to 550 nm; dichroic mirror, 505 nm). The resultant axenic lysates are referred to as the clonal viruses CtenDNAV type II (isolate name, SS10V-8V) and CtenRNAV type II (isolate name, SS10V-16V), respectively. Each milliliter of clonal viral lysates was inoculated into 25 ml of an exponentially growing host culture, and they were incubated at 20°C, under the lighting conditions described above. After host cells were decreased in number by viral infection, each culture lysate was used for fresh viral lysates for subsequent experiments.

Host range. The interspecies host specificity of viruses CtenDNAV type II and CtenRNAV type II was tested by the addition of 5% (vol/vol) aliquots of fresh lysate that had been passed through 0.2-μm-pore-size filters (Nuclepore) into duplicate cultures of 17 exponentially growing clonal algal strains: *C.* cf. *affinis* IT07-C40, *C. debilis* Ch48, *C. lorenzianus* IT-Dia51, *C. tenuissimus* 2-6, *C. tenuissimus* 2-10, *C. setoensis* IT07-C11, *C. socialis* f. *radians* L-4, *C.* sp. strain SS628-11, *C.* sp. strain TG07-C28, *C.* sp. strain SS08-C03, *Eucampia zodiacus* EzB, *Asterionellopsis glacialis* Ast K25, *Thalassiosira gravida* It-Dia1 (Bacillariophyta), *Teleaulax amphioxeia* Tel5W4 (Cryptophyceae), *Heterocapsa circularisquama* HU9433-P, *Karenia mikimotoi* KmY7 (Dinophyceae), and *Heterosigma akashiwo* HaSS12-1 (Raphidophyceae) (Table 1). Diatoms were cultured at 15°C under the conditions described above, and the other cultures were maintained at 20°C. Growth and evidence of lysis were monitored in each algal culture using optical microscopy and were compared with the results from control cultures that had been inoculated with SWM3 medium. Cultures that were not lysed at 14 days postinoculation (dpi) were considered to be unsuitable hosts for the pathogens.

Transmission electron microscopy. Exponentially growing cultures of *C. tenuissimus* 2-10 were inoculated with CtenDNAV type II or CtenRNAV type II suspensions (5% [vol/vol]). As a control, a *C. tenuissimus* 2-10 culture was inoculated with autoclaved SWM3 culture medium. An aliquot (10 ml) of the cell suspension was sampled at 3 dpi. *C. tenuissimus* cells were fixed with 3% glutaraldehyde–2% paraformaldehyde–0.1 M cacodylate buffer (pH 7.2) containing 33% SWM3 medium for 2 h at 4°C. The cells were collected by centrifugation at 2,500 × *g* for 5 min at 4°C. After the cell pellets were washed with 0.1 M cacodylate buffer (pH 7.2) containing 33% SWM3 medium, they were embedded in agarose (type IX-A; Sigma-Aldrich, St. Louis, MO) and then postfixed with 2% OsO_4 for 2 h on ice. Subsequently, the samples were washed, prestained with 2% uranyl acetate, dehydrated using an acetone series, and embedded in Spurr's resin (Nisshin EM, Tokyo, Japan) (19). Ultrathin sections were prepared using an Ultracut R microtome (Leica, Wetzlar, Germany) and stained with 4% uranyl acetate and 3% lead citrate. The sections were observed under a JEOL JEM-1010 transmission electron microscope (TEM; JEOL, Tokyo, Japan).

Virions that were negatively stained with uranyl acetate were also observed using TEM. Briefly, fresh lysate of a host culture inoculated with the subcultured clonal virus was concentrated using an Amicon Ultra-15 30K filter unit (Merck) and then mounted on a grid (no. 780111630; Nisshin EM) for 30 s. Excess water was removed using filter paper (no. 2; Advantec). Subsequently, 4% uranyl acetate was applied for 10 s and any excess dye was removed using a filter paper. After the grid was dried,

TABLE 1 Infection specificities of CtenDNAV type-II and CtenRNAV type-II for 17 strains of marine phytoplankton[a]

Family	Species	Strain code	Temp (°C)	Strains lysed by CtenDNAV type-II	Strains lysed by CtenRNAV type-II
Bacillariophyceae	*Chaetoceros* sp.	SS628-11	20	−	++
	Chaetoceros sp.	TG07-C28	15	−	++
	Chaetoceros sp.	SS08-C03	15	−	−
	Chaetoceros cf. *affinis*	IT07-C40	15	−	−
	Chaetoceros debilis	Ch48	15	−	−
	Chaetoceros lorenzianus	IT-Dia51	15	−	−
	Chaetoceros tenuissimus	2-6	15	++	−
	Chaetoceros tenuissimus	2-10	15	++	++
	Chaetoceros setoensis	IT07-C11	15	−	++
	Chaetoceros socialis f. *radians*	L-4	15	−	++
	Eucampia zodiacus	EzB	15	−	−
	Asterionellopsis glacialis	Ast K25	15	−	−
	Thalassiosira gravida	It-Dia1	15	−	−
Cryptophyceae	*Teleaulax amphioxeia*	Tel5W4	20	−	−
Dinophyceae	*Heterocapsa circularisquama*	HU9433-P	20	−	−
	Karenia mikimotoi	KmY7	20	−	−
Raphidophyceae	*Heterosigma akashiwo*	HaSS12-1	20	−	−

[a] ++, lysed; −, not lysed.

negatively stained CtenDNAV type II and CtenRNAV type II particles were observed under a TEM at an acceleration voltage of 80 kV. Particle diameters were estimated from the negatively stained images.

Virus purification. Exponentially growing *C. tenuissimus* cultures (500 ml) were inoculated with 5 ml of virus suspensions of CtenDNAV type II or CtenRNAV type II and lysed. Each lysate was passed through a 0.4-μm-pore-size polycarbonate Nuclepore filter (Isopore; Merck, Darmstadt, Germany) to remove cellular debris. Polyethylene glycol 6,000 (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the filtrate to achieve a final concentration of 10% (wt/vol), and the suspension was stored at 4°C in the dark overnight. After centrifugation at 57,000 × *g* at 4°C for 1.5 h, each pellet was washed with 10 mM phosphate buffer (pH 7.2) and added to an equal volume of chloroform. After vigorous vortex mixing, the suspensions were centrifuged at 2,200 × *g* for 20 min at room temperature to remove the chloroform. Each water phase was collected and ultracentrifuged at 217,000 × *g* for 4 h at 4°C to collect the virus particles. The virus particles were resuspended in 300 μl of ultrapure water (i.e., virus suspension) and were used for following viral nucleic acid, genomic, and protein analyses.

Viral nucleic acids. RNA and DNA were extracted from aliquots (100 μl) of purified viral samples from 500 ml of lysates of CtenDNAV type II and CtenRNAV type II using a DNeasy plant minikit and an RNeasy Plus minikit (Qiagen, Valencia, CA), respectively. In the case of CtenDNAV type II, to determine the nucleic acid type, aliquots (2 μl) of the nucleic acid solution were boiled at 100°C for 5 min, digested with DNase I (TaKaRa Bio) (0.5 U · μl^{-1}) at 37°C for 1 h, incubated with RNase A (Nippon Gene) (0.025 μg · μl^{-1}) at 37°C for 1 h, or digested with S1 nuclease (TaKaRa Bio) (0.7 U · μl^{-1}) at 23°C for 15 min. In the case of CtenRNAV type II, aliquots (2 μl) of the nucleic acid solution were digested with DNase I at 37°C for 1 h or incubated with RNase A at 37°C for 1 h. Each nucleic acid sample was electrophoresed on denatured agarose gels (1.5% SeaKem gold agarose; Lonza, Tokyo, Japan) at 50 V for 1 h. The nucleic acids were visualized using SYBR-gold staining (Life Technologies). As a control, 2 μl of each extracted nucleic acid sample was used directly for electrophoresis.

Viral genome sequencing. To determine the sequences of viral genomes of CtenDNAV type II and CtenRNAV type II, nucleic acids were extracted from 100 μl of each purified virus using a DNeasy plant minikit and an RNeasy Plus minikit (Qiagen), respectively. For analyzing the mutations in each viral group, two additional ssDNA virus isolates, SS10V-24V and SS10V-35V, and two additional RNA virus isolates, SS10V-39V and SS10V-45V, were also used for genome sequence analysis. They were isolated from sediment samples which were collected from a jetty of FEIS on 18 August 2010, 13 September 2010, 22 September 2010, and 1 October 2010, respectively. The procedures used for their isolation, purification, and genome extraction were as described above.

Sequences of each viral genome were determined using a GS FLX sequencer (Roche, Basel, Switzerland), according to the protocol of the manufacturer (Hokkaido System Science, Sapporo, Japan). Briefly, for RNA viruses, cDNA and double-stranded DNA (dsDNA) were synthesized from fragmented RNAs by the use of a cDNA synthesis system kit (Roche) with random primes. As for the DNA viruses, DNAs were fragmented by the use of a GS FLX Titanium Rapid Library Preparation kit (Roche). Then, DNA adopters designed to identify each sample were added by using a GS FLX Titanium Rapid Library MID Adaptors kit (Roche). Each viral genome was sequenced by the multiplex sequence method with an identification adopter using a GS FLX sequencer (Roche). The sequence data were automatically assembled for each virus using a v2.3 GS De Novo Assembler (Roche) and manually reassembled with Sequencher v4.9 (Hitachi Software, Tokyo, Japan). Putative open reading frames (ORFs) were identified by analysis using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Automated comparisons of the viral sequences with genetic databases were performed using the Basic Local Alignment Research Tool (BLAST) program.

The S1 nuclease-resistant fragment (~1 kbp) in the CtenDNAV type II genome was purified using a QIAquick PCR purification kit (Qiagen). It was then blunt-ended using T4 DNA polymerase (TaKaRa Bio) and ligated into the pUC 118 DNA HincII/BAP vector (TaKaRa Bio). Sequencing reactions were performed using universal primers (M13 forward and M13 reverse) and BigDye Terminator v3.1 (Life Technologies). Sequencing was performed using an ABI Prism 3730xl DNA analyzer (Life Technologies).

Viral proteins. One hundred microliters of purified viral samples from 500-ml lysates of CtenDNAV type II and CtenRNAV type II were mixed with the same volume of denaturing sample buffer (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 20% glycerol, and 0.005% bromophenol blue) and boiled for 5 min. The proteins were then separated using SDS-polyacrylamide gel electrophoresis

(PAGE) (12% polyacrylamide gel; 40 mA, 50 min) and a mini-Protean system (Bio-Rad, Richmond, CA). The proteins were visualized by staining with Coomassie brilliant blue (CBB) stain. Precision Plus protein standards (Bio-Rad) were used for size calibration.

Phylogenetic analysis. We identified a conserved putative viral protein (VP3) related to DNA replication in the genomic sequences of CtenDNAV type II, SS10V-24V, and SS10V-35V using BLAST. The deduced amino acid sequence of the corresponding region was compared with those for other viral VP3 sequences. Sequences were automatically aligned using ClustalW (20) and manually refined. For CtenRNAV type II, SS10V-39V, and SS10V-45V, we identified the RNA-dependent RNA polymerase (RdRp) gene region in the genomic sequence using BLAST. The deduced amino acid sequence of RdRp was compared with other viral RdRp sequences. Sequences were aligned using ClustalW and manually refined. Phylogenetic trees were constructed using the maximum likelihood (ML) method with the Jones-Taylor-Thornton matrix that is part of MEGA 5.2 (21). The amino acid sequences discussed in this work were used for comparison in our analyses (database accession numbers refer to the U.S. National Center for Biotechnology Information [NCBI] and the DNA Data Bank of Japan [DDBJ] databases).

Growth experiment. Exponentially growing cultures of *C. tenuissimus* 2-10 (100 ml) were inoculated with CtenDNAV type II and CtenRNAV type II at multiplicities of infection of 0.019 and 0.023, respectively. A host culture inoculated with autoclaved culture medium served as the control. Aliquots of the cell suspensions were sampled from each culture each day postinoculation (dpi), and the numbers of host cells and lytic agents were estimated. Cell counts were carried out with a Fuchs-Rosenthal hemocytometer using a Nikon inverted optical Ti-U microscope without fixation of the samples. The viral titers (i.e., the numbers of viral infectious units) were estimated using the extinction-dilution method. Briefly, the samples that were used for estimating the viral titer were passed through 0.8-μm-pore-size polycarbonate membrane filters (Nuclepore) to remove cellular debris. The filtrates were diluted with modified SWM3 medium in a series of 11-fold-dilution steps. Aliquots (100 μl) of each dilution were added to 8 wells of a 96-well flat-bottom plate that contained 150 μl of an exponentially growing culture of host algae. The cell culture plates were incubated at 15°C under a 12-h-light:12-h-dark cycle of 130 to 150 μmol photons $m^{-2} \cdot s^{-1}$ with cool white fluorescent illumination and were monitored by optical microscopy over 14 days for the occurrence of culture lysis. Culture lysis due to virus infection was usually observed as near-complete destruction of the host cells in a well. Using a BASIC program, we calculated the viral titer from the number of wells in which algal lysis occurred (22).

The latent period was estimated from the incubation period for the first viral titer increases after inoculations. The burst size was calculated on the basis of the increase in the infectious titer per decreased host cell number in a given period.

Nucleotide sequence accession number. Newly determined sequence data were deposited in GenBank under accession number AB971658.

RESULTS AND DISCUSSION

Isolation of viral pathogens and determination of their host range. Clonally isolated viruses retained their lytic activity (Fig. 1A to C) and were serially transferable to exponentially growing *C. tenuissimus* cultures. The cytoplasm and photosynthetic pigments were found to be degraded in the CtenDNAV type II-infected and CtenRNAV type II-infected cells, unlike those in the healthy cells (Fig. 1D to F). The host range of the virus was tested using 17 phytoplankton strains, including 13 diatom strains. CtenDNAV type II lysed only *C. tenuissimus* and no other microalgal species, indicating that CtenDNAV type II has high infection specificity (Table 1). CtenDNAV type I showed similar host species specificity (12). CtenRNAV type II lysed *C. tenuissimus* strain 2-10 but not strain 2-6, similarly to CtenRNAV type I (13). Our analyses also revealed an interesting host specificity characteristic of CtenRNAV type II. This RNA virus lysed four different diatom species in addition to *C. tenuissimus* 2-10: *C. setoensis* strain IT07-C11, *C. socialis* f. *radians* strain L-4, *Chaetoceros* sp. SS628-11, and *Chaetoceros* sp. TG07-C28 (Table 1). *Chaetoceros* sp. SS628-11 and *Chaetoceros* sp. TG07-C28 were species different from *C. tenuissimus* (23). Infection with and replication of CtenRNAV type II in *C. tenuissimus* 2-10, *C. setoensis* strain IT07-C11, and *Chaetoceros* sp. SS628-11 were also identified by the accumulation of the viral genome and its complementary strand of its genome in the host cells, measured using a Northern blot assay (see Fig. S1 in the supplemental material). CtenRNAV type II infects various *Chaetoceros* species, even though it has been reported that diatom ssRNA viruses infect only specific species (13, 24, 25, 26). Therefore, CtenRNAV type II may have an impact on the population dynamics not only of *C. tenuissimus* but also of various other *Chaetoceros* species. Additionally, in general, viral adsorption to the host cell surface is one of the most important steps in viral infection, and viral adsorption depends on the binding between the viral capsid proteins and host cell membrane proteins which play roles as viral receptors (27). The amino acid sequence and protein modification patterns of these proteins affect their binding. The diatom species susceptible to CtenRNAV type II may have receptor proteins similar to those of *C. tenuissimus*. Thus, CtenRNAV type II will be useful for improving our understanding of diatom-virus adhesion mechanisms in viral infection. Identification of the viral receptors will be necessary for further understanding diatom host-virus systems.

Morphological features. Thin sections of healthy *C. tenuissimus* cells exhibited the cytoplasmic organization and frustules that are typical of diatom cells seen under transmission electron microscopy (Fig. 2A). No virus-like particles (VLPs) were detected in healthy control cells (Fig. 2A). *C. tenuissimus* cells inoculated with CtenDNAV type II exhibited the presence of VLPs that were assembled in the host nucleus (Fig. 2B). VLPs formed random aggregations and paracrystalline arrays in the host nucleus (Fig. 2C and D). In addition, 22-nm-wide rod-shaped structures were observed in host cell nuclei (Fig. 2E and F). Similar structures have also been reported for other diatom DNA viruses: CtenDNAV, *C. lorenzianus* DNA virus (ClorDNAV), and *C.* sp. strain TG07-C28 DNA virus (Csp05DNAV) (12, 28, 29). These morphological observations may be common features of diatom DNA viruses and suggest that rod-like VLPs in virus-infected host cell nuclei may be precursors of mature virions. The rod-shaped structures observed in the thin sections of CtenDNAV type II-infected cells were not detected in the lysate in this study, which may support this hypothesis. Furthermore, in the present study, several virus particles were observed on the distal end of a rod-shaped structure (Fig. 2F and G). This might indicate that mature viruses were segregated from the rod-shaped precursors. However, Kimura and Tomaru (22) have suggested the possibility that the rod-shaped particles are a coinfecting virus for the ssDNA diatom virus *C.* sp. strain SS628-11 DNA virus (Csp07DNAV), infectious to *Chaetoceros* sp., because similar rod-shaped particles were observed in the lysate and the thin sections, along with mature virions. To elucidate the role of the rod-shaped structures, immunological methods and three-dimensional structural analysis may be necessary.

In CtenRNAV type II-inoculated host cells, many VLPs were randomly distributed throughout the cytoplasm (Fig. 2I). Several

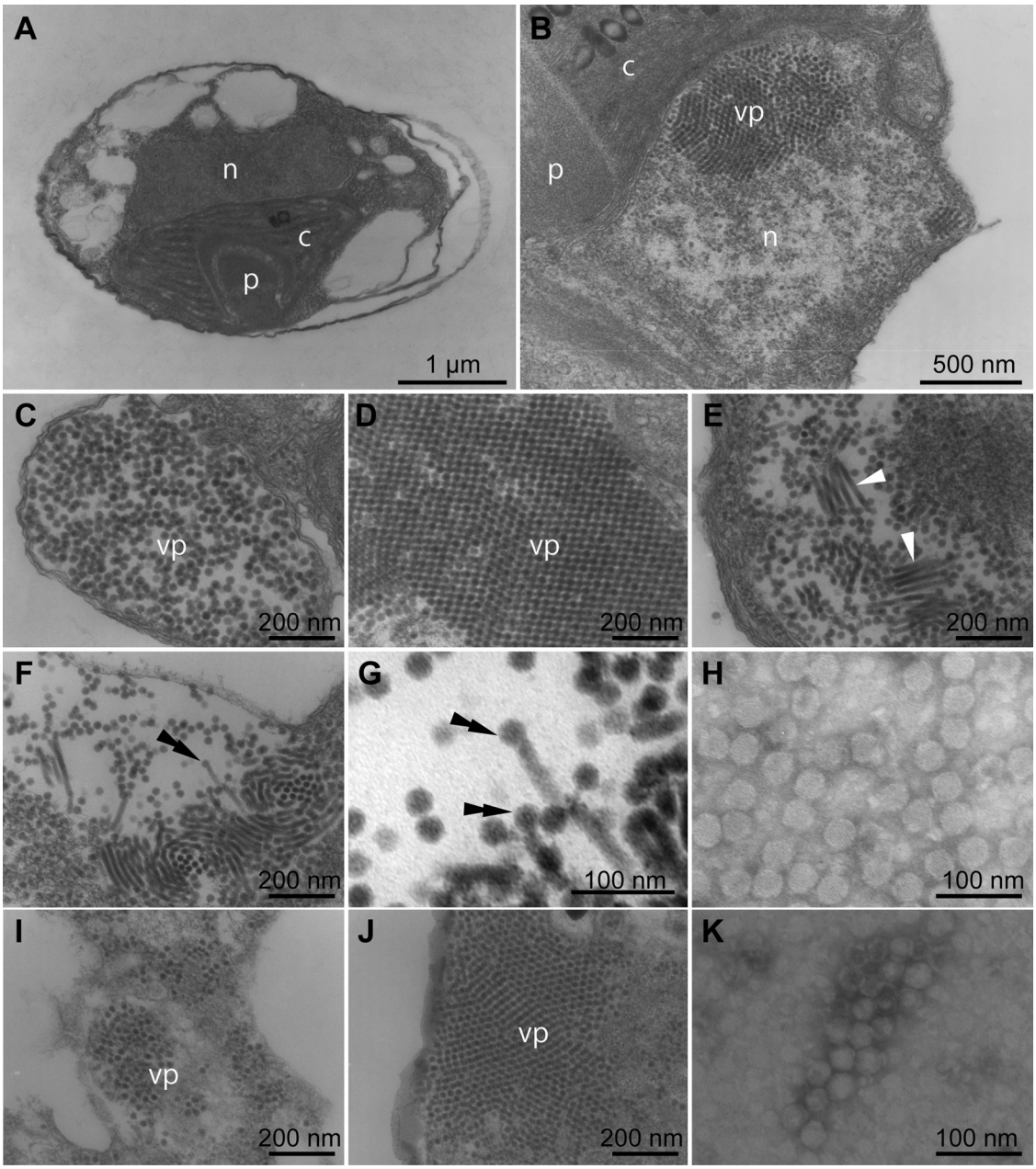

FIG 2 Transmission electron micrographs of ultrathin sections of *C. tenuissimus* strain 2-10 and negatively stained CtenDNAV type II and CtenRNAV type II particles. (A) A healthy cell. (B to G) Cells infected with CtenDNAV type II at 2 dpi. (B) Higher magnification of a nucleus with virus-like particles (VLPs). (C) Random aggregation of VLPs in host nuclei. (D) Crystalline array aggregation of VLPs in an infected host nucleus. (E) Rod-shaped particles in an infected host nucleus. (F and G) VLPs at the distal end of rod-shaped particles (F) and at high magnification (×60,000) (G). (H) Negatively stained CtenDNAV type II particles in culture lysate. (I and J) Cells infected with CtenRNAV type II at 3 dpi. (I) Random aggregated VLPs in the host cytoplasm at higher magnification. (J) Crystalline array aggregation of VLPs. (K) Negatively stained CtenRNAV type II particles in culture lysate. n, nucleus; c, chloroplast; p, pyrenoid; vp, virus particles. Arrowheads, rod-shaped particles; double arrowheads, the VLPs located at the distal end of the rod-shaped particles.

aggregates of the VLPs formed crystalline arrays (Fig. 2J), as observed in the previously isolated CtenRNAV type I (13).

In the culture lysates for both the viruses, VLPs similar to those observed in the thin sections were observed using negative staining. Virion sizes of CtenDNAV type II and CtenRNAV type II were 37 ± 2 nm (n = 100) and 35 ± 1 nm (n = 50), respectively, and both virions were icosahedral and lacked a tail and an outer membrane (Fig. 2H and K). Because both pathogens were transferable to a fresh host culture and VLPs were observed in the lysed culture and not in healthy cultures, we concluded that both observed VLPs were newly isolated viruses that are pathogenic to *C. tenuissimus*.

Viral genome and proteins of CtenDNAV type II. Denaturing agarose gel electrophoresis of CtenDNAV type II showed major nucleic acid bands at ca. 6 and 10 kb, with an additional small band at ca. 0.9 kb (Fig. 3A, lane 1). After heat treatment at 100°C for 5

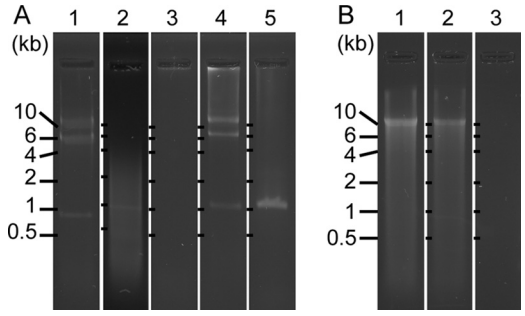

FIG 3 Nucleic acid analysis of CtenDNAV type II and CtenRNAV type II. (A) CtenDNAV type II genome. Nucleic acids of CtenDNAV type II without treatment (lane 1), treated at 100°C for 5 min (lane 2), treated with DNase I (lane 3), treated with RNase A (lane 4), and treated with S1 nuclease (lane 5) are shown. The samples were electrophoresed on a formaldehyde-agarose gel. (B) CtenRNAV type II genome without treatment (lane 1) and treated with DNase I (lane 2) and RNase A (lane 3).

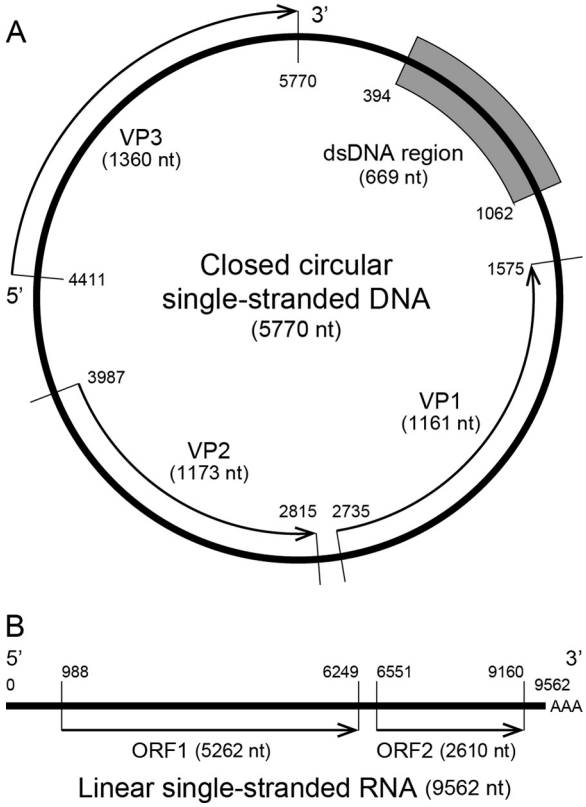

FIG 4 Schematic genomic structures of CtenDNAV type II and CtenRNAV type II. (A) CtenDNAV type II genome. The arrows indicate the locations for ORFs VP1 to VP3, and the shaded box represents the partially double-stranded DNA region. (B) CtenRNAV type II genome. The arrows indicate ORF1 and ORF2.

min, the intensity of the two larger bands disappeared (Fig. 3A, lane 2). All bands were digested by treatment with DNase I but not by RNase A treatment, indicating that the viral genome is composed of DNA (Fig. 3A, lanes 3 and 4, respectively). In addition, although the genome was digested by S1 nuclease, the band at ca. 0.9 kbp remained undigested (Fig. 3A, lane 5). The shorter nucleic acid, therefore, may be composed of double-stranded DNA (dsDNA). A genome structure composed of covalently closed circular ssDNA that contains a partially dsDNA region has typically been observed for most ssDNA diatom viruses reported to date (12, 23, 28, 29, 30, 31). Genome sequencing of CtenDNAV type II showed that the whole genome was 5,570 nt in length and that the short DNA fragment which remained after digestion with S1 nuclease was 669 nt. Therefore, it was concluded that the CtenDNAV type II genome was composed of 5,570-nt closed circular ssDNA harboring a region of 669-nt dsDNA (GenBank accession no. AB971658) (Fig. 4A). The sequenced ssDNA (5,570 nt) and a dsDNA fragment (669 nt) may correspond to the ~6-kb and ~1-kb bands of CtenDNAV type II DNA seen by agarose gel electrophoresis, respectively. The covalently closed circular DNA has lower mobility on agarose gel than linear DNA (30). Thus, the ~6-kb and ~10-kb (Fig. 3A, lane 1) bands may correspond to linear and closed circular viral DNAs, respectively.

On genome-wide analysis, nucleotide sequences of CtenDNAV type II (SS10-8V) were highly similar to those of other isolates, SS10-24V and SS10-35V (see Fig. S2 in the supplemental material). Compared to the CtenDNAV type II genome, only 3 and 204 single nucleotide mutations were identified in the SS10-24V and SS10-35V sequences, respectively. At least 40% of the genome sequences of CtenDNAV type I and type II differ (see Fig. S3). Therefore, the group of CtenDNAV type II, SS10-24V, and SS10-35V would be different from CtenDNAV type I. To understand the ssDNA viral species differentiations, mutations, and evolutions, further accumulations of this virus group information will be essential.

The CtenDNAV type II genome includes at least 3 major ORFs (≥300 amino acids [AAs]), designated VP1 to VP3 (Fig. 4A). The largest ORF, VP3, with 453 AAs, was predicted to encode a replication protein and showed high similarity to the putative protein of CtenDNAV type I (E-value, 0.00; identity, 78%) and lower similarity to the other eight diatom DNA viruses. It showed low similarity to the replication protein of circoviruses (family Circoviridae, genus Circovirus [32]), for example, a beak and feather disease virus (E-value, 4e−5), and to the replication protein of goose circovirus (E-value, 3e−5). VP1 (387 AAs) and VP2 (391 AAs) also had similarities to the putative proteins of CtenDNAV type I VP1 (E-value, 1e−28) and VP2 (E-value, 2e−69), respectively. Although the role of the VP1 is still unknown, our preliminary experiments, including analysis of the N terminus of the CtenDNAV type I protein, predicted that VP2 is a viral structural protein (unpublished data). The sizes and numbers of structural proteins of the virus particles were determined using SDS-PAGE. CtenDNAV type II expressed at least 1 protein at 39.0 kDa (Fig. 5, lane A).

On the basis of the morphological, genomic, and structural characteristics described above, CtenDNAV type II appears to belong to the genus *Bacilladnavirus*, recently accepted by the International Committee on the Taxonomy of Viruses (ICTV) (Table 2). For plant viruses, similarity of amino acid sequences of viral structural proteins is used for viral classification at the species and genus levels, as follows: viruses with >90% similarity are defined as the same species, those with 55% to 75% similarity are defined as different species, and those with <30% similarity are defined as different genera (33). In the present study, amino acid residues of the CtenDNAV type II VP2 protein which was predicted to be a structural protein showed only 38.3% similarity to those of the CtenDNAV type I VP2 protein (see Fig. S6A in the supplemental

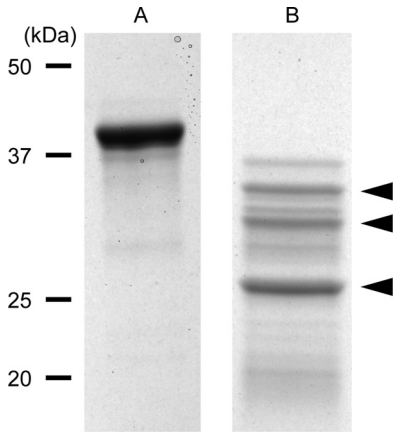

FIG 5 Patterns of SDS-polyacrylamide gel electrophoresis of viral structural proteins. (A) CtenDNAV type II. (B) CtenRNAV type II.

material), supporting the idea of the distinctness of the CtenDNAV type II virus.

Viral genome and proteins of CtenRNAV type II. Denaturing agarose gel electrophoresis showed a nucleic acid band of CtenRNAV type II of a fragment larger than 10 kb (Fig. 3B, lane 1). The genome treated with DNase I remained undigested (Fig. 3B, lane 2); treatment with RNase A resulted in complete digestion (Fig. 3B, lane 3). These results indicate that the genome of CtenRNAV type II is ssRNA. Sequence analysis showed that the genome is 9,562 nt in length, excluding the poly(A) tail (DDBJ accession no. AB971658) (Fig. 4B). This feature corresponded to the typical genomic structure of diatom RNA viruses, including *Rhizosolenia setigera* RNA virus (RsetRNAV), CtenRNAV, *C. socialis* f. *radians* RNA virus (CsfrRNAV), *C.* sp. strain SS08-C03 RNA virus (Csp03RNAV), and *Asterionellopsis glacialis* RNA virus (AglaRNAV) (13, 24, 25, 26, 31).

Nucleotide sequences of CtenRNAV type II (isolate name, SS10-16V), SS10-39V, and SS10-45V were highly similar to each other, with 99% similarity (see Fig. S4 in the supplemental material). The sequences of CtenRNAV type I, however, seemed not to be similar to that of CtenRNAV type II (similarity = 46%) (see Fig. S5). Even considering the differences of their sequences alone, the group of CtenRNAV type II would be different from CtenRNAV type I.

The CtenRNAV type II genome includes two ORFs (see Fig. 4B in the supplemental material). The forward ORF, ORF1, with 1,754 AAs, was predicted to encode a replication protein and showed highest similarity to the putative protein of another diatom RNA virus, Csp03RNAV, by BLAST search (E-value, 0.0; identity, 37%). The ORF also showed high similarity to sequences encoding the replication proteins of four other diatom RNA viruses, RsetRNAV, CtenRNAV, CsfrRNAV, and AglaRNAV. The other ORF, ORF2, with 870 AAs, was predicted to be a structural protein highly similar to that of Csp03RNAV (E-value, 0.0; identity, 54.7%). The structural proteins of CtenRNAV type II were separated using SDS-PAGE. Three major fragments were detected on the gel at 32.2, 29.0, and 26.1 kDa (Fig. 5, lane B). Multiple fragments of structural proteins have been detected in other diatom RNA viruses, using SDS-PAGE (13, 24, 25, 26). The molecular mass of the ORF2 protein, which was predicted from the viral genome, was ca. 96.2 kDa. This value was close to the total molec-

TABLE 2 Basic characteristics of known single-stranded DNA diatom viruses

Virus	Host	Isolation field	Isolation yr(s)	Particle characteristic(s)			Major protein(s) (kDa)	Latent period (h)	Burst size (no. of infectious units cell^{-1})	Genomic structure	Genome length (nt)	Complementary fragment length(s) (nt)	Database accession no.	Reference or source
				Size (nm)	Assembly site and aggregation pattern	Rod shape								
CdebDNAV	*Chaetoceros debilis*	Ariake Sound	2003–2005	32	Nucleus, random	ND[b]	37.5, 41	12–24	55	ND	ca. 7 kb (not fully sequenced)	ND	AB504376	42
ClorDNAV	*Chaetoceros lorenzianus*	Hiroshima Bay	2007	34	Nucleus, random, ring formation	Yes	<225[a]	48	2.2×10^4	Covalently closed circular	5,813	979	AB553581	28
CsalDNAV	*Chaetoceros salsugineum*	Ariake Sound	2003	38	Nucleus, random	ND	43.5, 46	12–24	325	Covalently closed circular	6,000	997	AB193315	30
CsetDNAV	*Chaetoceros setoensis*	Hiroshima Bay	2007	33	Nucleus, random	Yes	31, 37	48	2.0×10^4	Covalently closed circular	5,836	67, 70, 72, 76, 90, 107, 109, 145	AB781089	43
CtenDNAV type I	*Chaetoceros tenuissimus*	Ariake Sound	2005	37	Nucleus, random	Yes	38.5	96	320	Covalently closed circular	5,639	875	AB597949	11
CtenDNAV type II	*Chaetoceros tenuissimus*	Hiroshima Bay	2010	37	Nucleus, random, crystalline array	Yes	39.0	<24	1737	Covalently closed circular	5,570	669	AB971658	This study
Csp05DNAV	*Chaetoceros* sp. strain TG07-C28	Ago Bay	2008	33	Nucleus, random	Yes	40, 75	<24	ND	Covalently closed circular	5,785[a]	890[a]	AB647334	29
Csp07DNAV	*Chaetoceros* sp. strain SS628-11	Hiroshima Bay	2011	33.7	Nucleus, random, crystalline array	Yes	38.5	<12	29.1	Covalently closed circular	5,552	827	AB844272	22
TnitDNAV	*Thalassiosira nitzschioides*	Ariake Sound	2010	35	Nucleus, paracrystalline array	ND	ND	ND	ND	Covalently closed circular	5,573[a]	ca. 600 (not sequenced)	AB781284	31

[a] Unpublished data.
[b] ND, not determined.

TABLE 3 Basic characteristics of known single-stranded RNA diatom viruses[a]

Virus	Host	Isolation field	Isolation yr	Particle size (nm)	Major proteins (kDa)	Latency period (h)	Burst size (no. of infectious units cell^{-1})	Genome length (nt)	Database accession no.	Reference or source
CtenRNAV type I	*Chaetoceros tenuissimus*	Ariake Sound	2004	31	33.5, 31.5, 30.0	<24	1.0×10^4	9,431	AB375474	13
CtenRNAV type II	*Chaetoceros tenuissimus*	Hiroshima Bay	2010	35	32.2, 29.0, 26.1	24–48	287	9,562	AB971661	This study
CsfrRNAV	*Chaetoceros socialis* f. *radians*	Hiroshima Bay	2005	22	32.0, 28.5, 25.0	<48	66	9,467	NC_012212	25
Csp03RNAV	*Chaetoceros* sp. strain SS08-C03	Yatsushiro Sea	2009	32	42.0, 34.0, 28.0	<48	ND	9,417	AB639040	26
RsetRNAV	*Rhizosolenia setigera*	Ariake Sound	2002	32	41.5, 41.0, 29.5	48	3,100	8,877	NC_018613	24
AglaRNAV	*Asterionellopsis glacialis*	Ariake Sound	2009	31	ND	ND	ND	8,842[b]	AB973945	31

[a] For all of the viruses listed, the particle assembly site was the cytoplasm, the genomic structure was linear, and each had two ORFs. ND, not determined.
[b] Unpublished data.

ular mass of the fragments detected using SDS-PAGE. Therefore, the ORF2 protein may be a polyprotein that encodes three different viral structural proteins.

The structural features of the CtenRNAV type II genome were similar to those of the genomes of other diatom RNA viruses (Table 3). The amino acid sequences of the structural proteins of CtenRNAV type II showed 27.6% identity to those of CtenRNAV type I (see Fig. S6B in the supplemental material) (Table 4). According to a plant virus classification report (33), this result indicates that the two viruses belong to two different species. Thus, CtenRNAV type II appears to be a new virus species, distinct from CtenRNAV type I, in the genus *Bacillarnavirus*.

Phylogeny. ML methods were used to assess the phylogenetic relationships among the VP3 proteins of the diatom ssDNA viruses, which encode a putative replication-related protein and which showed higher similarity to diatom viruses. The monophyly of ClorDNAV, Csp05DNAV, Csp07DNAV, *C. salsugineum* DNA virus (CsalDNAV), CtenDNAV type I, and CtenDNAV type II was supported by a high bootstrap value (Fig. 6A). This result indicated that CtenDNAV type II belongs in the clade that includes the genus *Bacilladnavirus*, whereas TnitDNAV, CdebDNAV, and CsetDNAV may belong to separate groups. To understand the phylogenetic relationships of the ssDNA viruses which infect diatoms, it is necessary to reconsider the definition of the genus *Bacilladnavirus* on the basis of information from a greater diversity of diatom viruses.

Phylogenetic analysis of ssRNA viruses on the basis of the deduced amino acid sequence of RNA-dependent RNA polymerase (RdRp) showed that diatom RNA viruses are monophyletic; this result was supported by a high bootstrap value (100%) (Fig. 6B). In addition, the idea of the monophyly of CtenRNAV type II and Csp03RNAV was supported by a bootstrap value of 99% (Fig. 6B). In contrast, CtenRNAV type II and CtenRNAV type I were identified as different species, on the basis of the amino acid sequences of the viral structural proteins (see above), although they both infect host strain *C. tenuissimus* 2-10 but not strain 2-6 (Fig. S3B). Structural proteins of CtenRNAV type II and other centric diatom viruses, except Csp03RNAV, showed <30% similarity, according to the results of a BLAST search (Table 4) (33). In this report, it has been suggested that viruses with less than 30% similarity of amino acid sequences encoding viral structural proteins should be classified as different genera (33). Also, reconsideration of the taxonomy of ssRNA viruses infecting stramenopiles, including *Heterosigma akashiwo* RNA virus (HaRNAV) and *Aurantiochytrium* single-stranded RNA virus (AuRNAV), which infect the harmful bloom-forming phytoplankton *Heterosigma akashiwo* (Raphidophyceae) and the marine fungoid protist *Aurantiochytrium* sp. (Thraustochytriaceae), respectively, will be necessary in the near future to improve our knowledge of this viral group.

TABLE 4 Similarity of structural proteins of each diatom virus to those of CtenRNAV type-II

Virus name	Length (nt)	% identity	E-value
AglaRNAV	946	30.3	e−140
CsfrRNAV	897	24.7	2e−082
Csp03RNAV	791	54.7	0
CtenRNAV type-I	882	27.6	e−106
RsetRNAV	961	26.1	e−105
SS10V-39V	870	99.5	0
SS10V-45V	870	99.5	0

Replication. The host population in the culture without viral inoculation was maintained after it reached the stationary phase (Fig. 7A). The cell number in the CtenDNAV type II-inoculated culture during its early stationary phase rapidly decreased after 2 dpi (Fig. 7B). Viral titers rapidly increased after 1 dpi and reached a maximum of 5.10×10^8 infectious units · ml^{-1}. Thus, the latent period of CtenDNAV type II was estimated to be <24 h. The burst size was estimated to be approximately 1.70×10^3 infectious units · cell^{-1} from 3 to 4 dpi. The *C. tenuissimus* strain 2-10 culture inoculated with CtenDNAV type I at 20°C showed a significant cell number decrease at 6 dpi, and the viral titer gradually increased after 6 dpi (14). The maximum yield and burst size in that case were estimated to be 1.03×10^8 infectious units · ml^{-1} and 960 infectious units · cell^{-1}, respectively (14). Therefore, these proliferation features of CtenDNAV type I and type II seemed similar.

In the CtenRNAV type II-inoculated culture during its early stationary phase, the host cell number was maintained until 3 dpi, and then it decreased (Fig. 7C). An increase of the viral titer was most often observed between 2 to 4 dpi; the maximum observed was 3.85×10^8 infectious units · ml^{-1} at 9 dpi. The latent period of

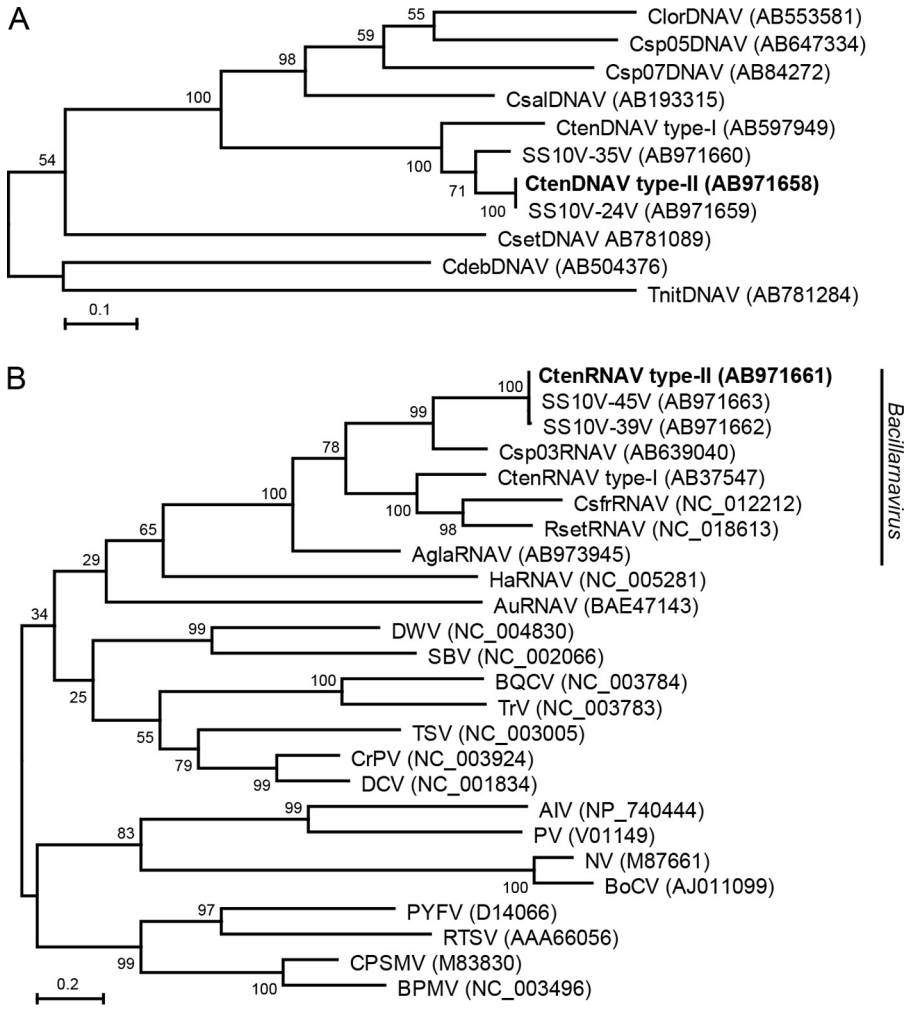

FIG 6 Phylogenetic analysis of diatom ssDNA viruses and ssRNA viruses. (A) Maximum likelihood (ML) trees of diatom viruses on the basis of amino acid sequences of VP3 which encoded viral replication genes. Bootstrap values (percent) from 1,000 samples are shown at the nodes. ML distance scale bars are shown under the ML tree. (B) ML trees of ssRNAV on the basis of amino acid sequences of structural protein encoded in ORF2. Bootstrap values (percent) from 1,000 samples are shown at the nodes. ML distance scale bars are shown under the ML tree. The bar on the right shows a genus of single-stranded diatom RNA virus, *Bacillarnavirus.* Virus names: AIV, Aichi virus; AuRNAV, *Aurantiochytrium* single-stranded RNA virus; BoCV, bovine enteric calicivirus; BMPV, bean pod mottle virus; BQCV, black queen cell virus; CPSMV, cowpea severe mosaic virus; CrPV, cricket paralysis virus; DCV, *Drosophila* C virus; DWV, deformed wing virus; HaRNAV, *Heterosigma akashiwo* RNA virus; NV, Norwalk virus; PV, Human poliovirus 1 Mahoney; PYFV, Parsnip yellow fleck virus; RTSV, Rice turgo spherical virus; SBV, sacbrood virus; TrV, Triatoma virus; TSV, Taura syndrome virus.

CtenRNAV type II was estimated to be 24 to 48 h. The burst size calculated from 2 to 3 dpi was 2.84×10^2 infectious units · $cell^{-1}$. According to a recent study of CtenRNAV type I proliferations performed using the 2-10 host strain, the latent period, maximum yield, and burst size at 20°C were 3 days, 2.55×10^{10} infectious units · ml^{-1}, and 1.86×10^4 infectious units · $cell^{-1}$, respectively (14). The latent period of CtenRNAV type I seemed longer than that of type II. However, both the maximum yield and burst size of the type I were 2 orders higher than those of the type II. Considering the differences between them with respect to the yields, the smaller burst size of CtenRNAV type II might be comprehensible. These differences might be reflected in their host-capturing strategies. The type II RNA viruses are not limited to infection of *C. tenuissimus* alone but also infect other *Chaetoceros* species; i.e., they are generalist viruses. The relatively smaller burst size of the type II RNA virus might be enough for its survival, considering the possibly greater opportunities for host capturing.

When the viral inoculations were conducted during the host logarithmic-growth phases, the latency periods and yields of both viruses seemed not greatly different from those seen in the early-stationary-phase cultures (Fig. 7E and F): <24 h and 5.10×10^8 infectious units · ml^{-1} for CtenDNAV type II and <48 h and 1.38×10^9 infectious units · ml^{-1} for CtenRNAV type II, respectively. Calculations of the burst sizes were difficult in these experiments, however, because the viral titer increased before the rapid decline in host cell numbers. An interesting phenomenon for comparing such logarithmic-phase or stationary-phase host cultures may be the differences in the time required for the host cultures to exhibit decreases in host cell numbers after viral inoculations. The logarithmic-growth-phase culture inoculated with CtenDNAV type II showed apparent cell number decreases after 5 dpi (Fig. 7E), whereas the stationary-phase culture did so only 2 dpi (Fig. 7B). In addition, the host population in the culture inoculated with CtenRNAV type II in the logarithmic phase was main-

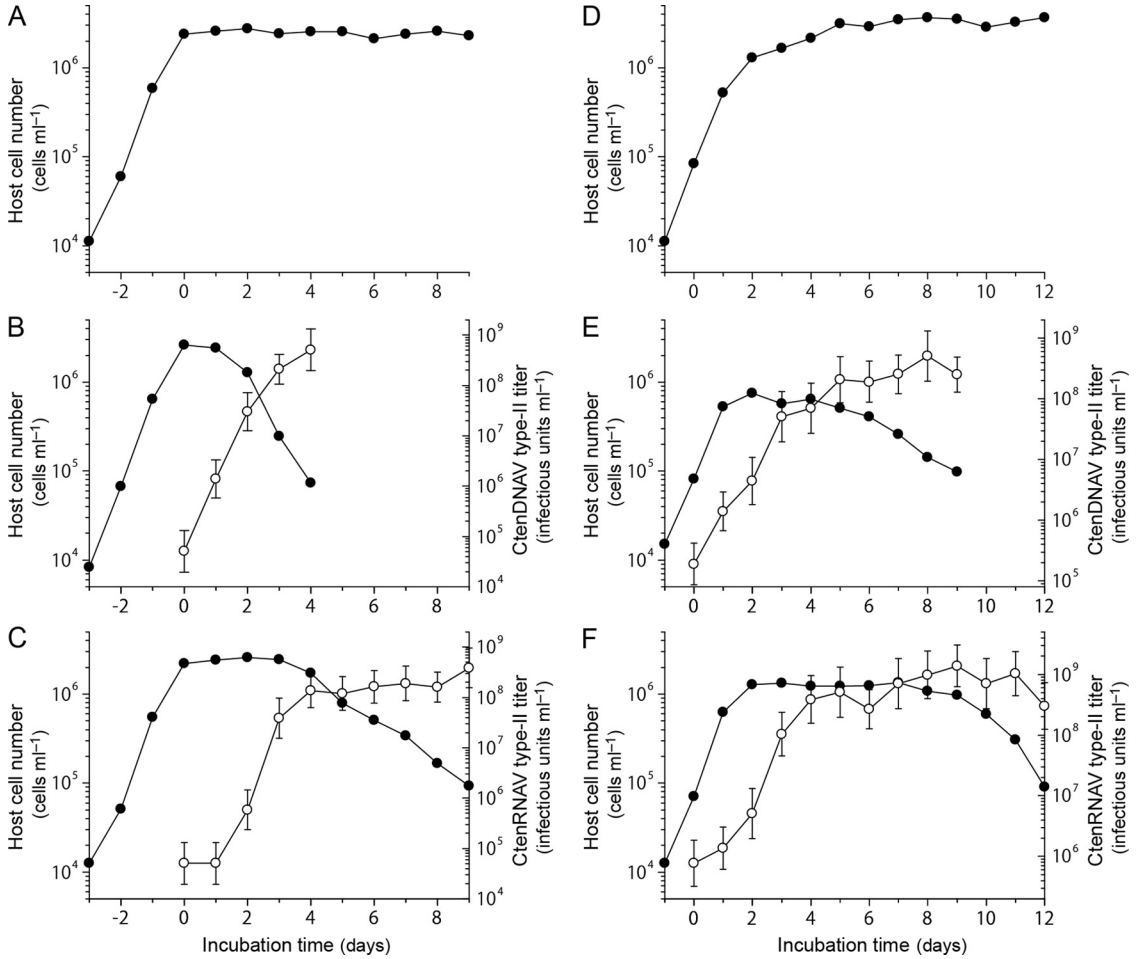

FIG 7 Growth experiments. (A to C) The results of analysis of stationary-phase cell cultures with viruses. (D to F) The results of analysis of logarithmic-phase cell cultures with viruses. (A and D) Plots of the cell density of *C. tenuissimus* strain 2-10 without virus inoculation (control culture). (B and E) Plots of the cell density of host cells with CtenDNAV type II inoculation (closed circles) and changes in viral titer (open circles). Virus was inoculated at day 0 at multiplicities of infection of 0.02 and 2.33 in experiments B and E, respectively. (C and F) Plots showing the cell density of host cells with CtenRNAV type II inoculation (closed circles) and changes in viral titer (open circles). Virus was also inoculated at day 0 at multiplicities of infection of 0.02 and 10.93 in experiments C and F, respectively.

tained until 7 dpi (Fig. 7F); however, the host population was evident only until 3 dpi in the stationary-phase culture (Fig. 7C). Similarly, in experiments performed using the *C. tenuissimus* CtenRNAV type I and RsetRNAV systems, the host populations during the logarithmic-growth phase showed continuous vigorous growth even after the viruses were inoculated, and the rapid decline in host cell numbers occurred after the cultures reached the stationary phase (13, 24). The viral susceptibility of the host cells has been considered to be related to host growth phases and conditions (5). The virus sensitivities of the diatom host cells might be also related to the growth phases. Considering that the virus titers rapidly increased even in the logarithmically growing host cultures for both the DNA and RNA viruses (Fig. 7E and F), the virus sensitivities of the host cells might not be uniform even in vigorous cultures; i.e., most cells might not permit the virus infection, but a part of the population might. The virus-sensitive cell percentage might increase when the host population reaches the stationary phase. Similar phenomena were previously observed in other diatom-virus systems (12, 13, 24, 25, 26, 28, 29, 30). The results of a recent preliminary study (14) may support the hypothesis that the viral sensitivities of diatom cells in a clonal population even under conditions of logarithmic growth are diverse. In that study, by the use of a semicontinuous culture method, they showed that the proportion of virus-sensitive cells in the *C. tenuissimus* strain 2-10 culture at 2 divisions · day^{-1} was 7% to 8%. Most of the diatom cells might be able to resist the viral infections under conditions of vigorous growth but not at lower growth rates. This would be reflected in the differences in the times required for the host cell numbers to decrease after viral inoculations.

The burst sizes determined by many diatom studies might be underestimated due to the problems presented by the presence of virus aggregations, readsorptions to viable cells, or cell debris. The virus particles are usually highly condensed aggregations such as paracrystalline arrays (13), and they might form aggregations >100 nm in diameter (34). The diatom virus numbers are now determined as viral infectious units by most probable number (MPN) method. The use of flow cytometry or transmission electron microscopy methods for counting viral particles may be possible; however, those methods also harbor technical problems:

there are no adequate dyes to detect small genomes (34, 35), and the analyses are labor consuming and less accurate (36, 37, 38). To increase the accuracy of measurements using any of the available methods, the viral aggregations should be disintegrated, and any associated problems might be overcome with cryopreservation and thawing (39). The most plausible way to determine the diatom virus burst sizes might be the use of a quantitative PCR (qPCR) method to detect the target copy number. Since determination of accurate viral burst sizes is important for understanding their ecology and impact on host organisms, future studies should address the problem by sophisticated methods.

Concluding remarks. The two novel viruses were isolated from the same sampling station in Hiroshima Bay in 2010. They are genetically distinct from viruses previously isolated from *C. tenuissimus* from western Japan, including Hiroshima Bay. The distribution of *C. tenuissimus* extends throughout western Japan, and strains are expected to possess a high degree of variation with respect to virus sensitivities (K. Kimura and Y. Tomaru, unpublished data). The genetic diversity of the viruses may correspond to variations in the host strain. Changes in environmental factors could have significant effects on host-virus systems, including host population dynamics and the dominant virus species. For example, water temperature and irradiance levels affect the relationship between *Phaeocystis globosa* (Prymnesiophyceae) and its lytic viruses (40, 41). Water temperature is also considered to be a significant factor controlling the *C. tenuissimus*, CtenDNAV type I, and CtenRNAV type I relationship (14). To date, at least four genetically distinct virus species have been identified that share a single diatom host, *C. tenuissimus*. Moreover, CtenRNAV type II was able to infect various diatom species. In general, diatom blooms are composed of multiple species which belong to the same taxonomic unit, and this is true in our study area. Such a multihost pathogen released from *C. tenuissimus* could affect other diatom populations and vice versa. Algal host-virus relationships in natural environments may be far more complex than expected. To improve our understanding of viral competition in host populations and viral impact on host dynamics in nature, we will need to conduct extensive physiological studies to analyze these systems under various environmental conditions in the field.

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K.K. and Y.T. designed the research, performed the research, analyzed the data, and wrote the paper.

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