

SHORT COMMUNICATION

First evidence for the existence of pennate diatom viruses

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Diatoms are considered the most successful and widespread group of photosynthetic eukaryotes. Their contribution to primary production is remarkably significant to the earth's ecosystems. Diatoms are composed of two orders: Centrales and Pennales. Thus far, viruses infecting centric diatom species have been isolated and characterized; however, viruses infecting pennates have not been reported. Here, we describe the first isolations and preliminary characterizations of two distinct pennate diatom viruses, AglaRNAV (31 nm in diameter, accumulates in the host cytoplasm) and TnitDNAV (35 nm in diameter, accumulates in the host nuclei) infecting *Asterionellopsis glacialis* and *Thalassionema nitzschioides*, respectively. Their genomes contain a single-stranded RNA of approximately 9.5 kb, and a closed, circular single-stranded DNA of approximately 5.5 kb harboring a partially double-stranded region, respectively. Further analysis of these viruses may elucidate many aspects of diatom host–virus relationships.

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Introduction

Diatoms constitute one of the major lineages of photosynthetic eukaryotes on earth, and there are probably more than approximately 10^5 species (Mann and Droop, 1996). Their contribution to global biogeochemical cycling is significant (Smetacek, 1999), and accounts for a large part of marine primary production, up to 35–75% in the oceans (Nelson *et al.*, 1995). On the basis of morphological features, diatom species are composed of two groups: centric and pennate. Complete genome sequences have recently been reported for members of these groups (Armbrust *et al.*, 2004; Bowler *et al.*, 2008), which revealed many aspects of diatom evolutionary history, ecology, metabolism and influence on biogeochemical cycling (Bowler *et al.*, 2010).

Marine virus studies during the last 2 decades have revealed the significance of viruses to host

population dynamics (Suttle, 2005). Diatom viruses are also considered to be important for the understanding of diatom life cycles and the impact of diatoms on biogeochemical cycling. So far, isolations of several viruses infecting centric diatoms have been reported (Tomaru *et al.*, 2011a). However, viruses have not been isolated from pennate species, constituting major components of marine environments (Kooistra *et al.*, 2007). Here, we report preliminary characterizations of viruses infecting the pennate diatoms *Asterionellopsis glacialis* (Castracane) Round and *Thalassionema nitzschioides* (Grunow) Mereschkowsky.

Materials, methods, results and discussion

The algal strains *A. glacialis* IT09-K25 (Figure 1a) and *T. nitzschioides* AR-TN01 (Figure 1b) were used for the experiments (Supplementary Table 1). Algal cultures were grown in modified SWM-3 medium (Itoh and Imai, 1987) at 15 °C under a 12/12 h light–dark cycle with a light intensity of 110–150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Surface waters from Ariake Sound, Japan (February 2010) and sediment samples (0–1 cm depth) from Ago Bay, Japan (March 2009) were used for isolating viruses infecting *T. nitzschioides* and *A. glacialis*, respectively. The water samples were

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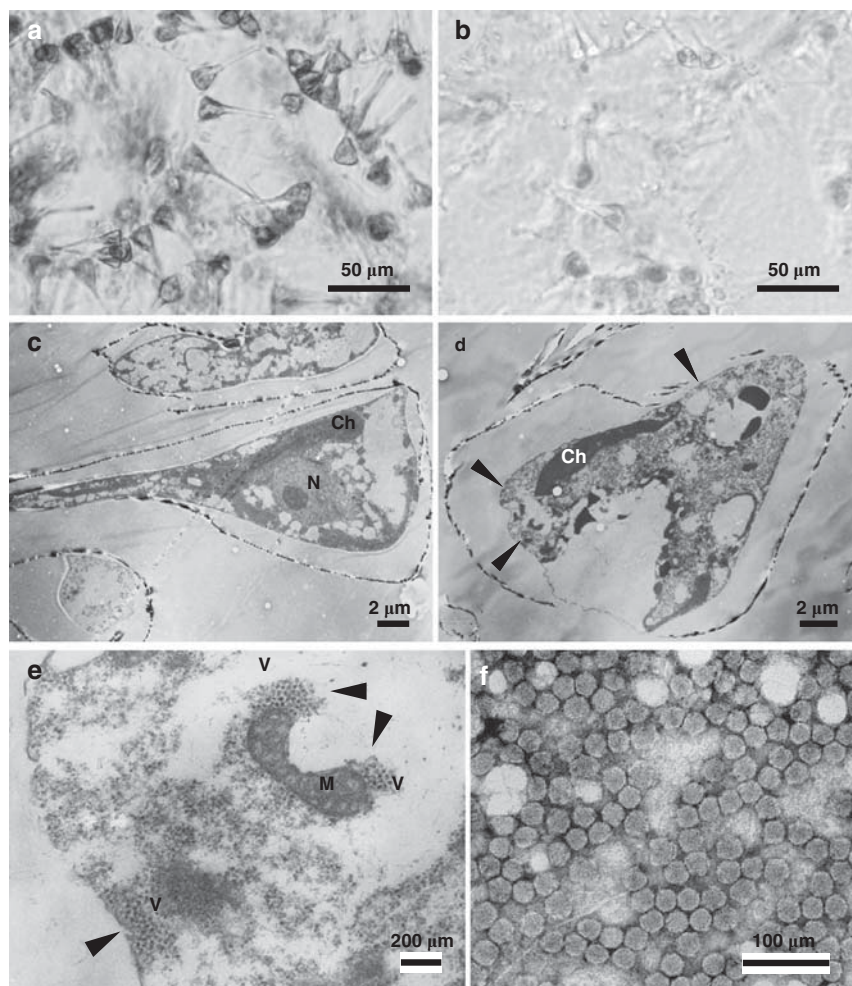


Figure 1 *Asterionellopsis glacialis* IT09-K25 isolated from surface water in Hiroshima Bay, Japan. (a) Optical micrograph of intact cells. (b) Cultures inoculated with AglaRNAV at 2 d.p.i.. Transmission electron micrographs of ultra-thin sections of *A. glacialis* and negatively stained AglaRNAV particles. (c) Healthy cell. (d and e) Cells infected with AglaRNAV at 2 d.p.i. (d) Degraded host cytoplasm and chloroplast. (e) Higher magnification of randomly aggregated VLPs in the host cytoplasm in panel d. Arrows denote the accumulation of VLPs. (f) Negatively stained AglaRNAV particles in purified culture lysate. N, nucleus; Ch, chloroplast; M, mitochondrion; V, virus-like particles.

filtered through 0.2- μm filters (Advantec, Tokyo, Japan) to remove microorganisms and the majority of bacteria. The filtrates were preserved at -20°C until the following isolation procedures. The sediment samples were collected using an Ekman-Birge bottom sampler (Rigo, Tokyo, Japan). Viruses in the sediments were extracted by the method described by Tomaru *et al.* (2011a).

Aliquots (0.5 mL) of the treated samples were inoculated into 1.0 mL of exponentially growing host cultures and incubated at 15°C using the light conditions described above. Algal cultures inoculated with SWM-3 served as controls. Cell condition and signs of lysis in each algal culture were monitored by optical microscopy (Tomaru *et al.*, 2011a). The lysed cultures were filtered through a 0.1- μm polycarbonate membrane filter (Whatman, Kent, UK) to exclude bacterial effects, and their lytic activity was reassessed. We assumed the pathogens were viruses and tentatively named the viruses

infecting *A. glacialis* and *T. nitzschioides* as AglaRNAV and TnitDNAV, respectively.

AglaRNAV was cloned through two extinction dilution cycles (Suttle, 1993). Cloning of TnitDNAV was unsuccessful because the host culture did not grow well in the microplates used for the cloning procedures and we, therefore, used non-cloned TnitDNAV in this study. Transmission electron microscopy observations for ultra-thin sections, negatively stained virions and viral genome analysis were performed using the method described by Tomaru *et al.* (2009, 2011a).

The occurrence of host cell lysis was usually observed 2 and 10 days post inoculation (d.p.i.) in *A. glacialis* and *T. nitzschioides*, respectively (Figures 1b and 2b). Thin sections of healthy host cells showed the cytoplasmic organization and frustules that are typical of the diatoms (Figures 1c and 2c). In contrast, electron micrographs of thin-sectioned *A. glacialis* cells at 2 d.p.i. revealed

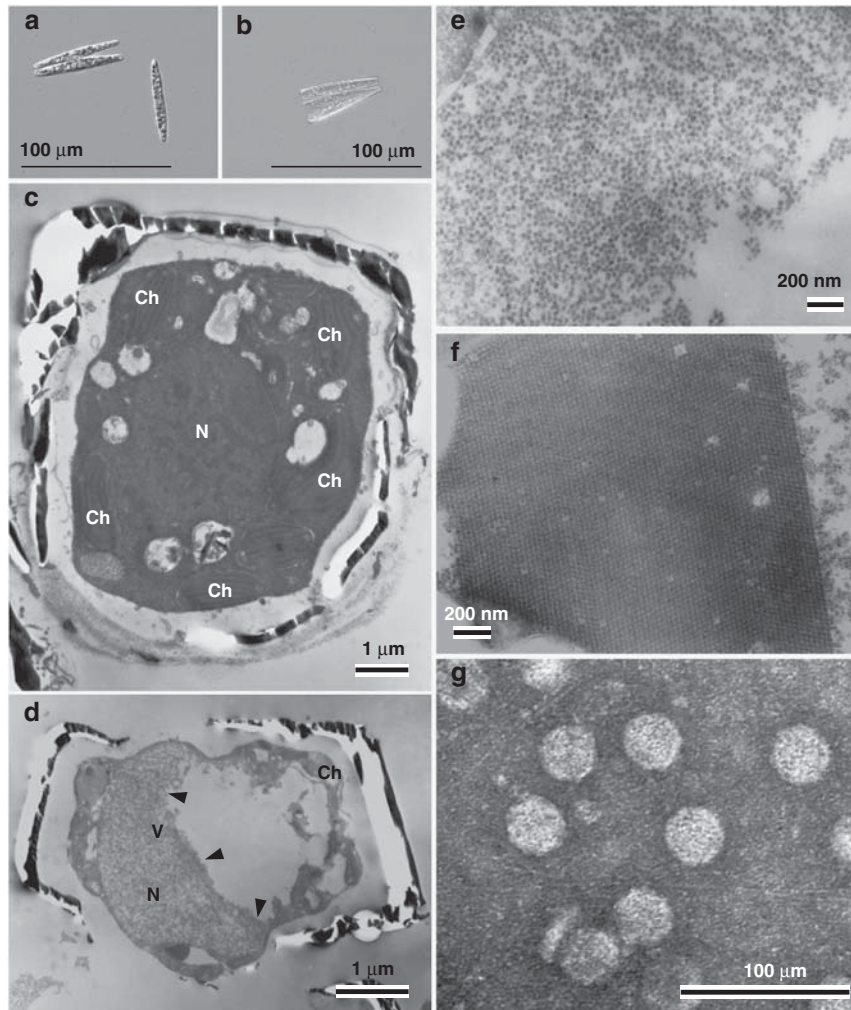


Figure 2 *Thalassionema nitzschioides* AR-TN01 isolated from surface water in Ariake Sound, Japan. (a) Optical micrograph of intact cells. (b) Cultures inoculated with TnitDNAV at 15 d.p.i.. Transmission electron micrographs of ultra-thin sections of *T. nitzschioides* and negatively stained TnitDNAV particles. (c) Healthy cell. (d, e and f) Cells infected with TnitDNAV at 8 d.p.i. (d) Degraded host cytoplasm and chloroplast. (e) Higher magnification of randomly aggregated VLPs in the host nucleus in panel d. (f) Para-crystalline array aggregation of VLPs in an infected host nucleus. (g) Negatively stained TnitDNAV particles in purified culture lysate. Arrows denote the accumulation of VLPs. N, nucleus; Ch, chloroplast; V, virus-like particles.

randomly assembled virus-like particles (VLP) in the host cytoplasm (Figures 1d and e). In *T. nitzschioides* cells at 8 d.p.i., the presence of randomly assembled or para-crystalline-arrayed VLP was observed in the host nuclei (Figures 2d, e, and f). VLPs were not found in healthy cells of either host species (Figures 1c and 2c). The diameters of VLPs in *A. glacialis* and *T. nitzschioides* were $31 (\pm 2)$ nm ($n=40$) and $34 (\pm 1)$ nm ($n=40$), respectively (Figures 1e and 2f).

VLPs were also observed in culture lysates by negative staining transmission electron microscopy. VLPs in the lysate of *A. glacialis* and *T. nitzschioides* were $31 (\pm 1)$ nm ($n=47$) and $36 (\pm 2)$ nm ($n=40$) in diameter, respectively (Figures 1f and 2g). They appeared similar to the VLP observed in their respective host cytoplasm and/or nuclei (Figures 1e and 2e). The particle diameters of these viruses were within a range of those of the centric diatom

viruses, 22–38 nm (Tomaru *et al.*, 2009; Tomaru *et al.*, 2011a).

The basic genome characters of the two viruses were examined by preliminary tests. Denaturing gel electrophoresis showed that the nucleic acids extracted from AglaRNAV were 9.5 kb, 4.0 kb and 2.5 kb (Supplementary Figure 1). These bands were sensitive to RNase A, both under high- and low-salt concentrations, but not to DNase I (Supplementary Figure 1), suggesting that AglaRNAV genome constituted a single-stranded RNA (ssRNA) molecule of approximately 9.5 kb. The lower bands were considered to be components of the viral genome, but not revealed in this study. Similar results were reported on ssRNA centric diatom virus CtenRNAV (Shirai *et al.*, 2008).

In a gel electrophoresis of closed circular single-stranded DNA (ssDNA) diatom viral genome harboring a complementary sequence fragment, two

larger bands with intact genome and single lower band with S1 nuclease treatment are usually observed at 5.0–6.0 kb and 0.8–1.0 kb, respectively (for example, Tomaru *et al.* (2011a)). The intact TnitDNAV genome exhibited two bands of nucleic acids of approximately 5.5 and 5.0 kb, and double-stranded DNA of 0.6 kbp remained undigested after S1 nuclease treatment (Supplementary Figure 2), which were considerably similar to the features of the ssDNA diatom viral nucleic acids. The above results suggested that the genome of TnitDNAV consists of a closed circular ssDNA molecule of approximately 5.5 kb, which contains a complementary fragment of ca 0.6 kb.

Previously reported centric diatom ssRNA and ssDNA viruses accumulate in their host cytoplasm and nucleus, respectively (Tomaru *et al.*, 2009; Tomaru *et al.*, 2011b), which agrees to the present observations. On the basis of the above results, we concluded that the particles in the host cells and in the lysates were viruses.

Implication

Considering the potential similarities between pennate and centric diatom virus features, diatom host–virus relationships may have been established in the early evolutionary stage of the diatoms. A recent study showed evidence for a horizontal gene transfer event from non-retroviral RNA viruses into the genomes of distantly related plant lineages (Chiba *et al.*, 2011), suggesting that lateral gene transfer could occur through viral infections. The understanding of pennate and centric diatom viruses may elucidate their hosts' phylogenetic relationships and evolution. The genome sequences and the precise features of the viruses described in this study are currently under analysis, and will be reported in the near future.

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