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REVIEW

Dinoflagellate-infecting viruses

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Dinoflagellates (Dinophyceae) are considered to be one of the most abundant and diverse groups of phytoplankton; however, the viral impact on dinoflagellates was not studied until recently. This review shows the present information concerning the viruses infecting dinoflagellates and the ecology relationships between the host and the virus. So far, two viruses have been isolated and characterized: a large DNA virus (HcV: *Heterocapsa circularisquama* virus) and a small RNA virus (HcRNAV: *H. circularisquama* RNA virus); both of which are infectious to the harmful bloom-forming dinoflagellate *H. circularisquama*.

In the present review, we mainly discuss the relationship between HcRNAV and *H. circularisquama* from the viewpoint of physiology, ecology and genetics. It will help clarify the viral impact on dinoflagellate populations in marine environments to understand the host/parasite ecology.

Dinoflagellates (Dinophyceae) are single-celled, aquatic organisms bearing two dissimilar flagellae; most of them are microscopic and marine. They have characteristics of both plants and animals: some are autotrophic using photosynthesis; some are heterotrophic using endocytotic feeding; and some are mixotrophs having both metabolisms. Because dinoflagellates are one of the most abundant and diverse groups of micro-plankton, their contribution as the net primary producers and decomposers is considered to be of great significance (Graham & Wilcox, 2000). Dinoflagellates contain a large number of species; they include various harmful bloom-forming species such as *Karenia mikimotoi*, *Gonyaulax polygramma*, *Cochlodinium polykrikoides* and *Heterocapsa circularisquama*; and toxic species such as *Alexandrium tamarense*, *A. catenella*, *Gymnodinium catenatum*, *Dinophysis* spp., and *K. brevis*. These dinoflagellates are intensively studied; among them *H. circularisquama* is one of the most studied dinoflagellate species (Horiguchi, 1995). It forms a dense bloom that kills bivalves. Since 1988 when its first bloom was observed, *H. circularisquama* blooms in Japan have frequently caused mass mortalities in bivalves: e.g. pearl oyster *Pinctada fucata martensii*, short-necked clam *Ruditapes philippinarum*, mussel *Mytilus edulis*, oyster *Crassostrea virginica*. More than 40 cases of *H. circularisquama* red tides are recorded (previous to 2002) including 18 leading to damage to the fishery (Matsuyama, 2003). To determine the ecology of *H. circularisquama* (as with studies on the other phytoplankton species), a number of studies have been conducted concerning various factors determining its dynamics: physical factors such as temperature, salinity and irradiation; chemical factors e.g. nutrients and vitamins; and biological factors, i.e. predators, competition with other phytoplankton, the effects of algicidal bacteria and viral impacts.

Studies concerning viral infection in dinoflagellates were very rare in the 20th Century. Three findings of virus-like particles (VLPs) in dinoflagellates by transmission electron microscopy (TEM) were reported in the 1970s. Franca (1976) found small VLPs (35 nm in diameter) arranged in a crystalline array in the cytoplasm of *Gyrodinium resplendens* (Hulbert) but detected no cellular lesion. Soyer (1978) reported the presence of VLPs (30 nm in diameter) in the nucleus of a dinoflagellate, *Blastodinium* Chatton, which principally parasitizes copepods. Sicko-Goad & Walker (1979) found large VLPs (385 nm in diameter) budding from the viroplasmic area in a freshwater dinoflagellate, *Gymnodinium uberrimum*. The morphology of the VLP and budding from the viroplasm suggests this particle is a phycodnavirus or its closely related virus (Wilson et al., 2005). However, none of the VLPs discovered in these dinoflagellates were further studied because cultivation was unsuccessful.

Wilson et al. (2001) induced virus-like agents presumably latent in zooxanthellae (Dinophyceae) cells, a symbiont of the sea anemone *Anemonia viridis,* by elevating the temperature of cultivation. The virus-like agents were transferable causing lysis of fresh zooxanthellae cultures. Based on these observations, they concluded that the virus-like agents fulfilled Koch's postulate. They proposed viral induction due to high water temperatures might be one of the causes of bleaching of coral reefs assuming that the zooxanthellae in *A. viridis* are taxonomically close to those in reef corals. Further investigation is expected for this host/virus relationship.

The first and the second cultured dinoflagellate-infecting viruses that were characterized were HcV (Figure 1A,B) (Tarutani et al., 2001) and HcRNAV (Figure 1C,D) (Tomaru et al., 2004b), respectively; both of which are infectious to *H. circularisquama*. This allowed study of the host/virus relationship between the viruses and dinoflagellates. In the present review, studies concerning the dinoflagellate-infecting viruses are shown and the ecological relationship between dinoflagellates and their viruses are discussed using *H. circularisquama* and its RNA virus (HcRNAV) as an example.

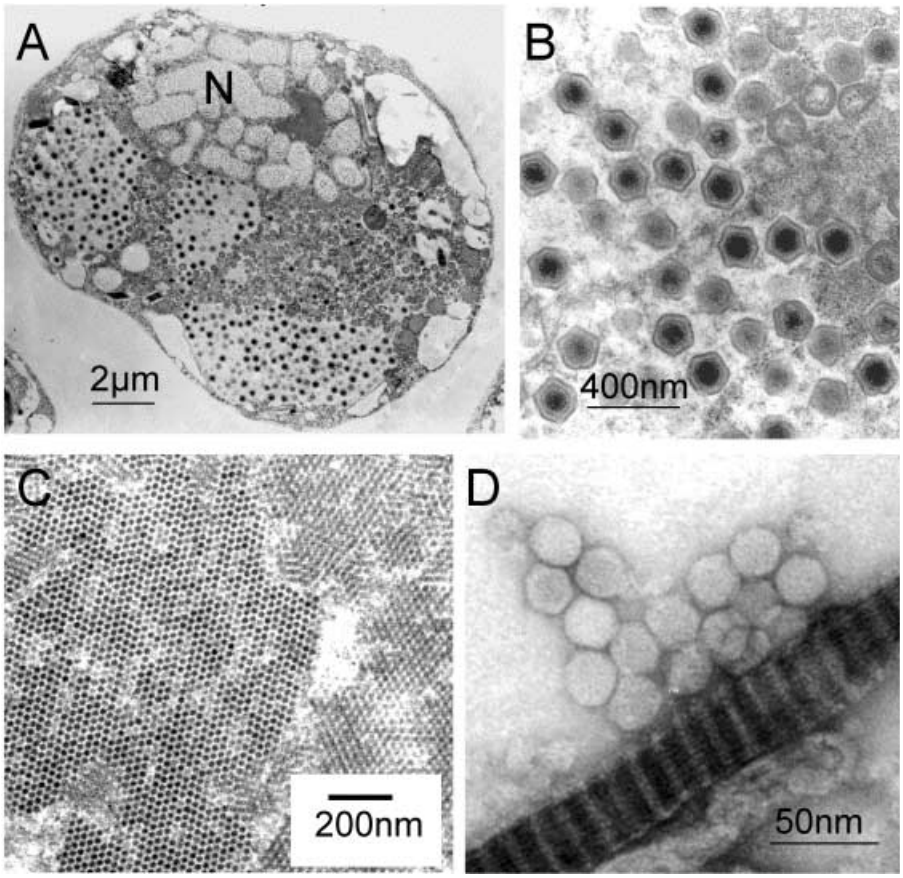

Figure 1. Transmission electron micrographs of viruses infecting *Heterocapsa circularisquama*. (A) Thin section of a *H. circularisquama* cell infected with HcV (N, nucleus); (B) thin section of HcV particles propagating in the host's cytoplasm; (C) thin section of crystalline arrays of HcRNAV particles propagating in the host's cytoplasm; and (D) negatively-stained HcRNAV particles.

Characteristics of HcV

HcV is an icosahedral virus 180–210 nm in diameter (Figure 1B) with a double-stranded DNA (dsDNA) genome ~356 kbp in length. This virus is propagated within the cytoplasm of its host cell (Figure 1A) where the virion is associated with the viroplasmic regions (Nagasaki et al., 2003). Based on these features, HcV is considered to be a member of the family Phycodnaviridae (Wilson et al., 2005) although phylogenetic analysis on its DNA polymerase amino acid sequence (generally regarded as a key in molecular-based classification for dsDNA viruses) has not been conducted. It infects *H. circularisquama* in a wide range of temperatures (15–30°C). The latent period and the burst size were respectively estimated at 40–56 h and 1800–2440 infectious particles cell^{-1} using one-step growth experiments (Nagasaki et al., 2003). In the cross-reactivity test, viral lysis was observed in ~95% of the combinations between HcV strains and *H. circularisquama* strains ([55+429]/[60+450]; Table 1) (Nagasaki et al., 2003). In contrast to HcRNAV that shows apparent strain-specific infectivity, HcV likely does not distinguish host ecotypes UA and CY (Table 1; see below).

Characteristics of HcRNAV

Morphological and physiological features

HcRNAV is an icosahedral virus ~30 nm in diameter (Figure 1D) harbouring a single-stranded RNA (ssRNA) genome ~4.4 kb long. The virus replicates in the cytoplasm of *H. circularisquama* (Figure 1C) (Tomaru et al., 2004b). The cross-reactivity test using 56 clonal host strains and 107 clonal HcRNAV strains isolated from various sources (i.e. 5992 host/virus combinations) show the viruses were roughly divided into two ecotypes, UA and CY that have a strain-specific infectivity complementary to each other (Table 1). Simultaneously, the host strains were also divided into two ecotypes based on the virus sensitivity spectra; i.e. type UA hosts were only sensitive to type UA HcRNAV, and type CY hosts were

Table 1. *Proportion of the host–virus strain combinations in which apparent viral lysis of* Heterocapsa circularisquama *cells was observed. (Reproduced and summarized from figure 7 in Nagasaki et al. [2003] and figure 2 in Tomaru et al. [2004b] with permission of the publishers.)*

	Viruses		
Host type	HcV	Type UA HcRNAV	Type CY HcRNAV
H. circularisquama Type UA	55/60 (91.7%)	345/469 (73.6%)	3/280 (1.1%)
H. circularisquama Type CY	429/450 (95.3%)	0/3149 (0%)	1806/1880 (96.1%)

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only sensitive to type CY HcRNAV (Tomaru et al., 2004b). Because the randomly isolated *H. circularisquama* strains showed specific sensitivity to either type CY or type UA HcRNAV, two host ecotypes having distinct virus sensitivity may occupy a large portion in natural *H. circularisquama* populations. However, the two ecotypes of *H. circularisquama* strains are indistinguishable by optical or transmission electron microscopy because of their high resemblance in morphology. Even by comparing the nucleotide sequences of the ribosomal DNA internal spacer regions which is assumed to be relatively variable at the subspecies level, they are not distinguished (Shirai et al., 2006). To summarize, these observations indicate multiple ecotypes of *H. circularisquama* and HcRNAV coexist in natural waters.

Ecological impact on Heterocapsa circularisquama

Nagasaki et al. (2004) conducted a field survey in Ago Bay, Japan to compare the dynamics of *H. circularisquama* and its infectious viruses. Through the survey, they observed a specific increase in abundance of HcRNAV-like viral agents *in situ* in and just after the bloom of *H. circularisquama*. Each virus ecotype independently fluctuated in abundance. Because the strain-specific infectivity of HcRNAV is highly strict (Table 1), each HcRNAV ecotype is considered to be propagated and released to the environment only by its suitable host ecotype. Hence, the dynamics of each HcRNAV ecotype should reflect the changes in abundance of its host ecotype in natural waters. There may be at least two distinct and independent host/virus systems between *H. circularisquama* and HcRNAV that can coexist within a natural bloom of *H. circularisquama* (Nagasaki et al., 2004; Tomaru et al., 2004b).

Another point of interest is the dynamics and ecological roles of HcRNAV in sediments. Following *H. circularisquama* blooms, the abundance of HcRNAV in the water column rapidly decreased; whereas, in the sediment it showed a much more gradual decrease. Hence, sediments may be the reservoir suitable for HcRNAV (Nagasaki et al., 2004). In a continuous field survey from 2001 through 2005 studying the ecological relationship between *H. circularisquama* and its viruses in Ago Bay, a tendency was found that large-scale blooms occurred when the abundance of HcRNAV in the sediment was low (Tomaru et al., unpublished data). The sediment viruses may be responsible for determining the size and term length of *H. circularisquama* blooms.

Based on these observations, HcRNAV is considered to be a remarkable factor affecting the population dynamics of *H. circularisquama*. Considering that two independent host/virus systems coexist in a bloom, the impact of HcRNAV on *H. circularisquama* population may be not only quantitative but also qualitative; i.e. it may affect the changes in the host clonal composition within the population as well as the host population's biomass.

Recent dominance of HcRNAV in Heterocapsa circularisquama *blooms*

Natural *H. circularisquama* cells harboring HcV-like particles or HcRNAV-like particles were found to be coexisting in a bloom (Tomaru & Nagasaki, 2004). Although further ecology studies are required, the presence of the viruses in natural host cells suggests both viruses may be potentially important in controlling *H. circularisquama* blooms (a host/parasite disease relationship).

In spite of this prediction, recent survey suggests the dominance of HcRNAV in its host blooms. Authors' group has isolated viruses infectious to *H. circularisquama* since 1999 from the western part of Japan where *H. circularisquama* often causes blooms. The ten HcV strains were isolated only in the summer of 1999 (Nagasaki et al., 2003); however, since then, no HcV strains have been isolated despite repeated isolation trials. We generally use an extinction dilution method to isolate viruses, where viral isolates were selected from the highest dilution well (Tomaru et al., 2004b). Hence, by this method, the dominant viruses infecting the *H. circularisquama* strains (presumably having a higher lytic activity to the host strains tested) tend to be selected. It may be that HcV was lower in number than HcRNAV in natural waters except in the cases in 1999 where HcV was presumably dominant. This hypothesis rationally explains the reason why HcV has not been isolated using our method since 2000. In addition, HcRNAV is less light-sensitive and temperature-sensitive than HcV (Tomaru et al., 2005); the burst size of HcRNAV is much larger than that of HcV; and the latent period of HcRNAV is shorter than that of HcV. Because of these characteristics, HcRNAV may have become the dominant virus infecting *H. circularisquama* in natural environments. We may be seeing in our field surveys since 1999 a succession process of the major viruses affecting *H. circularisquama*. Now we regard HcRNAV as the major virus influencing the dynamics and degradation of *H. circularisquama* blooms *in situ*. This is also supported by the results of dot-blot analysis using a HcRNAV-specific probe, which reacted with more than 95% of the viral agents causing lysis of *H. circularisquama* isolated since 2002 (Tomaru et al., unpublished data).

Genome structure

To further characterize HcRNAV, the typical HcRNAV strains, HcRNAV109 and HcRNAV34 were selected as type CY HcRNAV and type UA HcRNAV, respectively (Tomaru et al., 2004b).

The full genome sequence of HcRNAV109 (4391 bases) and HcRNAV34 (4375 bases) were determined and compared (DDBJ Accession numbers are AB218608 and AB218609, respectively). They are ~97.0% similar at the nucleotide sequence level and each genome has two open reading frames: ORF-1 and ORF-2 putatively encoding a polyprotein and a capsid protein, respectively (Nagasaki et al., 2005; see below). The 3′ ends of both viral genomes have stem-loop structures; however, they are different in stem length and loop size that may affect the replication efficiency in their hosts' cytoplasm (Buck, 1996; Nagasaki et al., 2005).

The deduced amino acid sequence of ORF-1 contains two conserved domains presumably coding for essential enzymes in replication; one is a serine protease domain and the other is the RNA-dependent RNA polymerase

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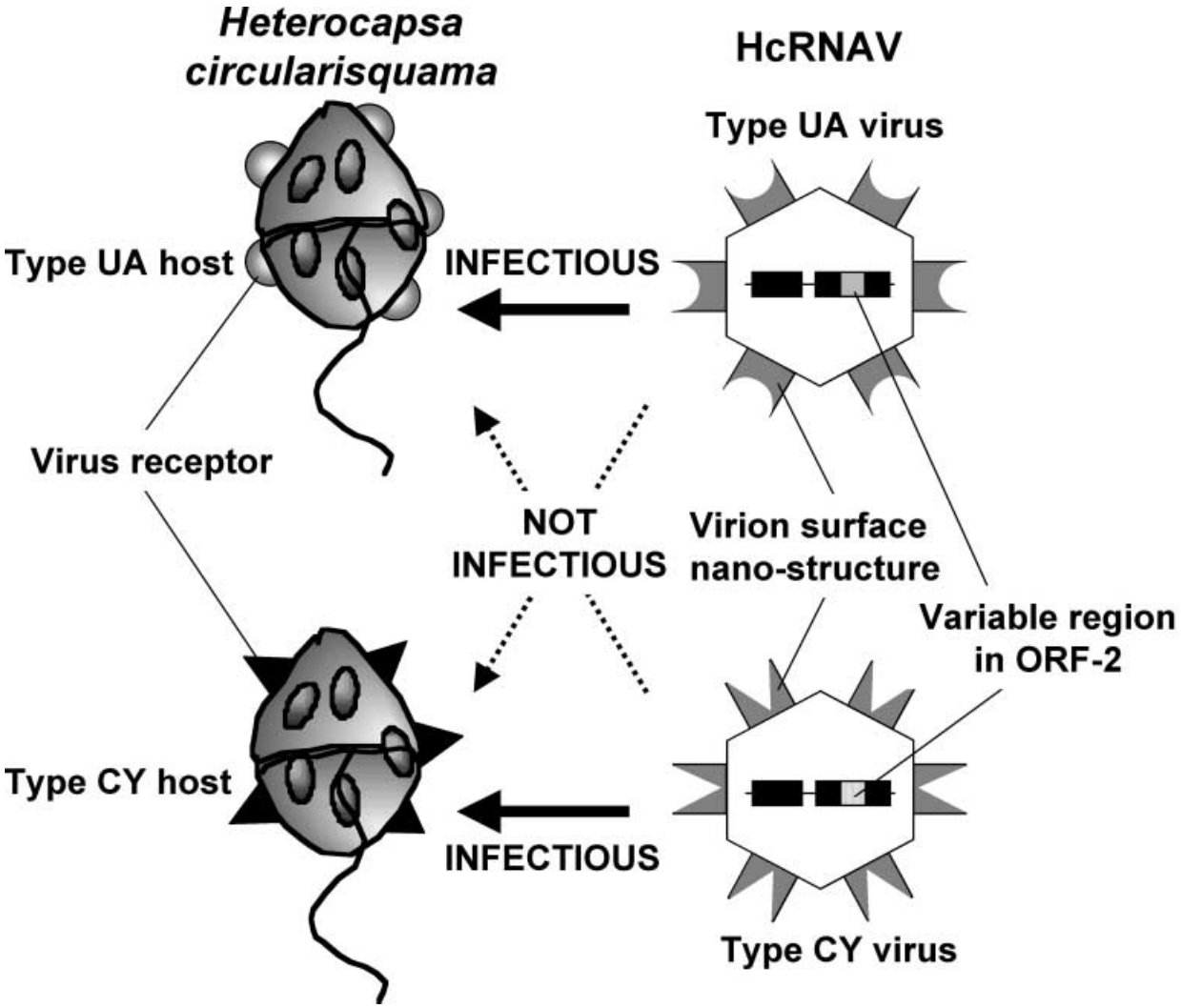

Figure 2. Schematic relationship between *Heterocapsa circularisquama* and HcRNAV. Note the affinity between the hosts' virus receptors and the surface nano-structures of HcRNAV and the difference in the variable region in HcRNAV ORF-2 encoding the capsid proteins. These two host-virus systems coexist in *H. circularisquama* blooms.

(RdRp) domain. This suggests ORF-1 codes for a polyprotein that is translated and cleaved into smaller functional proteins including RdRp (Nagasaki et al., 2005). Other ssRNA viruses such as poliovirus do this (Jacobson & Baltimore, 1968; Dormer et al., 1982). Similarity analysis for the deduced amino acid sequences of ORF-1 found a very low BLAST number for fungi viruses and plant viruses. The best fit BLAST comparison of the serine protease domain was for the Lucerne transient streak virus (e-value=2e-4) and those of the conservative RdRp domain were for the Mushroom bacilliform virus (2e-11) and Poinsettia latent virus (2e-11) (Nagasaki et al., 2005). These significantly high e-values apparently show that HcRNAV is evolutionarily quite distant from any of the land and aquatic viruses that have been genetically studied. This prospect is also supported by the results of phylogenetic analyses of the RdRp domain of HcRNAV and the other ssRNA viruses (Nagasaki et al., 2005; Shirai et al., 2006).

A large proportion of the present database of virus sequences is for land viruses, especially viruses harmful to human, animals, and plants. The database of aquatic viruses is very small; moreover, there are no viruses infecting Dinozoa (a higher classification rank including dinoflagellates) that were genetically examined except for HcRNAV. By expanding the database of aquatic viruses and/or protozoa-infecting viruses, we will be able to discuss the relationships of land viruses and aquatic viruses, their history and evolution.

ORF-2 most likely codes for HcRNAV's single major structural protein (i.e. capsid protein). The molecular weights predicted from the deduced amino acid sequences were 38.2 kDa in HcRNAV34 and 38.3 kDa in HcRNAV109 which are similar to the size evaluation by SDS–PAGE (Nagasaki et al., 2005). Based on these observations and the results of tertiary structure prediction for the capsid plate (Nagasaki et al., 2005), HcRNAV is assumed to have a T=1 capsid structure, and ORF-2 very likely is not a polyprotein gene (i.e. encoding only one protein). Using similarity analysis to other viruses, ORF-2 products of the two HcRNAV strains only showed low homology (~10%) to Black beetle virus capsid protein (data not shown). This also supports the idea that HcRNAV is evolutionarily distant from any viruses that have been genetically studied.

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Mechanism determining intraspecies host specificity of HcRNAV

Four regions of ORF-2 have remarkable amino acid substitutions when comparing HcRNAV34 and HcRNAV109 genomic sequences (Nagasaki et al., 2005). Testing additional three type UA HcRNAV strains and three type CY HcRNAV strains, they showed the intraspecies host specificities may be related to the site-specific amino acid substitutions in ORF-2. By phylogenetically analysing the deduced amino acid sequences of ORF-2, type UA HcRNAV sequences and type CY HcRNAV sequences were divided into distinct clusters, which was supported with a bootstrap value of 100% (Nagasaki et al., 2005). Furthermore, the predicted tertiary structure of the capsid proteins of HcRNAV109 and HcRNAV34 showed that three ORF-2 products form one of the 20 equilateral triangle capsid plates where many of the amino acid substitutions were located in regions on the outside of the viral capsid proteins exposed to the ambient water environments (Nagasaki et al., 2005). This suggests that the intraspecies host specificity of HcRNAV is determined by nano-structures on the virus surface that may affect its binding affinity to the host cell (Figure 2).

HcRNAV was propagated even in the cytoplasm of an inappropriate host ecotype (i.e. host ecotype not lysed by the virus ecotype) when the viral RNA is directly introduced into the cell using a particle bombardment method (Mizumoto et al., unpublished data). This indicates the processes in intracellular viral RNA replication are not directly related in determining the intraspecies host specificity of HcRNAV. Considering that viral replication requires host cellular function as well as viral function (Buck, 1996), the intracellular condition of *H. circularisquama* strains may fulfill the requirements essential for RNA replication and morphogenesis of HcRNAV, which is common between the two host ecotypes. This also emphasizes the above hypothesis that the capsid protein is responsible in determining the intraspecies host specificity of HcRNAV. To summarize, the capsid protein of HcRNAV may have at least three important roles: (1) to establish an attachment of the virion to the cell surface of its suitable host ecotype; (2) to prevent an attachment of the virion to an unsuitable host ecotype; and (3) to defend the genomic RNA from ambient enzymatic activities (digestion by environmental RNases).

Evolution history of HcRNAV ecotypes

Owing to the strictly strain-specific infectivity (intraspecies host specificity) of HcRNAV, the two ecotypes of HcRNAV may have been positively selected due to the dominance of the two host ecotypes having distinct virus sensitivity spectra in natural waters. By genetically comparing the two typical virus ecotype strains, no significant differences are found in the amino acid sequence of ORF-1 that presumably encodes a replicase polyprotein; on the other hand, remarkable amino acid substitutions are located in the amino acid sequence of ORF-2 presumably coding for the capsid protein, especially in regions predicted to be on the outside (hydrophilic side) of the virion (Nagasaki et al., 2005). Assuming that the nanostructure difference on the capsid surface determines the viral adsorption to host cells, it may be that the virus-receptors of the two *H. circularisquama* ecotypes are different in determining their virus sensitivity spectra (Figure 2). However, the virus-receptors of *H. circularisquama* have not been identified yet.

It is believed that the RNA viruses' replication is accompanied with a rapid increase in diversity because RdRp is not equipped with a proof-reading activity (Domingo et al., 1996). Looking at the high similarity and the site-specific amino acid substitutions, one rational explanation is that type UA HcRNAV and type CY HcRNAV originated from a common ancestor virus having a RNA replication mechanism which is similar to that of HcRNAV and later they were differentiated evolutionarily to yield nano-structural differences in the capsid protein that resulted in the different host ranges. Although further study is required to verify this hypothesis, these molecular-ecological studies of the relationship between *H. circularisquama* and HcRNAV show the possibility that capsid alterations can change the phytoplankton-virus (host/parasite) ecological interactions.

Open questions

Investigation of dinoflagellate-infecting viruses is now a new study. The relationship between *H. circularisquama* and its viruses shows there may be a variety of molecular mechanisms involved. While there still remain a number of questions: (1) what was the ancestral virus of HcRNAV? (2) Does HcRNAV (or its ancestral virus) have other hosts in the marine environments? (3) How large is the viral impact on *H. circularisquama* bloom dynamics compared to the other factors? (4) What are the host-virus relationships? (5) Do similar host-virus relationships exist in the other dinoflagellate-virus systems? (6) How diverse are the viruses in the marine environment and how are they related to known viruses? (7) What is the mechanism involved in the viral replication process, especially in the process for translation of essential replicases encoded by HcRNAV ORF-1? (8) How is the HcRNAV downstream ORF (capsid protein gene) translated? (9) Is it possible for HcRNAV to propagate in other algal host species or genera? (10) What molecules function as receptors of *H. circularisquama* to HcV and HcRNAV? More study will be required to answer these questions: the authors are now performing several experiments to examine these.

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