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Widespread occurrence of viruses lytic to the bivalve-killing dinoflagellate *Heterocapsa circularisquama* along the western coast of Japan

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Abstract: Blooms of the bivalve-killing dinoflagellate *Heterocapsa circularisquama* Horiguchi have occurred since 1988 along the western coast of Japan. Two distinct viruses infectious to *H. circularisquama*, a large double-stranded DNA virus HcV (*H. circularisquama* virus) and a small single-stranded RNA virus HcRNAV (*H. circularisquama* RNA virus) have been found and intensively studied. In the present study, the abundances of viruses infecting *H. circularisquama* were examined at six sites along the western coast of Japan in 2001. Viral agents lytic to *H. circularisquama* were detected from all water samples tested in the present study, and the maximum abundance was 2.08×10^5 infectious units ml^{-1}. Transmission electron microscopy revealed the coexistence of two distinct virus-like particles (VLPs) in a *H. circularisquama* bloom that occurred in Fukura Bay (Hyogo Prefecture): large (210±17 nm) and small (28±2 nm) VLPs that were morphologically quite similar to HcV and HcRNAV, respectively. These results suggest a close relationship between *H. circularisquama* blooms and the lytic viruses in natural environments.

Key words: *Heterocapsa circularisquama*, harmful algal bloom, red tide, dinoflagellate, algal virus

Introduction

Heterocapsa circularisquama Horiguchi is a small thecate dinoflagellate (20–29 μm in length, 14–20 μm in width), that was described from Ago Bay, central Japan (Horiguchi 1995). Since this dinoflagellate was recorded for the first time in 1988 in Uranouchi Inlet, located in Kochi Prefecture, Japan, the distribution area has expanded rapidly into embayments throughout central and western Japan (Matsuyama 1999). With the expansion of its distribution, this species has formed large-scale red tides and caused mass mortalities of bivalves such as pearl oyster *Pinctada fucata martensii* Dunker, oyster *Crassostrea gigas* Thunberg, and short-necked clam *Ruditapes philippinarum* Adams & Reeve (Matsuyama et al. 1995, Nagai et al. 1996, Matsuyama 1999). Until 2002, more than 40 cases of *H. circularisquama* red tide had been recorded, including 18 incidences leading to fisheries damage (Matsuyama 2003).

A large icosahedral double-stranded DNA (dsDNA) virus HcV (Tarutani et al. 2001, Nagasaki et al. 2003) and a small icosahedral single-stranded RNA (ssRNA) virus HcRNAV (Tomaru et al. in press) infecting *H. circularisquama* have been isolated from the western part of Japan, and intensively studied. Just recently, Nagasaki et al. (in press) reported a possible relationship between the termination of a *H. circularisquama* bloom and its viruses in Ago Bay in 2001, and suggested that viral infection had a significant impact on the dynamics of the *H. circularisquama* bloom. However, there have been few reports on the occurrence of viruses lytic to *H. circularisquama* in natural environments. To elucidate the ecological implications of viral infection on *H. circularisquama* populations, basic assessments of viral abundances and distributions are essential. Thus, in the present study, the relationship between *H. circularisquama* blooms and lytic viruses were examined at 6 different sites in western Japan. Furthermore, in the present paper, the coexistence of two distinct virus-like particles (VLPs) in a *H. circularisquama* bloom is also reported, which supports the idea that different viruses co-affect within a single bloom of *H. circularisquama*.

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Materials and Methods

Study site

Water samples were collected from *H. circularisquama* blooms at 6 sampling sites, Obama Bay (Fukui Prefecture), Fukura Bay (Hyogo Pref.), Shido Bay (Kagawa Pref.), Uchino-umi (Tokushima Pref.), Uranouchi Inlet (Kochi Pref.) and Kame-ura (Kumamoto Pref.) (Fig. 1). The abundance of *H. circularisquama* cells in each water sample is shown in Table 1.

Measurement of viral abundance

The water samples were processed to measure viral abundance within 24 h of sampling. Each sample was fil-

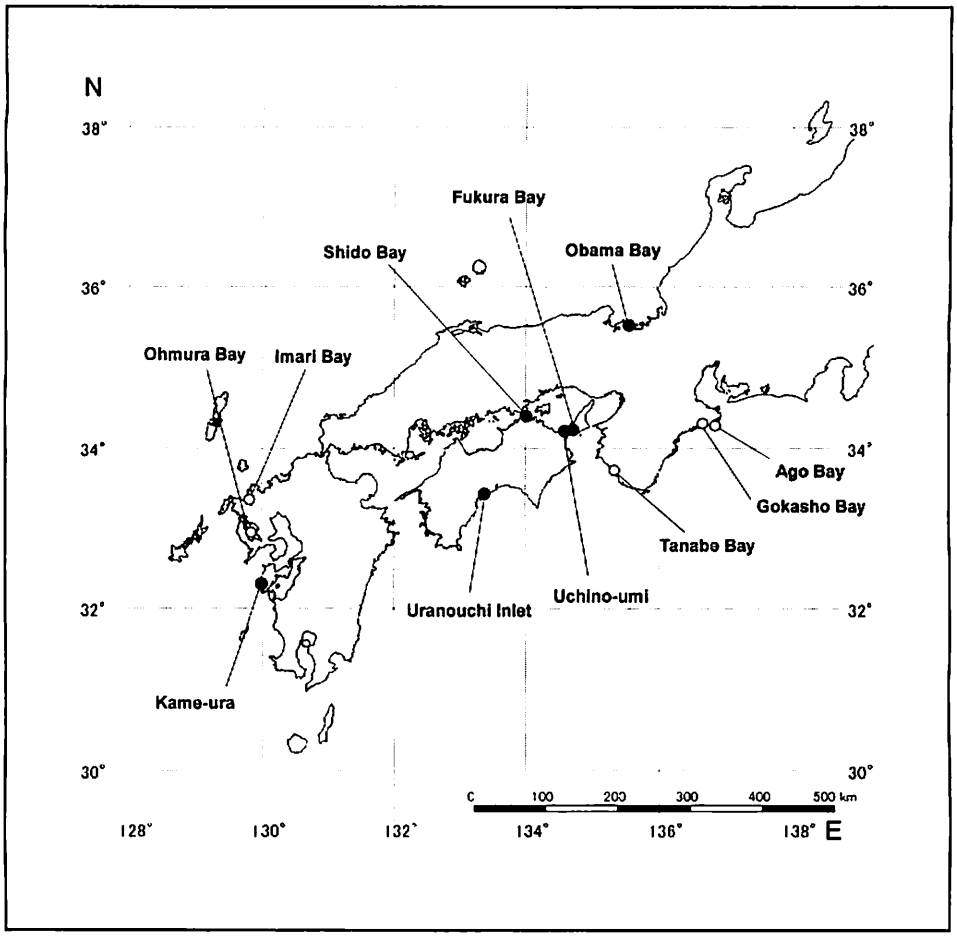

Fig. 1. A map showing the locations (closed circles) from which seawater samples were collected in the present experiment. Open circles indicate the regions where the HcRNAV-like virus has been isolated so far (Tomaru et al. in press, Tomaru et al. unpublished data).

Table 1. *Heterocapsa circularisquama* abundance in water samples.

Sample	Date	Location	Depth (m)	*Heterocapsa circularisquama* (cells ml^{-1})
A	27 Aug. 2001	Uranouchi Inlet (Stn. A219)	0	no data
B	27 Aug. 2001	Uranouchi Inlet (Stn. A913)	0	no data
C	29 Aug. 2001	Fukura Bay	0	2500
D	4 Sep. 2001	Fukura Bay	0	not detected
E	12 Sep. 2001	Obama Bay (Mamiya-Shinju)	2	10
F	12 Sep. 2001	Obama Bay (Ohtsuki-Shinju)	2	10
G	15 Oct. 2001	Uchino-umi (Stn. 5)	1	373
H	15 Oct. 2001	Uchino-umi (Stn. 6)	0–5	405
I	12 Sep. 2001	Shido Bay	0	no data
J	10 Dec. 2001	Kame-ura	2	35

tered through a 0.8 μm nominal pore-size polycarbonate membrane filter (Nuclepore). The titer of infectious viruses in the filtrated water sample was measured by means of the extinction dilution method (=MPN method: Suttle 1993, Imai et al. 1998a, Imai et al. 1998b, Nagasaki et al. in press) using 4 extracellularly axenic clonal strains of *H. circularisquama*, HU9433-P, HA92-1, HCLG-1 and HY9423 (details were shown in Nagasaki et al. (2003)) as hosts. The sensitivities to viruses of HU9433-P and HA92-1 are different to those of HCLG-1 and HY9423 (Tomaru et al. in press). *H. circularisquama* strains were grown in modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987) enriched with 2 nM Na_2SeO_3 (Imai et al. 1996) under a 12 hL : 12 hD cycle of 130 to 150 μmol photons $m^{-2}s^{-1}$ with cool white fluorescent illumination at 20°C.

TEM observation

Five hundred ml of the Fukura Bay water sampled on 29 August 2001 was incubated at 25°C under a 12-h and 12-h light–dark cycle of ca. 140 μmol photons $m^{-2}s^{-1}$ for 24 h, and then the *H. circularisquama* cells in the water were fixed with 1% glutaraldehyde. Sample preparation for transmission electron microscopy (TEM) observation was conducted by following the methods given by Tarutani et al. (2001).

Results and Discussion

Abundance of viruses lytic to *H. circularisquama*

From Obama Bay (Fukui Pref.), Fukura Bay (Hyogo Pref.), Shido Bay (Kagawa Pref.), Uchino-umi (Tokushima Pref.), and Uranouchi Inlet (Kochi Pref.), viral abundances were scored as MPN using the four *H. circularisquama* strains (Fig. 2). Among them, the maximum titer was detected in the water sample collected from Uchino-umi Stn. 5 on 15 October 2001: 2.08×10^5 infectious units ml^{-1} for the host strain HA92-1 (Fig. 2G). From the Kame-ura water sample, viruses lytic to HU9433-P and HA92-1 were detected by means of the MPN method, but those to HCLG-1 and HY9423 were not (Fig. 2J). However, Tomaru et al. (in press) succeeded in isolating a virus infectious to HCLG-1 from the same water sample. This is presumably because the abundance of the viruses infectious to HCLG-1 in the Kame-ura water sample was lower than the detection limit of the MPN method employed in the present experiment (<3.18 infectious units ml^{-1}).

Viral abundances were variable and the maximum was 5 orders of magnitude higher than the minimum (Fig. 2). Based on previous studies on viral impacts on algal blooms, the abundance of viruses infecting bloom-forming microalgae increases during the mid-bloom period, reaches a maximum at bloom termination stages, and then gradually decreases, as was observed in the interactions between *Heterosigma akashiwo* (Hada) Hada and its viruses (Tarutani et al. 2000) or *H. circularisquama* and its viruses (Nagasaki et al. in press). The *H. circularisquama* bloom in Fukura Bay examined in the present study was considered to be in its decay phase because host abundance decreased from 6.5×10^3 cells ml^{-1} to 7.5 cells ml^{-1} from 27 to 30 August (data not shown), ending below the detection limit (<1 cells ml^{-1}) on 4 September, 2001. The abundance of viruses lysing each *H. circularisquama* strain in Fukura Bay decreased by 3 orders of magnitude from 29 August to 4 Sep, 2001 following bloom termination (Fig. 2C, 2D). The relationship between the host and virus abundance patterns during the late bloom disintegration stage in Fukura Bay was similar to that observed in previous studies (Tarutani et al. 2000, Nagasaki et al. in press). Thus, the differences in viral abundances detected in the present survey might reflect the developmental stage of the blooms.

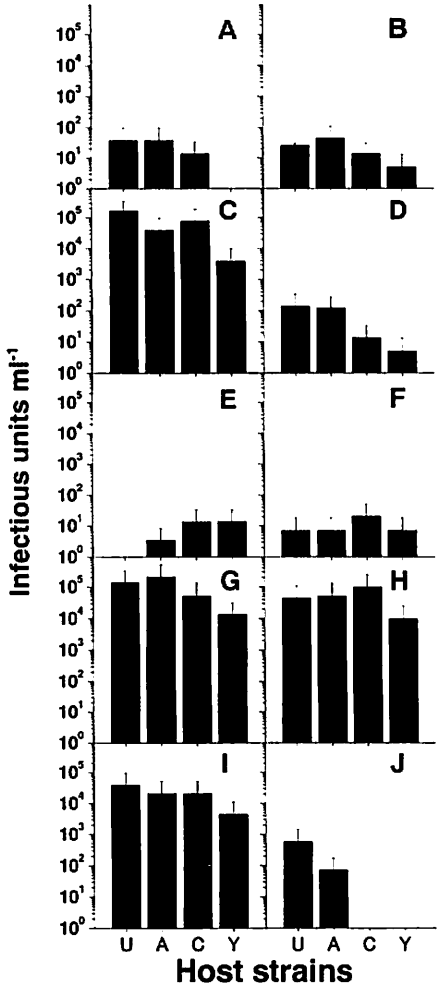

Fig. 2. Abundance of viruses infectious to *Heterocapsa circularisquama* strains HU9433-P (U), HA92-1 (A), HCLG-1 (C), and HY9423 (Y) in western Japan, A) Uranouchi Inlet Stn. A219 on 27 Aug. 2001, B) Uranouchi Inlet Stn. A913 on 27 Aug. 2001, C) Fukura Bay on 29 Aug. 2001, D) Fukura Bay on 4 Sep. 2001, E) Obama Bay Stn. M on 12 Sep. 2001, F) Obama Bay Stn. O on 12 Sep. 2001, G) Uchino-umi Stn. 5 on 15 Oct. 2001, H) Uchino-umi Stn. 6 on 15 Oct. 2001, I) Shido Bay on 12 Sep. 2001, and J) Kame-ura on 10 Dec. 2001.

Two distinct viruses infectious to *H. circularisquama*, HcV and HcRNAV have been isolated and characterized so far. HcV is a large icosahedral virus 197 nm in diameter, which harbors a dsDNA 356 kbp in length (Tarutani et al. 2001, Shirai pers. comm.). HcV was first isolated from Wakinoura Fishing Port (Fukuoka Pref.) in 1999 (Tarutani et al. 2001). Based on its morphology, genome type, and pathology, it is most probable that HcV belongs to the family *Phycodnaviridae* (Van Etten 2000). On the other hand, HcRNAV is a small roundish virus 30 nm in diameter, which harbors a ssRNA 4.4 kb in length (Tomaru et al. in press). HcRNAV was isolated from several sampling sites in western Japan (Tomaru et al. in press). HcRNAV consists of 2 distinct types of virus clones that have a complementary host range to each other. HcRNAV clones infectious to *H. circularisquama* strains HU9433-P or HA92-1 (UA type-HcRNAV) are not infectious to HCLG-1 and HY9423, and those infectious to *H. circularisquama* strains HCLG-1 or HY9423 (CY type-HcRNAV) are not infectious to HU9433-P and HA92-1 (Tomaru et al. in press). The characteristics of the viral clones isolated from the most diluted wells in which host cell lysis occurred in the present study were reported by Tomaru et al. (in press). None of the viral clones isolated from the six sites were DAPI-stainable, suggesting that they are more likely HcRNAV, either UA type-HcRNAV or CY type-HcRNAV, based on their infection specificity spectra (Tomaru et al. in press). Thus, the MPN value in the present survey presumably represented the abundance of the most dominant virus HcRNAV. On the other hand, these results also demonstrate that 2 distinct types of HcRNAV and 2 distinct types of *H. circularisquama* coexisted in these locations.

The occurrence of a *H. circularisquama* bloom in Tolo Harbour, Hong Kong, in 1986 is the oldest record of this species (Iwataki et al. 2002). Since the first *H. circularisquama* bloom in Japan was recorded in Uranouchi Inlet, Kochi Prefecture in 1988, outbreaks of *H. circularisquama* red tides have increased in western Japan (Matsuyama 1999). Considering the result that HcRNAV-like viruses infectious to *H. circularisquama* were detected from all of the 6 sampling locations, and in addition that HcRNAV-like viruses have been isolated from Ago Bay, Gokasho Bay (Mie Pref.), Tanabe Bay (Wakayama Pref.), Ohmura Bay (Nagasaki Pref.) and Imari Bay (Saga Pref.) waters during *H. circularisquama* bloom periods so far (Tomaru et al. in press, Tomaru et al. unpublished data) (Fig. 1), the relationship between HcRNAV-like viruses and the host alga *H. circularisquama* appears to be widely distributed throughout western Japan. It is most interesting that HcRNAV clones having similar properties have been independently isolated from distant sites (Tomaru et al. in press), suggesting that they have the same original ancestor. However, detailed comparisons using molecular techniques of HcRNAV-like virus clones with different distributions would be required to determine their origin and the reason for their widespread occurrence.

Viruses in Fukura Bay

TEM images from the cultured water sample collected in Fukura Bay on 29 August 2001 are shown in Fig. 3. In the cultured Fukura Bay water sample, 74% of the *H. circularisquama* cells harbored large mature VLPs (172–237 nm, 210±17 nm in diameter, n=20) (Fig. 3A, 3B), while cells having small VLPs (24–31 nm, 28±2 nm in diameter, n=20) (Fig. 3C, 3D) comprised 3% of the total population. Both kinds of VLPs were found within the cytoplasm, and co-infection within a single cell was not observed. Based on their morphological characteristics, the large and the small VLPs were considered to be HcV and HcRNAV, respectively (Tarutani et al. 2001, Nagasaki et al. 2003, Tomaru et al. in press).

The existence of VLPs has been reported in many eukaryotic phytoplankton species (Van Etten et al. 1991) and some authors have suggested the importance of viruses for the termination of microalgal blooms, based on transmission electron microscopic work (Bratbak et al. 1993, Nagasaki et al. 1994, Brussaard et al. 1996). Considering the latent periods of HcV (40–56 h: Nagasaki et al. 2003), it is presumable that a large proportion of *H. circularisquama* cells in Fukura Bay might have already been infected by HcV. Although an accurate estimation of the VLP-harboring cell ratio within the *H. circularisquama* population under natural conditions was not possible because the water sample collected in Fukura Bay was incubated at 25°C for 24 h prior to TEM preparation, the proportion of virus-harboring cells was probably high in Fukura Bay water on 29 August 2001.

In spite of the probably high frequency of HcV-infected cells within the *H. circularisquama* population, all of the viruses isolated using the extinction dilution method were DAPI-negative HcRNAV-like viruses (Tomaru et al. in press). With respect to this apparent paradox, some possible explanations are given below: (1) *H. circularisquama* cells infected by the HcRNAV-like virus were more delicate than HcV-infected cells and lysed during TEM preparation (thin sectioning, concentration, dehydration and embedding) (Bratbak et al. 1993), (2) since TEM observation of a cell gives only an image of a cell section, cells with a few small HcRNAV-like particles were difficult to detect, (3) since two extinction dilution series were used in the virus isolation procedure (Nagasaki and Yamaguchi 1997), which enables isolation of the most abundant viruses in a water sample, only HcRNAV-like viruses, which were more abundant than HcV, were obtained; i.e., most cells infected by HcRNAV-like viruses had already lysed and released their virus particles, (4) viruses within host cells were trapped in the filtration process; thus, most HcV were excluded. Although the third explanation suggests that distinct types of virus act during a single algal bloom at different stages, a more refined design for viral monitoring is required in order to assess this possibility. In order to detect or isolate minority viruses from the major ones, isolation methods should be

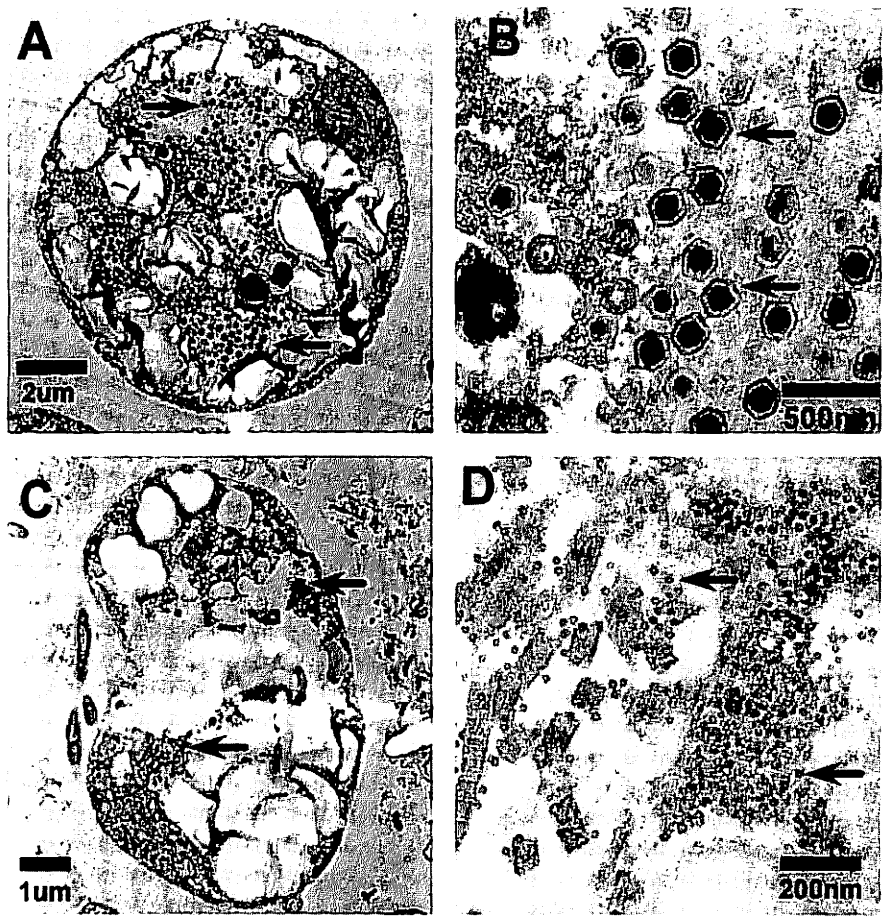

Fig. 3. Transmission electron micrographs of *Heterocapsa circularisquama* cells collected from Fukura Bay on 29 August 2001. TEM preparation was conducted following incubation at 25°C for 24 h. A) a *H. circularisquama* cell harboring large VLPs (arrows) showing degradation of cell organelles within the cytoplasm, B) a higher magnification of the large VLPs within *H. circularisquama* cytoplasm (arrows), C) a *H. circularisquama* cell harboring small VLPs (arrows) showing degradation of cell organelles within the cytoplasm, and D) a higher magnification of the small VLPs (arrows).

redesigned using fractionation techniques, i.e., filtration or density gradient centrifugation.

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