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Co-occurrence of *Heterocapsa Circularisquama* Bloom and Its Lytic Viruses in Lake Kamo, Japan, 2010

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Abstract

In autumn 2009, the first outbreak of the shellfish-killing dinoflagellate *Heterocapsa circularisquama* bloom was recorded in a small lagoon, Lake Kamo, located on Sado Isl., Niigata Prefecture, Japan. At the time, the fisheries damage on the oyster culture due to the bloom was estimated at about JPY 190 million. Also in summer 2010, *H. circularisquama* formed a bloom in Lake Kamo, but in this case, no oyster kill was recorded. In the present study, we measured the temporal change in the abundance of viruses causing lysis of *H. circularisquama* and compared it with the host dynamics. We checked the host specificity and virus sensitivity of the isolated virus clones and host clones, respectively. Different types of host and virus clone dominantly coexisted in this lake, and the intraspecies host specificity of each HcRNAV clone isolated in Lake Kamo, 2010, agreed well with the host range predicted based on the deduced amino acid sequence of the major capsid protein (MCP). Considering the contemporary appearance of the host and virus, viral infection was assumed to be one of the factors affecting the dynamics of *H. circularisquama* population in this lake.

Discipline: Fisheries
Additional key words: harmful algal bloom, HcRNAV, population dynamics, virus infection

Introduction

Lake Kamo is a brackish lagoon (486 ha in area) located on Sado Isl., Niigata Prefecture, Japan (Fig. 1), which is famous for Pacific oyster (*Crassostrea gigas*) cultivation. The oyster aquaculture production has been estimated at 1,500-2,000 tons per year. This lake is highly enclosed and eutrophic (Fig. 1), and the main damage on the oyster cultivation in this lake has usually been caused by extremely low oxygen concentration (especially at the lower stratum) and shell-covering due to Sea-squirt, *Styela plicata*. However, in October 2009, a bloom of the shellfish-killing dinoflagellate *Heterocapsa circularisquama* first occurred and inflicted destructive damage on the oyster culture of Lake Kamo, the cost of which was estimated at *ca.* JPY 190 million[4]. This is one of the most serious incidents of fisheries damage in the history of the aquaculture industry in Niigata Prefecture. *H. circularisquama* also formed a bloom in the following summer in this lake (in 2010), but it was not accompanied by fisheries damage due to the bloom[4].

H. circularisquama is one of the most noxious bloom-forming dinoflagellates, which specifically kills bivalves[2]. Since the first bloom recorded in Uranouchi Bay (Kochi Prefecture, Japan) in 1988, *H. circularisquama* has frequently caused mass mortalities of bivalves such as pearl oyster (*Pinctada fucata*), Manila clam (*Ruditapes philippinarum*), Pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus galloprovincialis*) in western Japan[2,5]. Prior to the first outbreak in Lake Kamo (38°06'N, 138°44'E), the northern limit of its distribution range had been Obama Bay, Fukui Prefecture, Japan (35°50'N, 135°72'E), hence this phenomenon should be noted as a typical case involving the northward expansion of harmful algae in the North Hemisphere. Although the original entry of *H. circularisquama* to Lake Kamo has not been assured, considering the

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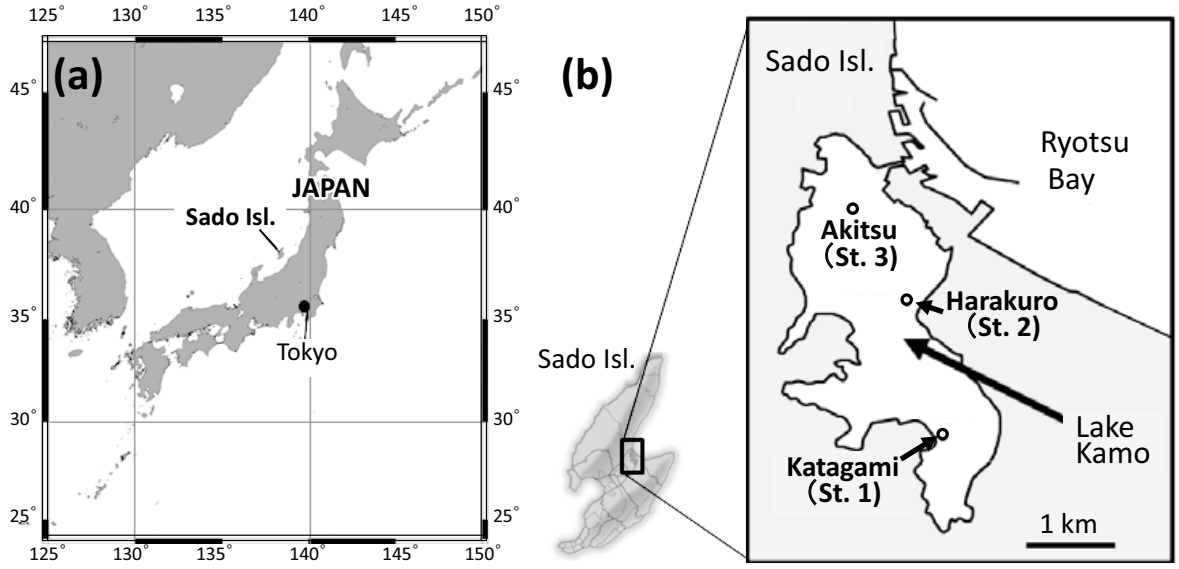

Fig. 1. Locations of Lake Kamo (a) and the sampling stations in Lake Kamo (b)

isolated locality of Lake Kamo (Fig. 1), it is most likely that the harmful organism was transplanted due to the artificial import of bivalve culture(s) to this lake.

Because *H. circularisquama* is a photosynthetic microalga, various physical factors (e.g. water temperature, salinity, irradiation), chemical factors (e.g. nutrients, minerals, vitamins), and biological factors (e.g. predation, bacterial attack) are considered related in determining its population dynamics. Besides, two distinct virus species have been found to date infecting *H. circularisquama*. One is the *Heterocapsa circularisquama RNA virus* (HcRNAV), a small icosahedral virus *ca.* 30 nm in diameter, harboring a linear single-stranded RNA genome *ca.* 4.4 kb in length[9,14], while the other is the *Heterocapsa circularisquama virus* (HcDNAV), a large icosahedral virus (*ca.* 0.2 μm in diameter) harboring a double-stranded DNA genome *ca.* 356 kb in length[10,13]. HcRNAV in particular has been assumed to be one of the key factors controlling the host population dynamics[8,15]. HcRNAV infection is strain-specific[14]; i.e. in the previous study, HcRNAV clonal strains were roughly divided into two types (UA- and CY-types) based on their intraspecies infection specificity[14]. Still, most of the host clonal isolates were also revealed to be susceptible to either of the two virus types[14]. The data imply that at least two independent host-virus systems coexist in a natural *H. circularisquama* population, where UA-type HcRNAV clones infect only UA-type host clones but not the CY-type, and CY-type HcRNAV clones infect only CY-type host clones but not the UA-type[14]. Hence, HcRNAV infection can affect not only the quantity (biomass) but also the quality (clonal composition) of the host population[15].

The mechanism supporting the intraspecies host specificity has been partially revealed. The genomes of the two typical HcRNAV strains (HcRNAV34 and HcRNAV109, respectively belonging to UA- and CY-types) were fully sequenced, and the deduced amino acid sequence of their MCP and the predictable tertiary structure were compared. Consequently, the difference in virus capsids' surface nanostructure was predicted to be the key factor regulating the affinity between the host- and virus clones[9]. In particular, four variable regions were found in the MCP gene, generating 29 amino acid substitutions between the two HcRNAV strains' MCP, which were considered to determine their intraspecies host specificity. Therefore, the host range type of each HcRNAV clonal strain has been considered predictable based on the deduced amino acid sequence of the MCP.

In the present study, we measured the changes in the abundance of viruses causing lysis of *H. circularisquama* in Lake Kamo, 2010, and characterized the host- and virus clones isolated during the survey. The main objectives of this study were to assess the possible virus-host relationship in the *H. circularisquama* population in Lake Kamo, and elucidate the diversity of *H. circularisquama* clones and its virus clones isolated from Lake Kamo, 2010.

Materials and Methods

1. Field survey and sampling

Sample waters and sediments were collected at three stations in Lake Kamo (Fig. 1): Katagami (St. 1; 6.8 m in depth), Harakuro (St. 2; 7.8 m in depth), and Akitsu (St. 3; 5.5 m in depth), where the ebb and flow were scarcely perceptible during the survey. Water samples were collected from the surface layer (0 m) and 0.5 m above the sediment-water interface (B-0.5 m) using a Kitahara's water bottle.

Sediment samples (0-1 cm in depth) were collected using an Ekman-Birge bottom sampler. Subsequently, those samples were immediately sent to the laboratory without fixation. Samplings were conducted from Jul through 11 Nov, 2010 once a week in principle. We referred to data of the *H. circularisquama* cell abundance and environmental parameters (temperature, salinity, dissolved oxygen concentration etc.) during the survey period from the most recent article describing the *H. circularisquama* blooms in Lake Kamo[4].

2. Host isolation and characterization

H. circularisquama cells were isolated from the water samples under the Nikon Eclipse TE300 Inverted Microscope (Nikon, Tokyo, Japan) using a microcapillary. Subsequently, the *H. circularisquama* clonal isolates were incubated in a modified SWM3 medium enriched with 2 nM Na_2SeO_3[1,3] under a 12/12-h light-dark cycle of ca. 130 to 150 μmol of photons m^{-2} s^{-1} using cool white fluorescent illumination at 20°C.

To test the intraspecies virus sensitivity type of each isolated host clone, HcRNAV34 and HcRNAV109 were respectively used as typical lytic virus clones of UA- and CY-types. Each established host clone (1 mL) was inoculated with either of the two virus clones (500 μL) and incubated under the condition mentioned above[14]. The occurrence of viral lysis was judged by optical microscopy principally every other day for 7-10 days after virus inoculation. Algal cultures without virus inoculation (i.e. added with sterilized SWM3 medium) served as negative controls.

3. Virus titration

The virus abundance in both seawater and sediment samples was measured according to the method given by Nagasaki et al.[8] Briefly, the seawater samples were prefiltered through 0.8 μm pore size polycarbonate nuclepore membrane filters (Whatman, Kent, UK) to remove larger organisms such as zooplankton and phytoplankton. Each 5-g sediment sample was then shaken with 5 mL of sterilized SWM3 medium enriched with 2 nM Na_2SeO_3[1,3] at 400 rpm for 30 min, and centrifuged at 2,000 rpm for 10 min at 4˚C. Subsequently, the supernatant was filtered through a 0.2 μm pore-sized Dismic-25cs filter (Advantec, Tokyo, Japan) to obtain virus fraction. The filtrates were diluted with SWM3 medium in a series of 10-fold dilution steps, and aliquots (100 μL) of each dilution step were added to 8 wells in cell culture plates with 96 round-bottom wells (BD-Falcon, Franklin Lakes, NJ, USA) containing 150 μL of an exponentially growing host culture of *H. circularisquama* strains HU9433-P, HCLG-1, 05HC06, and HCKM02, which were respectively isolated from Uranouchi Bay (Kochi Prefecture), Gokasho Bay (Mie Prefecture), Ago Bay (Mie Prefecture), and Lake Kamo (Niigata Prefecture). Among them, HU9433-P, HCLG-1 and 05HC06 are typical host clones which exhibit sensitivity to HcRNAV34 (UA-type), HcRNAV109 (CY-type), and resistance to both HcRNAV strains, respectively[6,14]. HCKM02 (CY-type) was used as a typical host clonal isolate originated from Lake Kamo. The cell culture plates were incubated and the occurrence of algal lysis in each well was monitored as mentioned above. The most probable number of viruses causing lysis of *H. circularisquama* was then calculated using the computer program given by Nishihara et al.[11]

4. Virus isolation and characterization

Through the titration procedure, viruses lytic to *H. circularisquama* strains were screened, and cloned from water and sediment samples by repetitive extinction dilution procedures[7]. The virus suspensions were filtered through a 0.2 μm pore-sized Dismic-25cs filter to remove bacteria, centrifuged at 10,000 ×*g* for 1 min to remove cell debris, and each supernatant was preserved at 4˚C until use.

Subsequently, each clonal virus suspension (500 μL) was inoculated to the vigorously growing cultures (1 mL) of *H. circularisquama* strains HU9433-P, HCLG-1 and 05HC06 to determine the intraspecies infection specificity. The cell culture plates were then incubated and checked as mentioned above. Algal cultures inoculated with sterilized SWM3 medium served as negative controls, while those inoculated with each compatible virus clone served as positive controls.

5. MCP sequence analysis

A PCR designed to amplify the fragments of HcRNAV ORF2 (MCP gene) was conducted. Forward and reverse primers were 5'- TTT CAC CCT GAG CAC CTT CCG C -3' and 5'- CGC CAT GCA ACG CAT TAA GCA GC -3', respectively, which were designed based on the nucleotide sequences of HcRNAV34 and HcRNAV109[9].

Genomic RNAs of newly-isolated HcRNAV clones were extracted with TRIzol LS reagent (Life Technologies, California, USA). For RNA isolation, 0.25 mL of each fresh virus suspension was used. The resulting crude RNAs were reverse-transcribed to obtain cDNAs with Superscript III Reverse Transcriptase (Life Technologies) using random primers according to the manufacturer's recommendation. The cDNAs were used as templates for the PCR with a High Fidelity RNA PCR kit (TaKaRa Biomedicals, Otsu, Japan). Subsequently, PCR amplification was performed using a 20 μL mixture containing 1 μL of cDNA solution (prepared as described above), 2 μL of 10×Buffer for Blend Taq, 2 μL of 2 mM dNTPs, 0.5 μL of 2.5 units μL^{-1} Blend Taq polymerase and 1 μL of 10 μM primers mentioned above. The PCR was conducted using a GeneAmp PCR System 9700 (Applied Biosystems, California, USA) with the following cycles: 1 cycle of denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at

68.3°C for 30 s, extension at 72°C for 1 min; and a final extension cycle of 68ºC for 10 min. The resultant PCR products were purified using Exo-Sap IT (USB, Ohio, USA) before direct sequencing. DNA sequencing was performed using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

6. Phylogenetic analysis

Phylogenetic analysis was performed using the deduced amino acid sequences based on the consequently obtained fragment nucleotide sequences of the MCP gene. They were aligned with HcRNAV MCP amino acid sequences of previously analyzed HcRNAV clones, including HcRNAV34 and HcRNAV109 (DDBJ accession numbers AB218608 and AB218609, respectively). Subsequently, a phylogenetic tree was constructed via the Maximum Parsimony method using MEGA 5[12]. Refer to Table 1 showing the origin of each of the virus clonal strains used in this analysis.

Table 1. HcRNAV strains used in the present study

Virus strain	Isolation locality	Year	Host strain used for isolation	MCP type*
HcRNAV15-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV28-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV46-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV62-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV63-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV75-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV79-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV80-kamo10	Lake Kamo, Niigata, Japan	2010	05HC06	UA
HcRNAV82-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV85-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV86-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV89-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV90-kamo10	Lake Kamo, Niigata, Japan	2010	05HC06	UA
HcRNAV92-kamo10	Lake Kamo, Niigata, Japan	2010	05HC06	UA
HcRNAV99-kamo10	Lake Kamo, Niigata, Japan	2010	05HC06	UA
HcRNAV103-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV107-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV110-kamo10	Lake Kamo, Niigata, Japan	2010	HU9433-P	UA
HcRNAV106-kamo10	Lake Kamo, Niigata, Japan	2010	HCLG-1	CY
HcRNAV13	Gokasho Bay, Mie, Japan	2000	HCLG-1	CY
HcRNAV34	Ago Bay, Mie, Japan	2001	HA92-1	UA
HcRNAV109	Obama Bay, Fukui, Japan	2001	HY9423	UA
HcRNAV136	Ago Bay, Mie, Japan	2001	HU9433-P	UA
HcRNAV141	Ago Bay, Mie, Japan	2001	HCLG-1	CY
HcRNAV142	Ago Bay, Mie, Japan	2001	HCLG-1	CY
HcRNAV616	Ago Bay, Mie, Japan	2007	05HC05	UA
HcRNAV626	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV630	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV631	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV633	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV635	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV637	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV639	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV640	Kusuura Bay, Kumamoto, Japan	2007	HCLG-1	CY
HcRNAV641	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV643	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV644	Kusuura Bay, Kumamoto, Japan	2007	HCLG-1	CY
HcRNAV645	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV647	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV649	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV650	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV653	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV654	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV655	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV658	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV659	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV660	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV662	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV663	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV664	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV665	Kusuura Bay, Kumamoto, Japan	2007	HCLG-1	CY
HcRNAV667	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV668	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA

Table 1. (Continued)

Virus strain	Isolation locality	Year	Host strain used for isolation	MCP type*
HcRNAV669	Kusuura Bay, Kumamoto, Japan	2007	HCLG-1	CY
HcRNAV671	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV672	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV674	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV676	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV678	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV679	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV680	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV683	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA
HcRNAV684	Kusuura Bay, Kumamoto, Japan	2007	HU9433-P	UA
HcRNAV686	Kusuura Bay, Kumamoto, Japan	2007	05HC05	UA
HcRNAV687	Kusuura Bay, Kumamoto, Japan	2007	05HC06	UA

* Refer Fig. 5.

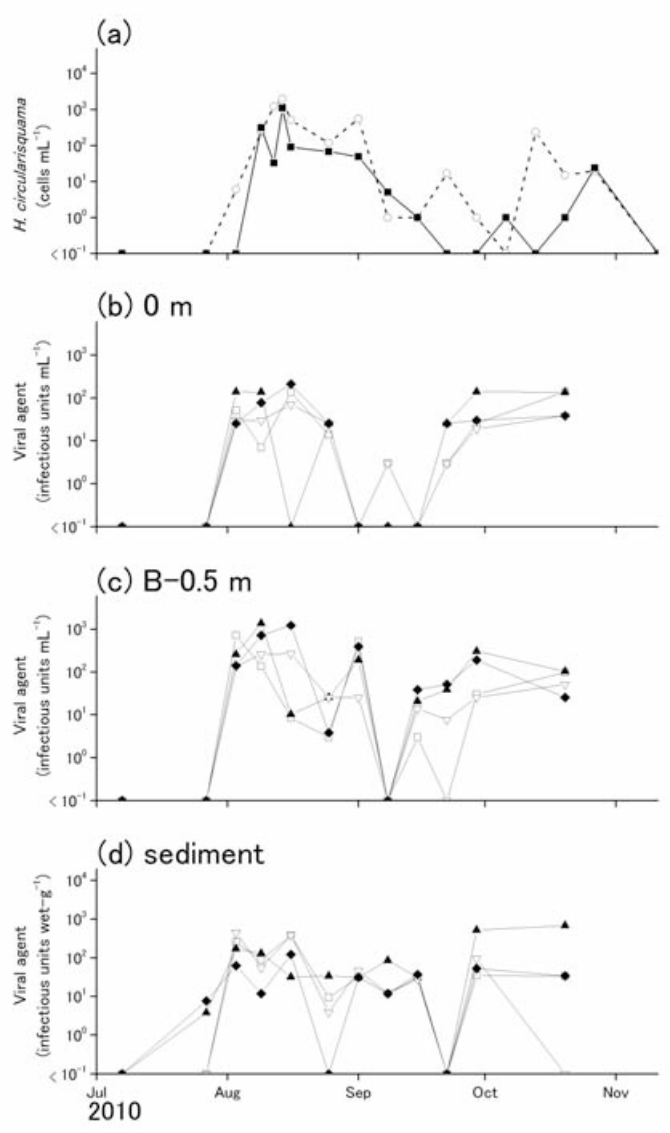

Fig. 2. Changes in abundance of *Heterocapsa circularisquama* cells (a) in the surface layer (■) and 0.5 m above the sediment-water interface (○), its lytic viral agents in the surface layer (b), 0.5 m above the sediment-water interface (B-0.5 m) (c), and sediment (d) at Katagami (St. 1)

Density of viral agent lytic to *H. circularisquama* was enumerated by the MPN method using the four typical host strains: HU9433-P (□), 05HC06 (▲) HCKM02 (▽), and HCLG-1 (◆). (The data of *H. circularisquama* cell abundance was reproduced with copyright permission from the Japanese Society of Fisheries Science: Kondo, S. et al. *Nippon Suisan Gakkaishi*, **78,** 719-725 [published in August 2012])

Results and Discussion

1. Possible ecological relationship between *Heterocapsa circularisquama* and its viruses

Throughout the duration of the present field survey, both *H. circularisquama* and its viruses were detected at all three sampling stations (Fig. 1). Their temporal change in abundance at Katagami (St. 1), Harakuro (St. 2) and Akitsu (St. 3) stations are respectively shown in Figs. 2, 3, and 4. In 2010, *H. circularisquama* cells were first detected on 27 Jul at the bottom layer of St. 2 and 3. Subsequently, a peak of the bloom was observed on 12-16 Aug, when the cell density scored >1,000 cells mL^{-1} at any layer of the three stations. The maximum cell abundance (*ca.* 20,000 cells mL^{-1}) was recorded at the surface layer of St. 3[4]. The transition trend in abundance of *H. circularisquama* and its viruses almost coincided at the three sampling stations. After the first moderate peak of *H. circularisquama* in early to mid-August, the population showed steep decrease over September, whereupon the second smaller peak was detected in October (Figs. 2, 3, and 4). It was noticeable that a considerable proportion of cells in the *H. circularisquama* population likely remained in the bottom layer; especially at St. 1, while the highest cell density was estimated at 240 cells mL^{-1} in the bottom layer. This may reflect the loss of motility of *H. circularisquama* cells due to viral infection[8]

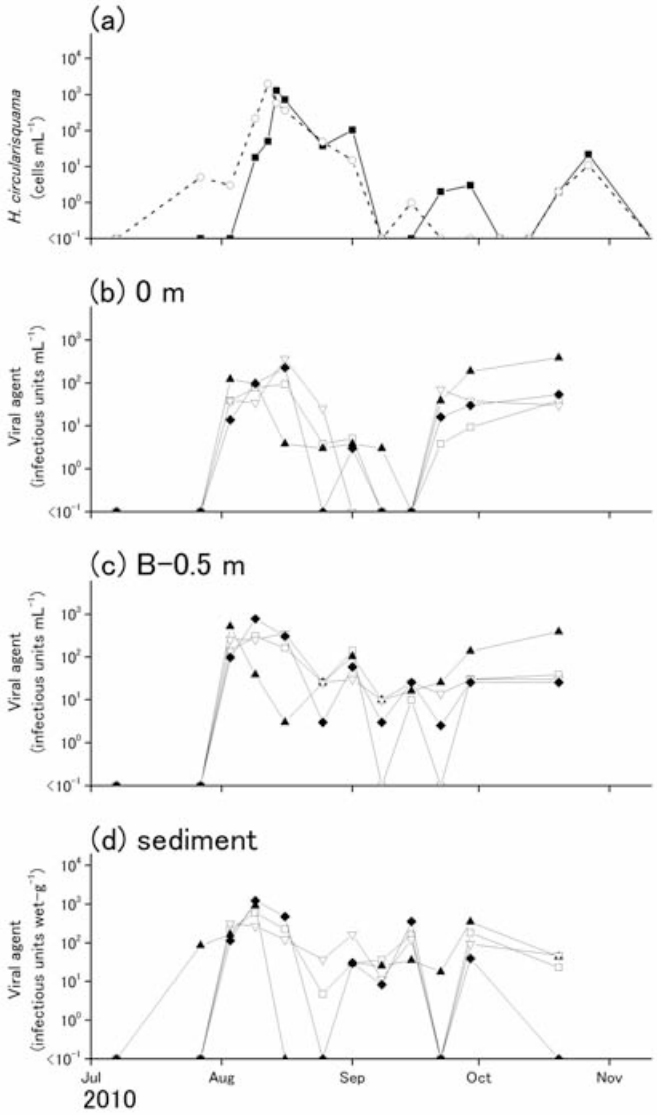

Fig. 3. Changes in abundance of *Heterocapsa circularisquama* cells (a) in the surface layer (■) and 0.5 m above the sediment-water interface (○), its lytic viral agents in the surface layer (b), 0.5 m above the sediment-water interface (B-0.5 m) (c), and sediment (d) at Harakuro (St. 2)

Density of viral agent lytic to *H. circularisquama* was enumerated by the MPN method using the four typical host strains: HU9433-P (□), 05HC06 (▲), HCKM02 (▽), and HCLG-1 (◆). (The data of *H. circularisquama* cell abundance was reproduced with copyright permission from the Japanese Society of Fisheries Science: Kondo, S. et al. *Nippon Suisan Gakkaishi*, **78,** 719-725 [published in August 2012])

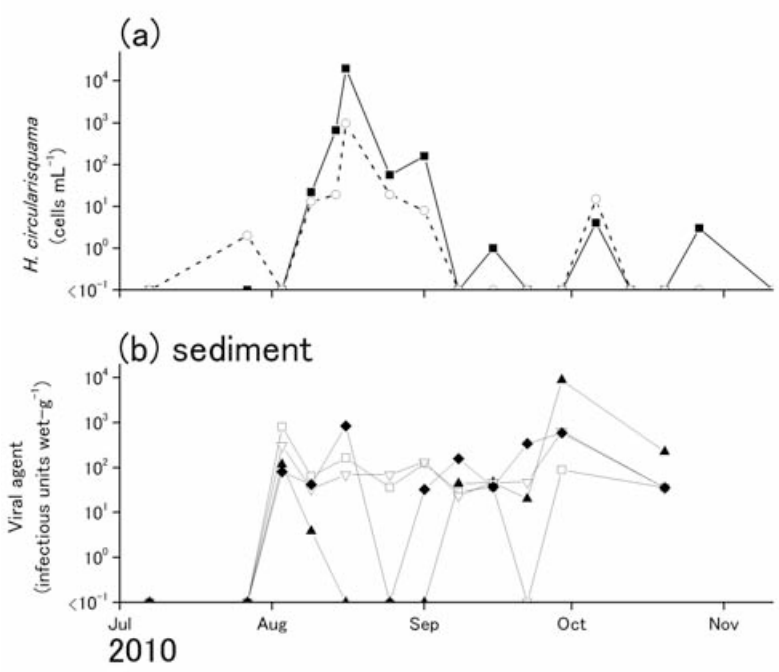

Fig. 4. Changes in abundance of *Heterocapsa circularisquama* cells (a) in the surface layer (■) and 0.5 m above the sediment-water interface (○), and its lytic viral agents in sediment (b) at Akitsu (St. 3)

Density of viral agent lytic to *H. circularisquama* was enumerated by the MPN method using the four typical host strains: HU9433-P (□), 05HC06 (▲), HCKM02 (▽), and HCLG-1 (◆). (The data of *H. circularisquama* cell abundance was reproduced with copyright permission from the Japanese Society of Fisheries Science: Kondo, S. et al. *Nippon Suisan Gakkaishi*, **78,** 719-725 [published in August 2012])

(N. Hata, unpubl. data). Considering the co-occurrence of the host bloom and increase in virus titer, these data suggest an ecological relationship between *H. circularisquama* and its viruses in Lake Kamo. Another point of interest is the increased virus abundance observed from September over October when host cells were not so abundant, both in the water column and bottom layer (Figs. 2, 3, and 4). One possible explanation is that the increase in viral titer in the water column was due to disturbance of sediment viruses caused by the foul weather in mid-September (refer to the following data provided by the Japan Meteorological Agency: http://www.jma-net.go.jp/niigata/menu/sokuhou/20100911.pdf).

The peak period of the *H. circularisquama* bloom in 2010 was shorter than that in 2009. Formerly, a similar trend was also observed in Ago Bay; i.e. occurrence of the first large bloom in 1993 was followed by the small bloom occurrence in 1994 (data not shown). The impact of viruses accumulated in this lake in 2009 on the *H. circularisquama* bloom, which occurred in 2010, is of considerable interest. Now studies on the activity of sediment viruses as a biological suppressor against *H. circularisquama* are underway.

2. Typing of host- and virus clones isolated from Lake Kamo

In the present survey, 62 clones of *H. circularisquama* were isolated from the water samples collected from Lake Kamo, and their virus sensitivity was examined (Table 2). Twenty and 26 clones were shown to be sensitive to HcRNAV34 (UA-type) and HcRNAV109 (CY-type), respectively. The remaining 16 host clones were not apparently lysed by either of them, suggesting that these differ from ever-known types of *H. circularisquama*. Mizumoto et al. (2008) reported that some of the *H. circularisquama* strains originating from Ago Bay showed very low or no sensitivity to HcRNAV34 and HcRNAV109, hence the present results suggest the existence of unidentified host-virus group(s) differing in intraspecies specificity or efficiency of intracellular virus replication[6]. The 16 unlysed host clones tested in the present study may belong to the undiscovered group(s).

Nineteen virus clones were isolated through extinction dilution procedures using *H. circularisquama* strains HU9433-P, HCLG-1, and 05HC06 as hosts. Through the characterization, all the clones were revealed to be HcRNAV (not HcDNAV). Among them, 8 and 7 virus clones were respectively lytic to UA- and CY-type typical host clones, i.e. respectively lytic to HU9433-P and HCLG-1. The other 4 clones were lytic to the strain 05HC06 resistant to HcRNAV34 and HcRNAV109[6], and are thus considered to differ in host range. Furthermore, even within the 4 clones lytic to 05HC06, two were infectious to HU9433-P (Table

Table 2. List of *Heterocapsa circularisquama* clones isolated from Lake Kamo, 2010, and their virus sensitivity

H. circularisquama clones (No.)	Virus sensitivity		
	sensitivity to HcRNAV 34	sensitivity to HcRNAV 109	infection type
1	-	-	n.d.*
3	-	+	CY
4	+	-	UA
5	-	+	CY
6	-	+	CY
7	+	-	UA
9	+	-	UA
10	+	-	UA
11	-	-	n.d.
12	-	-	n.d.
13	+	-	UA
15	+	-	UA
16	+	-	UA
17	+	-	UA
19	-	+	CY
20	+	-	UA
21	-	+	CY
22	-	-	n.d.
23	-	+	CY
25	+	-	UA
28	-	+	CY
29	-	-	n.d.
30	-	-	n.d.
32	+	-	UA
34	-	+	CY
35	-	+	CY
36	-	-	n.d.
37	-	+	CY
38	-	+	CY
39	-	+	CY
40	-	+	CY
41	-	+	CY
42	-	-	n.d.
43	-	-	n.d.
44	+	-	UA
45	-	+	CY
46	+	-	UA
47	+	-	UA
48	-	+	CY
49	-	+	CY
51	+	-	UA
53	-	+	CY
55	+	-	UA
56	-	-	n.d.
60	-	-	n.d.
62	-	+	CY
64	-	-	n.d.
65	+	-	UA
70	-	+	CY
71	+	-	UA
73	+	-	UA
77	+	-	UA
n1	-	+	CY
n2	-	-	n.d.
n3	-	+	CY
n4	-	-	n.d.
n5	-	+	CY
n6	-	+	CY
n8	-	-	n.d.
n9	-	+	CY
n10	-	-	n.d.
n11	-	+	CY

* not determined.

3: HcRNAV90-kamo10 and HcRNAV99-kamo10) but the other two were not (Table 3: HcRNAV80-kamo10 and HcRNAV92-kamo10). Accordingly, HcRNAV clones are assumed to be more diverse than we previously evaluated.

3. Phylogenetic analysis of HcRNAV MCP

Each of the 19 HcRNAV clones isolated from Lake Kamo was designated with "kamo10" in its code name (Table 1). Part of the major capsid protein (MCP) gene (ORF2) of each HcRNAV clone was PCR-amplified, sequenced, and the deduced amino acid sequence was phylogenetically compared with the previously isolated HcRNAV strains differing in localities and years of isolation (Table 1). Consequently, they were phylogenetically separated into two groups; i.e. one comprising 12 isolates showing lytic activity to HU9433-P and/or 05HC06 (UA-type), and the other composed of 7 isolates lytic to HCLG-1 (CY-type) (Fig. 5). The present results supported the previous report whereby the difference in the amino acid sequence of MCP effectively coincides with the infection specificity type[9] (Table 3). The bootstrap value supporting the CY-type clade was as high as 99%; in contrast, the UA-type clade was supported with a bootstrap value lower than 70%. Further, it is to be noted that HcRNAV clones that showed lytic activity to 05HC06 were not phylogenetically separated from those only lytic to HU9433-P in the tree (Table 3, Fig. 5). Through the analysis, the clonal composition of HcRNAV was considered diverse also in Lake Kamo, as was observed in the cases of Ago Bay, Kusuura Bay and the other coastal waters of western Japan (Table 1)[6].

4. Implication

Lake Kamo is separately from the main islands of Japan (Fig. 1), where the first outbreak of *H. circularisquama* bloom was recorded in 2009. It is most probable that the host and its viruses were simultaneously transplanted to this highly enclosed lagoon[4]. Through the present study, we found that *H. circularisquama* and its viruses may be ecologically related to each other. Viral infection is most likely one of the factors affecting the dynamics of the *H. circularisquama* population also in Lake Kamo. In addition, the host- and virus clones were shown to be highly diverse. A continuous field survey is important to further comprehend the ecological relationship between *H. circularisquama* and its viruses in natural waters.

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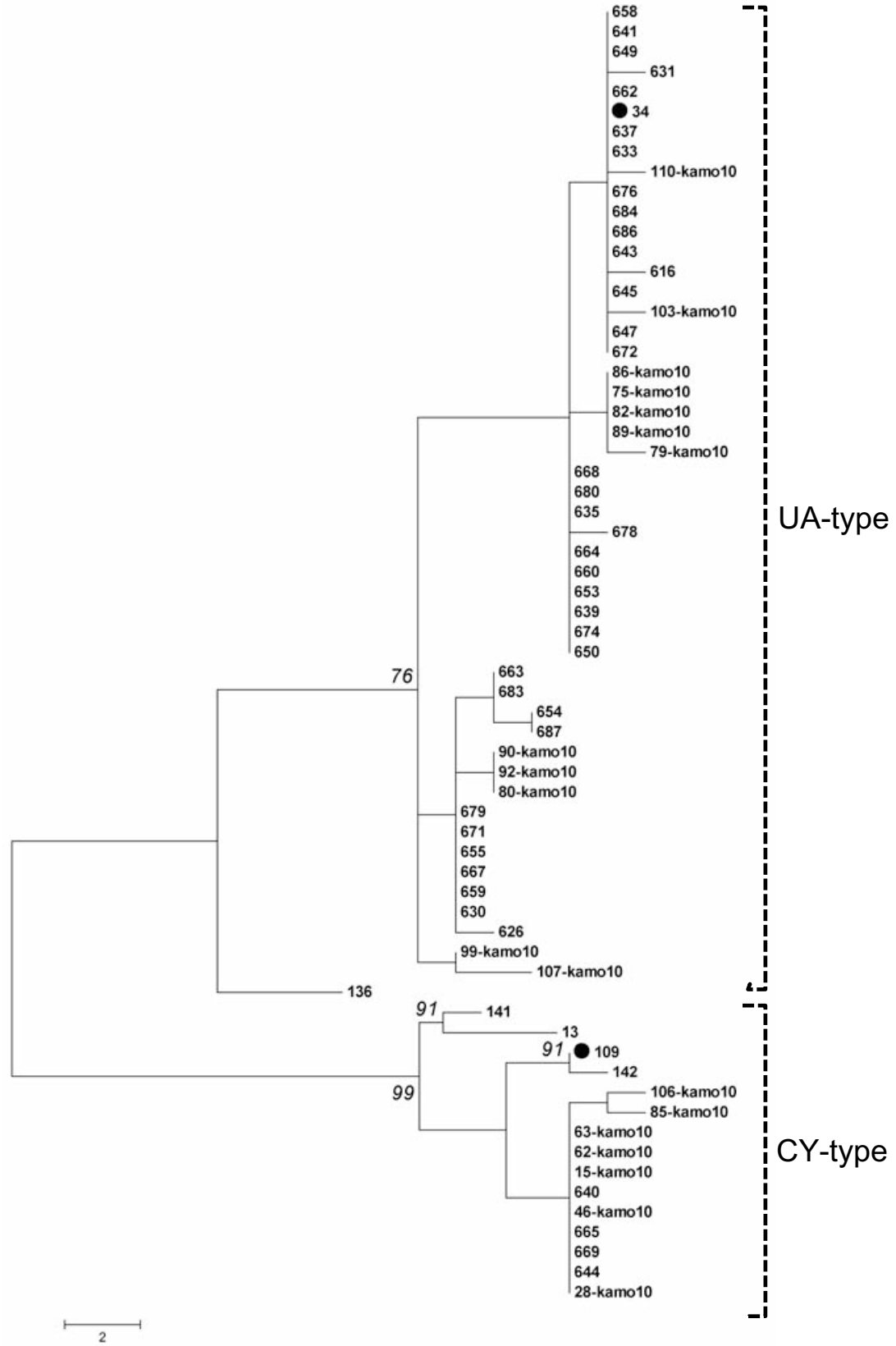

Fig. 5. Phylogenetic tree calculated from confidently aligned regions of amino acid sequences of the major capsid protein of various HcRNAV strains

Note that the HcRNAV clones were roughly separated into two clades, UA- and CY-types, in this tree. Nodes with bootstrap values (in Italic) below 70% were collapsed. The bar indicates two fixed mutations per amino acid position. HcRNAV34 and HcRNAV109 are the representative strains of UA- and CY-types, respectively, as mentioned in the text (●).

Table 3. List of HcRNAV strains isolated in Lake Kamo, 2010, and their MCP type

Virus strain	Infectivity to *Heterocapsa circularisquama* strains			Host strain used for virus isolation	MCP type*
	HU9433-P	05HC06	HCLG-1		
HcRNAV75-kamo10	+	-	-	HU9433-P	UA
HcRNAV79-kamo10	+	-	-	HU9433-P	UA
HcRNAV82-kamo10	+	-	-	HU9433-P	UA
HcRNAV86-kamo10	+	-	-	HU9433-P	UA
HcRNAV89-kamo10	+	-	-	HU9433-P	UA
HcRNAV103-kamo10	+	-	-	HU9433-P	UA
HcRNAV107-kamo10	+	-	-	HU9433-P	UA
HcRNAV110-kamo10	+	-	-	HU9433-P	UA
HcRNAV80-kamo10	-	+	-	05HC06	UA
HcRNAV90-kamo10	+	+	-	05HC06	UA
HcRNAV92-kamo10	-	+	-	05HC06	UA
HcRNAV99-kamo10	+	+	-	05HC06	UA
HcRNAV15-kamo10	-	-	+	HCLG-1	CY
HcRNAV28-kamo10	-	-	+	HCLG-1	CY
HcRNAV46-kamo10	-	-	+	HCLG-1	CY
HcRNAV62-kamo10	-	-	+	HCLG-1	CY
HcRNAV63-kamo10	-	-	+	HCLG-1	CY
HcRNAV85-kamo10	-	-	+	HCLG-1	CY
HcRNAV106-kamo10	-	-	+	HCLG-1	CY

* Refer Fig. 5.

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