

## Preliminary Analysis of Diatom-infecting Viruses in Ariake Sound, Japan

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### Abstract

Certain marine diatoms can be harmful to seaweed cultivation farms, resulting in loss of yield and quality. Diatoms are exposed to viral attack; to date, more than 20 diatom-infecting viruses have been isolated and characterized to different extents. Nevertheless, no data have been reported on viruses infecting the harmful bloom-forming diatom *Asteroplanus karianus*, which causes bleaching of cultured *Pyropia*. In this study, we successfully isolated a novel virus that infects *A. karianus* from the sediment of the Ariake Sound, which is famous for its nori cultivation. The virion was 21 nm in diameter and exhibited apparent host cell lysis over 10 days post inoculation. Moreover, using degenerate PCR targeting the RNA-dependent RNA polymerase (RdRp) domain, ssRNA virus sequences were detected from a natural diatom bloom population, which included *A. karianus*, collected in the Ariake Sound. The phylogenetic analysis showed that the detected sequence was monophyletic with diatom-infecting ssRNA virus members at a bootstrap value of 85%. These results indicated that viruses infecting *A. karianus* are present in the Ariake Sound and may play a role in decreasing the diatom populations in natural environments.

**Discipline:** Fisheries

**Additional key words:** algal culture, *Asteroplanus karianus*, RdRp domain, virus isolation

### Introduction

The marine bloom-forming pennate diatom *Asteroplanus karianus* (Grunow) Gardner & Crawford, formerly described as *Asterionella kariana* Grunow and *Asterionellopsis kariana* (Grunow) Round (Crawford and Gardner 1997), is a cosmopolitan species, being distributed in Europe, Asia, the Atlantic Islands, South America, Africa, Australia, and New Zealand (Guiry and Guiry 2017). In the last decade, in Japan, this diatom has been reported as a bloom-forming species that is harmful to seaweed cultivation. In February 2008, *A. karianus* formed massive blooms ( $10^4$  cells mL<sup>-1</sup>) in seaweed, *Pyropia*, cultivation farms in the Ariake Sound

(Matsubara et al. 2014). Since then, winter blooms of *A. karianus* have occurred annually in this region (Yamaguchi et al. 2017). The blooms of this species, which occurred during winter season from December 2011 through January 2012, caused heavy discoloration of cultivated *Pyropia*, probably caused by nutrient deficiency, resulting in decreases in nori production and/or quality (Yamaguchi et al. 2014). Therefore, understanding the dynamics of *A. karianus* in Japan is important for the management of *Pyropia* cultivation.

As many studies have suggested, diverse physical and chemical factors affect diatom dynamics (Tilstone et al. 2000, Sarthou et al. 2005). Several researchers reported the effects of water temperature, salinity,

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nutrients, and light intensity on *A. karianus* (Yamaguchi et al. 2014, Shikata et al. 2015). Biological factors, such as predators and algicidal bacteria, are also considered important factors that determine diatom dynamics. Recent studies have shown the potential significance of viruses infecting the diatoms (Bettarel et al. 2005, Tomaru et al. 2011a), however, viruses lytic to *A. karianus* have not been reported so far. Here, we report the detection of viruses associated with diatom blooms in the Ariake Sound, as well as the challenges faced in the isolation of novel viruses that infect *A. karianus*. This research will aid our further understanding of *A. karianus* dynamics.

## Materials and methods

### 1. Algal cultures and growth conditions

A clonal *Asteroplanus karianus* strain, AK13-02, was isolated from surface water in the Ariake Sound off the Saga prefecture (mouth of the Shiota River, Stn. 1; 33°04'N, 130°11'E), Japan, on 3 January 2013. Algal cultures were grown in modified SWM3 medium enriched with 2 nM Na<sub>2</sub>SeO<sub>3</sub> (Chen et al. 1969, Itoh and Imai 1987) under a 12/12-h light-dark cycle of *ca.* 110 to 150  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ , using cool white fluorescent illumination at 15°C.

### 2. Virus isolation

Sediment samples (0-1 cm depth) were collected from the same sampling station mentioned above on 28 January 2013. During January 2013, a winter diatom bloom comprising *Skeletonema* spp., *A. karianus*, and *Thalassiosira* spp. occurred in this area (Yamaguchi et al. 2017). The sediment sample (3 g) was shaken with 3 mL of medium (400 rpm at room temperature for 30 min) and centrifuged at  $860 \times g$  at 4°C for 10 min. The supernatants were passed through 0.2- $\mu\text{m}$  Dismic-25cs filters (Advantec, Toyo Roshi, Ltd., Tokyo, Japan). Aliquots (0.2 mL) of the filtrates obtained from water and sediment samples were inoculated into exponentially growing *A. karianus* cultures (0.8 mL), followed by incubation at 15°C, using the light/dark cycle conditions described above. Algal cultures inoculated with SWM3 alone served as controls.

From the *A. karianus* cultures that showed an apparent crash after inoculation of the filtrates (e.g. Fig. 1), the pathogenic factors were cloned through two extinction dilution cycles (Suttle 1993, Tomaru et al. 2004). The resultant lysate was referred to as a clonal pathogen, AkarV strain Mss12V-75.

### 3. Inoculation test

An exponentially growing culture of *A. karianus* AK13-02 (2 mL) was inoculated with 30  $\mu\text{L}$  of the AkarV suspension, which was passed through a 0.1- $\mu\text{m}$  filter (Millex filter, Millipore, MA, USA). A host culture inoculated with an autoclaved culture medium served as the control. The cultures were then incubated at 15°C and 20°C under a 12/12-h light-dark cycle of *ca.* 110 to 150  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ , using cool white fluorescent illumination. This experiment was conducted in duplicate. The host cell condition was monitored using optical microscopy (ECLIPSE Ti, Nikon, Tokyo, Japan), without fixation of the samples.

### 4. TEM observation

The pathogenic particles negatively stained with uranyl acetate were observed using transmission electron microscopy (TEM). Briefly, a drop of the lysate was mounted on a grid (no. 78011630; JEOL Datum, Tokyo, Japan) for 30 s, and excess water was removed using filter paper (no. 1; Advantec). Then, 4% uranyl acetate was applied for 10 s, and excess dye was removed using filter paper. After the grid was dried in a desiccator for >12 h, negatively stained virus-like particles were observed using TEM at 80 kV. Particle diameters were estimated using the negatively stained images.

### 5. Viral RdRp detection in a bloom population

Water samples at the surface and at 1 m from the bottom obtained from the mouth of the Shiota River (Stn. 3; 33°06'N, 130°08'E) during a diatom bloom on 7 January 2015 were pooled together and a 100-mL portion was passed through a 3- $\mu\text{m}$  polycarbonate filter. The dominant species in the water were *Skeletonema* spp. (19,500 cells  $\text{mL}^{-1}$ ) and *A. karianus* (2,890 cells  $\text{mL}^{-1}$ ) (Yamaguchi et al. 2017). RNA was then extracted from the suspensions retained on the filter using an RNeasyPlus Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed to construct cDNAs, using a SuperScript III First-Strand Synthesis System with random 6-mer (Thermo Fisher Scientific, MA, USA). To confirm the presence of possible ssRNA virus infections in the natural diatom bloom population, including *A. karianus*, the degenerate primer pair, Mplsc2F: 3'-ITWGCIGGIGATTWCA-5' and Mplsc2R: 3'-CKYTTTCARRAAWTCAGCATC-5', which amplifies the RNA-dependent RNA polymerase (RdRp) of ssRNA viruses (Culley et al. 2014), was used. PCR amplification was conducted using 20- $\mu\text{L}$  mixtures containing 5  $\mu\text{L}$  of cDNA,  $1 \times$  BlendTaq buffer (Toyobo, Tokyo, Japan), 200 nM of deoxynucleoside triphosphate (dNTP), 10 pmol of Mplsc2, and 1 U of BlendTaq DNA polymerase, using a

GeneAmp PCR system 9700 (Thermo Fisher Scientific) according to the following cycle parameters: 40 cycles of denaturation at 94°C (30 s), annealing at 43°C (45 s), and extension at 72°C (1 min). The PCR products were then electrophoresed through 1% (w/v) agarose ME gels (WAKO Pure Chemical Industries, Osaka, Japan), and the nucleic acids were visualized using Midori green nucleic acid stain (NIPPON Genetics, Tokyo, Japan). Consequently, PCR amplicons of approximately 0.5 kb were excised, and their nucleic acids were extracted (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel GmbH and Co. KG, Germany). The PCR products were ligated into a pGEM-T Easy vector (Promega, WI, USA) and transformed in *Escherichia coli* DH5 $\alpha$ -competent cells (Toyobo). Sequencing was conducted using the dideoxy method with an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific). The resultant sequences were analysed using GENETYX v12 (GENETYX, Tokyo, Japan).

## 6. Phylogenetic analysis

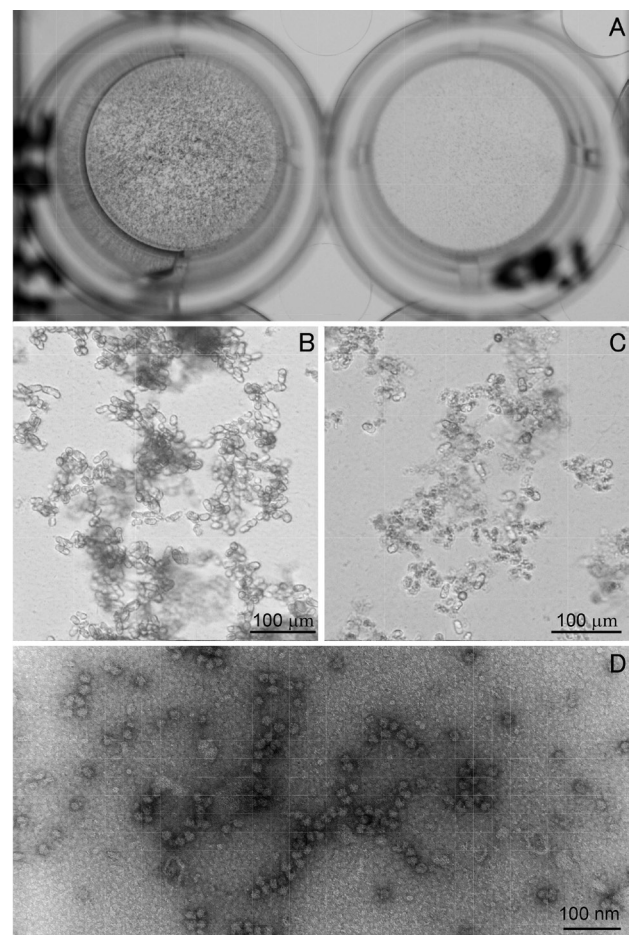
An amplified DNA fragment was identified as a partial conserved RNA-dependent RNA polymerase (RdRp) domain, using BLAST. The deduced amino acid sequence was compared with the RNA virus RdRp domain dataset (Kimura & Tomaru 2015). The RdRp sequence derived from the environmental sequence of the diatom bloom sample was automatically aligned to the RNA virus RdRp database, using Clustal X profile alignment (Thompson et al. 1997), and manually refined. Phylogenetic trees based on the deduced amino acid sequences of the RdRp domain were constructed using the maximum likelihood (ML) method with the Jones-Taylor-Thornton matrix (JTT model) packaged in the MEGA7 Program (Kumar et al. 2016). The JTT model is widely used for phylogenetic analysis for various viruses other than algal viruses, for example in Cholleti et al. (2018).

## Results and Discussion

The isolated pathogen AkarV retained its lytic activity after filtration through a 0.1- $\mu$ m filter. The lytic activity was serially transferable to *Asteroplanus karianus* AK13-02 cultures. The cytoplasm and photosynthetic pigments in the virus-infected cells were degraded compared to those of uninfected cells (Fig. 1A). Apparent cell degradations in the viral-infected cultures occurred almost 10 days post inoculation (Fig. 1B, C). Virus-like particles (VLPs) were not detected in the healthy control cultures. The VLPs observed in the culture lysates, using negative staining, were  $21 \pm 2$  nm

( $N = 105$ ) in diameter, and lacked a tail and outer membrane (Fig. 1D). The virions of previously reported diatom viruses are icosahedral (22-38 nm in diameter) and lack a tail (Tomaru et al. 2015). The virion size of 21 nm is similar to that of the diatom ssRNA virus CsfrrNAV (22 nm), which infects *Chaetoceros socialis* f. *radians* (Tomaru et al. 2009). Based on these results, the VLPs observed in the present study are considered to fulfil Koch's postulates, i.e. (1) the pathogenic effect was transferable to a fresh algal culture, (2) VLPs appeared in lysed cultures, and (3) VLPs were not detected in healthy cultures. Therefore, we concluded that the VLPs were lytic viruses pathogenic to *A. karianus* strain AK13-02.

The lytic effects of diatom virus inoculations to their host cultures are usually significant. Inoculations of ClorDNAV and Csp05DNAV into their host culture, for example, result in apparent discolorations of the cultures within a week (Tomaru et al. 2011b, Toyoda et al. 2012).



**Fig. 1. *Asteroplanus karianus* strain AK13-02**

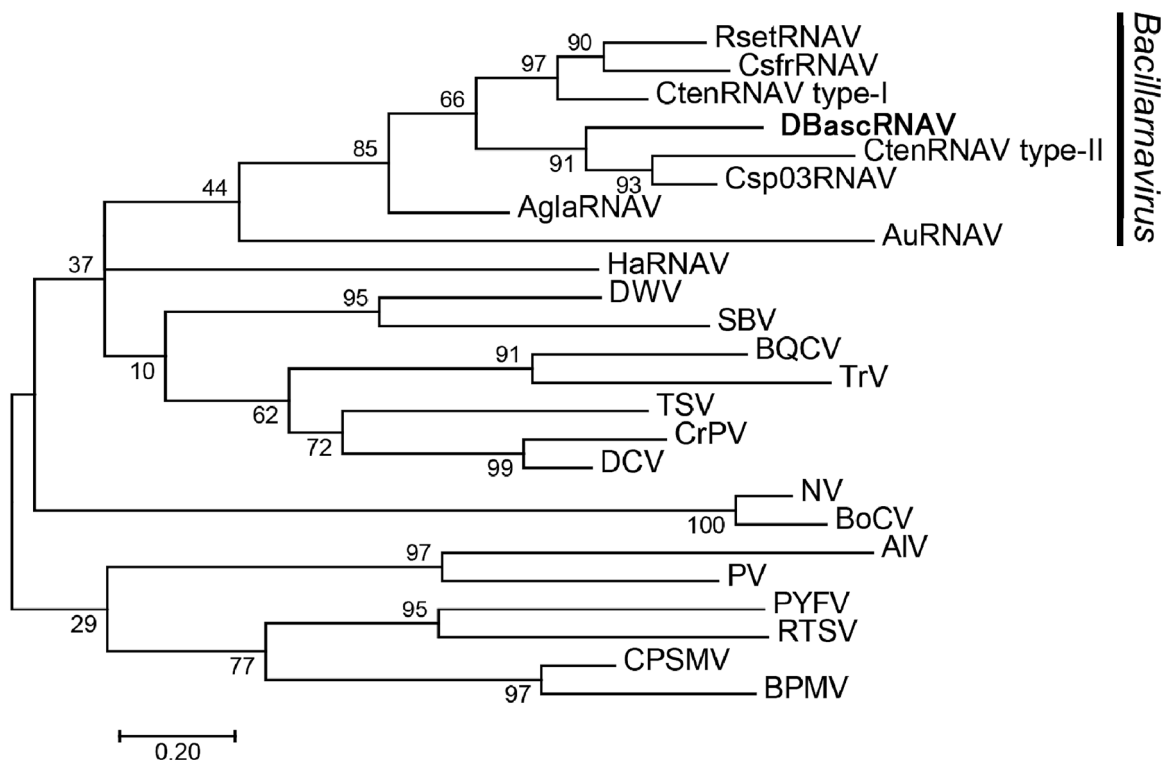
(A) *Asteroplanus karianus* cultures with (right) or without (left) AkarV inoculation; (B) Optical micrograph of intact cells; (C) optical micrograph of AkarV-infected *A. karianus* cells; (D) Transmission electron micrographs of negatively stained AkarV particles in the culture lysate.

Considering the period needed for host culture crashes after viral inoculations, the lytic activities of AkarV seemed to be slight relative to those of previously identified diatom viruses. Moreover, when we tried to retain the lytic activity of this virus by transferring it to fresh host cultures, we failed. Therefore, unfortunately, we were not able to conduct further characterizations of AkarV.

Using the degenerate primer Mplsc2, we successfully isolated a fragment corresponding to the RdRp domain of RNA viruses from the suspensions in a diatom bloom sample (Fig. 2). The fragment was predicted to be the RdRp domain based on BLAST analysis, which revealed it to be highly similar to that of an uncultured marine

AGDYSKYDVRMPAQVTIAAFDILI  
 DIAEKCDGYTADDIHLMKMVVHE  
 VVYPVMAYNGDLIQLFGTNPSTG  
 QNLTVIINSLVNSLLLRSCTFIYP  
 EKNFKENCSFLTYGDDVIGTVSA  
 ECEKFTHITYAEWLAEHDMKFTM  
 PDKESTPTHYMTENDADXLKR

**Fig. 2. Amino acid sequence of the isolated fragment corresponding to the RNA-dependent RNA polymerase (RdRp) domain of RNA viruses from the suspensions in a diatom bloom that occurred in the Ariake Sound on 7 January 2015**



**Fig. 3. Phylogenetic tree constructed using the maximum likelihood (ML) method with amino acid sequences of partial RNA-dependent RNA polymerase (RdRp) protein in ssRNA viruses**

Bootstrap values (%) 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 diatom single-stranded RNA virus, *Bacillarnavirus*. The amino acid sequences used in this analysis were as follows: Aichi virus (AIV), NP\_740444; *Asterionellopsis glacialis* single-stranded RNA virus (AglaRNAV), AB973945; diatom bloom-associated RNA virus (DBascRNAV), see Fig. 2; *Aurantiochytrium* single-stranded RNA virus (AuRNAV), BAE47143; bovine enteric calicivirus (BoCV), AJ011099; bean pod mottle virus (BPMV), NC\_003496; black queen cell virus (BQCV), NC\_003784; cowpea severe mosaic virus (CPSMV), M83830; cricket paralysis virus (CrPV), NC\_003924; *Chaetoceros socialis* f. *radians* single-stranded RNA virus (CsfrRNAV), NC\_012212; *Chaetoceros* sp. strain SS08-C03 single-stranded RNA virus (Csp03RNAV), AB639040; *Chaetoceros tenuissimus* single-stranded RNA virus type-I (CtenRNAV type-I), AB37547; *Chaetoceros tenuissimus* single-stranded RNA virus type-II (CtenRNA2V), AB971661; CtenRNA2V strain SS10V-39V, AB971662; CtenRNA2V strain SS10V-45V, AB971663; drosophila C virus (DCV), NC\_001834; deformed wing virus (DWV), NC\_004830; *Heterosigma akashiwo* RNA virus (HaRNAV), NP\_944776; Norwalk virus (NV), M87661; Human poliovirus 1 Mahoney (PV), V01149; parsnip yellow fleck virus (PYFV), D14066; rice turgo spherical virus (RTSV), AAA66056; *Rhizosolenia setigera* single-stranded RNA virus (RsetRNAV), NC\_018613; sacbrood virus (SBV), NC\_002066; *Triatoma* virus (TrV), NC\_003783; and Taura syndrome virus (TSV), NC\_003005.

RNA virus (AHE37887) detected from coastal waters in Hawaii (identities: 156/161, E-value:  $5e^{-114}$ ) and an ssRNA virus, CtenRNAV type-II, which is infectious to the marine planktonic diatom *Chaetoceros tenuissimus* (identities: 79/169, E-value:  $4e^{-42}$ ). Here, ML methods were used to assess the phylogenetic relationship among the positive-sense ssRNA viruses, including the environmental sequence from diatom bloom populations that included *A. karianus*, as a diatom bloom associated RNA virus (DBascRNAV). The monophyly of AglaRNAV, Csp03RNAV, CsfrRNAV, CtenRNAV type-I, CtenRNA type-II, RsetRNAV, and DBascRNAV was supported by an 85% bootstrap value (Fig. 3), suggesting that the presently identified sequence was an ssRNA diatom virus, i.e. a member of the genus *Bacillarnavirus*. The diatom blooms occurred on 7 January 2015 were composed of *A. karianus* and *Skeletonema* spp. (Yamaguchi et al. 2017). Taken together, the results indicated that the diatom bloom populations, including *A. karianus*, would be infected by diatom ssRNA viruses. Given that diatom ssRNA virus infections are considered to be ‘inter-species’ (Kimura and Tomaru 2015), the detected RNA viruses might be possibly transmitted among the diatom populations.

In the present study of samples from the Ariake Sound, a novel virus lytic to the harmful bloom-forming diatom *A. karianus* was discovered, and the existence of a possible virus member belonging to the genus *Bacillarnavirus* in the natural diatom bloom was detected. These results suggested that viruses act to decrease the diatom populations in the Ariake Sound.

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