Co-occurrence of DNA- and RNA-viruses infecting the bloom-forming dinoflagellate, *Heterocapsa circularisquama*, on the Japan coast

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Abstract: In western Japan, *Heterocapsa circularisquama* blooms occur almost every year. Two distinct viruses infectious to *H. circularisquama*, a large double-stranded DNA (dsDNA) virus, ‘HcV’, and a small single-stranded RNA (ssRNA) virus, ‘HcRNAV’, were found and characterized. Here we report the co-occurrence of the two viruses in Hiroshima Bay, Japan, during 2006. Viruses isolated using *H. circularisquama* strains HU9433-P and MZ2 (the hosts) were large dsDNA viruses (ca. 0.2 µm in diameter) that accumulated in the host cytoplasm. Pulsed-field gel electrophoresis analysis showed their genome sizes were ca. 388 kbp, comparable to the previously reported HcV strain. Small ssRNA viruses were also isolated (using the host strain HCLG-1) at ca. 30 nm in diameter and formed crystalline arrays and/or were randomly accumulated in the host cytoplasm, and their partial genome sequences were highly similar to a previously reported HcRNAV strain. This is the first report of the co-occurrence of HcV and HcRNAV in natural water and their successful isolation. The data suggest the two distinct viruses may cooperatively affect the dynamics of *H. circularisquama* blooms.

Key words: Algal virus, dinoflagellate, HcRNAV, HcV, *Heterocapsa circularisquama*

Introduction

Heterocapsa circularisquama Horiguchi is a bloom-forming small thecate dinoflagellate that kills bivalves (Horiguchi 1995); and is one of the most noxious marine microorganisms causing harmful algal blooms (HABs). Since the first occurrence of this alga was recorded in Urano Inlet, Kochi Prefecture, Japan, its distribution area has expanded rapidly in embayments throughout central and western Japan (Matsuyama 2003).

Two different viruses infecting *H. circularisquama* have been isolated from western Japan. A large icosahedral double-stranded DNA (dsDNA) virus, *H. circularisquama* virus (HcV), was isolated from Tanabe Bay, Wakayama Prefecture in 1999 (Tarutani et al. 2001). The other, a small single-stranded RNA (ssRNA) virus, *H. circularisquama* RNA virus (HcRNAV), was later isolated from most *H. cir-*

cularisquama bloom waters in western Japan (Tomaru et al. 2004). Simultaneous occurrence of *H. circularisquama* blooms and viruses (including unidentified species) in western Japan have been observed almost every year since 2001; and most of the viruses were recognized as HcRNAV using molecular biological methods (Tomaru et al. 2007). Therefore, HcRNAV infection is considered to be one of the most significant factors affecting the population dynamics of *H. circularisquama* (Nagasaki et al. 2004, Tomaru et al. 2007). On the other hand, the ecological relationship between *H. circularisquama* blooms and HcV is poorly understood. Using transmission electron microscopy, Tomaru & Nagasaki (2004) reported the detection of *H. circularisquama* cells harboring HcV-like particles during bloom disintegration in Fukura Bay on the south coast of Awaji Island, Hyogo Prefecture, Japan in 2002; however, HcV isolation from the natural water samples was unsuccessful.

To determine the ecological significance of the viruses in *H. circularisquama* blooms, further study of their relationships and occurrence using constant monitoring are important. Here we show the co-occurrence of HcV and HcRNAV in a *H. circularisquama* bloom that occurred in Hiroshima Bay. This supports the idea that two different

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viruses cooperatively affect the *H. circularisquama* bloom dynamics and disintegration. This is the first report of simultaneous isolation and identification of two different viruses both infecting *H. circularisquama* from the same waters.

Materials and Methods

Sampling

Water samples from the surface layer (0 m) and 0.2 m above the sediment-water interface (B-0.2 m) were collected at 9:30 a.m. on 19 October 2006 from a semi-enclosed basin in Itsukaichi Fishing Port (34°21.400'N, 132°21.864'E) (water depth=ca. 5 m) located in northern Hiroshima Bay, Japan (Fig. 1). In this area, heavy blooms of *Heterocapsa circularisquama* have occurred in 1995, 1997 and 1998 (Matsuyama 2003); since then, no large scale blooms have been reported. Water temperatures of the surface and bottom water samples were respectively 21.4°C and 22.4°C; and the salinities were respectively 28.8 psu and 31.4 psu. The dominant phytoplankton was *Prorocentrum triestinum* Schiller (Dinophyceae) with a cell density of 1.8×10^3 cells mL⁻¹ at 0 m. The abundance of *H. circularisquama* was below the detection limit (<50 cells mL⁻¹) where we could detect *H. circularisquama* in the surface water samples only after concentration using a 20 µm plankton filter.

Algal cultures

Three clonal strains of *H. circularisquama*, HU9433-P, MZ2 and HCLG-1, which have been previously reported in Tomaru et al. (2004), were used as hosts for virus isolation. Based on HcRNAV susceptibility spectra, *H. circularisquama*

strains are roughly divided into two-types: the UA-type and the CY-type, where they exhibit complementary host-type—virus-type specificities, i.e. UA-type *H. circularisquama* strains infect only UA-type HcRNAV but not the CY-type HcRNAV, and vice versa (Tomaru et al. 2004). The host strains HU9433-P and MZ2 belong to the UA-type *H. circularisquama*, and HCLG-1 belongs to the CY-type. Although the strain MZ2 is susceptible to the UA-type HcRNAV, its virus-induced cell lysis symptoms are faint and delayed (Mizumoto et al. 2008). All of the host strains are susceptible to the 10 strains of HcV isolated (HcV01–HcV10) (Nagasaki et al. 2003).

The *H. circularisquama* strains were grown in modified SWM3 medium (Itoh & Imai 1987) enriched with 2 nM Na₂SeO₃ under a 12 hL : 12 hD cycle with 130 to 150 µmol photons m⁻² s⁻¹ using cool white fluorescent illumination at 20°C.

Isolation of viruses

The water samples were filtered through 0.8 µm membrane filters (Nuclepore) to remove eukaryotic microorganisms and most bacteria. Aliquots (1.0 mL) of the filtrates obtained from water samples were inoculated into exponentially growing *H. circularisquama* cultures (1.0 mL) before being incubated. Algal cultures inoculated with SWM3 served as controls. We cloned the viruses using two cycles with the extinction dilution procedure (Suttle 1993, Tomaru et al. 2004) from lysed cultures of *H. circularisquama*. The cloned pathogens were made free of bacterial contamination by filtration through a 0.2 µm Dismic-25cs filter (Advantec Co. Ltd.); and then transferred to another exponentially growing host culture. The resultant lysate was regarded as a clonal virus suspension; and used for further experiments.

TEM

Transmission electron microscopy (TEM) was performed according to the method described by Tomaru et al. (2004). Vigorously growing cultures of *H. circularisquama* were inoculated with a viral suspension at a concentration of 5% (v/v). *H. circularisquama* cultures inoculated with SWM-3 medium served as the control. An aliquot of cell suspension was sampled at 30 h post-inoculation (pi) and processed for TEM observation (Tomaru et al. 2004). Thin sections of the cells were stained with 4% uranyl acetate and 3% lead citrate; and observed at 80 kV using a JEOL JEM-1010 transmission electron microscope. The viruses alone were negatively stained with 4% uranyl acetate and observed using TEM. Particle diameters were estimated using the negatively stained images.

Analysis of the virus genome

The virus particles of HcV and HcRNAV strains established in this study were collected according to the method

Fig. 1. Map showing the location of Itsukaichi Fishing Port, Hiroshima Bay where seawater samples were collected.

described by Tomaru et al. (2004). The genome size of HcV was estimated using pulsed-field gel electrophoresis (PFGE); and partial genome sequencing of HcRNAV and HcV were conducted according to the methods described by Nagasaki et al. (2005a) and Nagasaki et al. (2005b), respectively.

Infection specificities of viruses

To examine the intraspecies host specificity of the virus strains isolated in the present study, viral suspension was inoculated ($v/v=5\%$) independently into exponentially growing cultures of the three *H. circularisquama* strains (HU9433-P, MZ2 and HCLG-1). The culture plates were incubated under the same conditions of light and temperature as shown above, and the occurrence of algal lysis was monitored by optical microscopy. For comparison, growth of host cultures, not having undergone viral inoculation, were also monitored. Algal cultures in which the majority of cells lost mobility and were degraded were scored as lytic due to the inoculum.

Results and Discussion

Virus identification

We isolated viruses infectious to *Heterocapsa circularisquama* from water samples of the surface and bottom layers.

Two large dsDNA viruses, virus strains HcV-ITD01 and HcV-ITD02, were isolated only from surface waters using *H. circularisquama* strain HU9433-P and MZ2 as hosts, respectively. TEM observations showed the sizes of the viruses were 183–210 nm and 183–191 nm, respectively (Fig. 2A, B); and the daughter virus particles accumulated in the host cytoplasm at 30 h pi (Fig. 3A–D). The morphological and physiological characteristics of these isolates were similar to HcV (Tarutani et al. 2001, Nagasaki et al. 2003). PFGE analysis showed the genome sizes of these dsDNA viruses were comparable to that of HcV, ca. 388 kbp (Fig. 4) (Nagasaki et al. 2005b), and the partial genome sequences of these viruses (591 bp) were completely identical to the putative major capsid protein region of HcV (HcV03) (Table 1). In comparison, for plant viruses, they are considered to be the same virus species if the similarity in the amino acid sequences for the virus coat protein region is at $>90\%$ (Shukla et al. 1994). Integrating the physiology, morphology and genomic features, we concluded that both the large dsDNA viruses that were isolated in this study were HcV.

However, PFGE analysis showed a difference in genome structure between the large dsDNA virus HcV-ITD02 and the previously described HcV. Nucleic acid extracted from HcV-ITD01 exhibited a single band 388 kbp in length that is similar to previously reported HcV (388 kbp; Nagasaki et al. 2005b). Contrastingly, HcV-ITD02 genomic DNA showed two bands differing in size at 388 and 350 kbp (Fig.

Fig. 2. Transmission electron micrographs of negatively stained particles of HcV-ITD01 (A), HcV-ITD02 (B), HcRNAV-ITR01 (C) and HcRNAV-ITR02 (D) in the culture lysate.

4). Similarly, the circular genomic DNA of *Ectocarpus siliculosus* (Dillwyn) Lyngbye (Phaeophyceae) virus shows two prominent bands in PFGE gels corresponding to an intact circular DNA and a linear DNA where the latter had been generated presumably by artificial breakage (Lanka et al. 1993). Our PFGE analysis suggested the possibility that the HcV genome may be circular DNA. Further studies are essential to characterize the genome structure of this virus.

The small ssRNA viruses, virus strains HcRNAV-ITR01 and -ITR02, were isolated using *H. circularisquama* strain HCLG-1 as the host from both surface and bottom water samples, respectively. TEM observations showed the sizes of these viruses were 27–29 nm and 27–32 nm, respectively (Fig. 2C, D); and for both, the daughter virus particles accumulated in the host cytoplasm at 30 h pi (Fig. 3E–H). The accumulation patterns of these viruses in the host cell, random aggregations and crystalline array formations, were similar to HcRNAV (Tomaru et al. 2004). The partial genome sequence analyses revealed 97.6% of the partial nucleotide sequence and $>96\%$ of the amino acid sequence in the ORF-2 (capsid protein gene) of HcRNAV-ITR01 and -ITR02 coincided with the CY-type HcRNAV (HcRNAV109) (Table 1). From the above data, both RNA viruses isolated in this study were concluded to be HcRNAV.

Infection specificities of viruses

Both HcV strains caused lysis in all three *H. circularisquama* strains tested; whereas in contrast, the two HcRNAV strains were lytic in only one *H. circularisquama* strain, HCLG-1 (Table 2). This is similar to our previous

Fig. 3. Transmission electron micrographs of ultra thin sections of *Heterocapsa circularisquama*. A, C, E and G: cells infected with viruses at 30 h post-infection. B, D, F and H: higher magnification of the virus-like particles of A, C, E and G, respectively. A, B: host strain HU9433-P infected with HcV-ITD01; C, D: host strain MZ2 infected with HcV-ITD02; E, F: host strain HCLG-1 infected with HcRNAV-ITR01; and G, H: host strain HCLG-1 infected with HcRNAV-ITR02. Scale bars indicate 2 μ m in A, C, E and G; and 200 nm in B, D, F and H. Ch: chloroplast; N: nucleus; V: virus-like particles.

Fig. 4. Genome size of viruses as determined by pulsed-field gel electrophoresis. A single band (388 kbp) and two bands differing in size (388 and 350 kbp) are observed in lane 1 and lane 2, respectively. Lane 1, HcV-ITD01; lane 2, HcV-ITD02.

Table 1. Percentage of identical nucleotides and amino acids in isolated virus strains as compared to HcV or HcRNAV.

Virus strains	Nucleotides/amino acids (%)	
	591 nt in a putative major capsid protein of HcV03	678 nt in a capsid protein of HcRNAV109
HcV-ITD01	100/100	—
HcV-ITD02	100/100	—
HcRNAV-ITR01	—	97.6/96.9
HcRNAV-ITR02	—	97.6/97.3

results concerning strain-specificity of HcV, which is considered much lower than HcRNAV (Nagasaki et al. 2003, Tomaru et al. 2004). Our previous studies showed *H. circularisquama* strains HU9433-P and MZ2 belong to the UA-type and HCLG-1 belongs to the CY-type (Mizumoto et al. 2008, Tomaru et al. 2004) (see Materials and Methods). Thus, the small viruses HcRNAV-ITR01 and -ITR02 isolated in the present survey were considered to be members of the CY-type HcRNAVs.

Implications

Here we found the HcV strains were only able to be isolated using the UA-type host, HU9433-P and MZ2, but not with the CY-type host, HCLG-1. Based on the results of the infection specificity tests (Table 2), HcV could potentially be isolated using the host strain HCLG-1, but the actual re-

Table 2. Infection specificities of viruses to *Heterocapsa circularisquama* strains.

<i>H. circularisquama</i> strain	Virus strain			
	HcV-ITD01	HcV-ITD02	HcRNAV-ITR01	HcRNAV-ITR02
HU9433-P	+	+	—	—
MZ2	+	+	—	—
HCLG-1	+	+	+	+

+, algal lysis; —, no effect.

sult differed. In many cases, both the UA-type and CY-type HcRNAV are simultaneously isolated from natural waters (Tomaru et al. 2004), however, in the present study the CY-type HcRNAV strains were successfully isolated but no UA-type HcRNAV strain was. In order to interpret these results, the relationship between the host-strain specificity of infectious viruses and their viral abundances should be considered. The most plausible explanation for this phenomenon may be that the abundance of CY-type HcRNAV was much higher than HcV; thus only CY-type HcRNAV was isolated using the extinction dilution method where the most abundant viruses tend to be isolated. HcV, having a wide host range, may only have been isolated using UA-type hosts because the most abundant virus causing lysis of UA-type hosts was not UA-type HcRNAV but HcV. For further understanding of this host-virus ecological relationship, the development of a quantitative detection method for viral abundance will be essential at the species or type level.

The water of the Itsukaichi Fishing Port one week prior to sampling was discolored due to an algal bloom (data not shown); however, on the day of sampling the water was clear. Therefore, the red-tide bloom at this port may have collapsed during the 7 days prior to the sampling. We detected *H. circularisquama* cells at considerably lower concentrations from the water samples on the sampling day (see Materials and Methods). In our previous surveys, *H. circularisquama* blooms were accompanied by the occurrence of viruses lytic to *H. circularisquama* (Nagasaki et al. 2004, Tomaru et al. 2007). From the above results, the viruses isolated in this study were considered to be HcV and HcRNAV; and were propagated during the process of a *H. circularisquama* bloom that occurred just before our sampling in October 2006. This suggests two distinct virus species may have cooperatively affected the disintegration of the *H. circularisquama* bloom.

In Ago Bay, central Japan, where *H. circularisquama* blooms occur almost every year, where we have annually detected and isolated HcRNAV since 2001. In contrast, HcV has not been detected or isolated from this location (Nagasaki et al. 2004, Tomaru et al. 2007). HcRNAV has been isolated from a wide region of the western Japanese coast from central Japan to Kyushu Island (Tomaru et al. 2004). The first detection and isolation of HcV was from

Tanabe Bay on the east side of the Kii-Chanel in 1999 (Tarutani et al. 2001) although HcRNAV was not detected from the water samples. Our previous study showed that *H. circularisquama* cells intracellularly harboring HcV-like particles and cells harboring HcRNAV-like particles coexisted in a host bloom occurring in Fukura Bay on the south coast of Awaji Island in the Seto Inland Sea, 2002 (Tomaru & Nagasaki 2004). Integrating the previous observations and our present results (successful isolation of HcV and HcRNAV from the same waters), these viral infections are considered to be important factors functioning in the termination of *H. circularisquama* blooms. It is difficult to evaluate the contribution of each virus type in determining the outcome of the bloom dynamics. Possibly the two distinct virus species affect the dynamics of *H. circularisquama* blooms in natural water environments and are a significant factor determining the fate of blooms. To understand the interaction between *H. circularisquama* blooms and virus infections more precisely, further field surveys as well as physiological studies examining relationships with environmental factors should be performed.

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