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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

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動物プランクトンに感染する海洋性ウイルスの多様性と生態学的意義の解明

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（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6.研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

Diversity and ecological roles of viruses infecting marine zooplankton

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Marine viruses are the most abundant life forms in the ocean, and they possibly infect all marine organisms. A major cause of mortality of marine bacteria and phytoplankton is caused by viral infections, providing a large impact on food web structures and geochemical cycles in the ocean. Viral pathogens, which cause a significant loses in aquaculture, have been also well studied in commercially important organisms including fish and shrimp. Regardless of ecological importance of marine viruses, studies are mainly limited in microbial and industrially-important organisms. In particular, viruses associated with marine zooplankton are still poorly understood. Marine zooplankton play a key role as secondary or tertiary producer in marine ecosystems, linking primary producer to high trophic level including commercially important fish. Because ecological impacts of viruses infecting zooplankton have been overlooked, investigating their diversity and ecological roles are important for further understandings of marine ecosystem. During I stayed in Suttle's lab in University of British Columbia, the following research topics were carried out for accumulating a basic knowledge of previously unrecognized diversity and ecological roles of viruses associated with marine zooplankton.

1. RNA virosphere associated with zooplankton community using the FLDS method

As a first step to understand viruses infecting marine zooplankton, viral communities should be revealed comprehensively in marine zooplankton. RNA viruses are known to show high diversity in eukaryotes, and metatranscriptomics methods using high-throughput sequencer have been commonly used for detecting RNA viruses in these days. In particular, the fragmented and loop primer ligated dsRNA sequencing (FLDS) is a promising method to reveal RNA virosphere across various ranges of host species (Urayama et al. 2016). This FLDS method can efficiently purify long double-stranded RNA (dsRNA) including dsRNA viruses and replicating single-stranded RNA (ssRNA) viruses, and diverse RNA virosphere including new genetic lineages have been revealed from marine microbial communities (Urayama et al. 2018). Thus, I used the FLDS method for revealing RNA viroshere in marine zooplankton communities.

Zooplankton community sample was collected at 0–200 m depth using a NORPAC net with a 100-μm mesh in the western North Pacific (25º 59.5´N, 126º 26.8 ´E). Zooplankton community with approximately 2 g in wet weight was pulverized in liquid nitrogen. A part of pulverized sample was used for analysis of zooplankton community, and others were used for the FLDS analysis. In zooplankton community analysis, small subunit ribosomal RNA was assembled from total RNA sequences to reveal possible hosts. In the FLDS method to reveal RNA virosphere was followed by Urayama et al. (2016; 2018). Massive sequence data were obtained by Illumina MiSeq.

After quality-filtering and assemble, 53,946 sequence reads remained for Operational Taxonomic Units (OTUs) based on SSU in the analysis of zooplankton. Total 82/149 Operational Taxonomic Units (OTUs) and > 90% sequence reads were derived from metazoan zooplankton, in particular copepods with approximately 70% sequence reads. The metazoan zooplankton taxa were thus considered as possible hosts of viruses. In the FLDS analysis, 163,818 sequence reads after quality filtering were assembled to into total 2,857 contigs. In taxonomic assignment, 237 contings including 49,206 reads (approximately 30% of assembled reads) were classified into viruses by BLASTX analysis. The viral contings covered 5 and 8 taxonomic groups in ssRNA and dsRNA viruses, respectively. In ssRNA viruses, Narnaviridae showed the heist diversity (Figure 1). In dsRNA viruses, Partitiviridae showed the highest diversity of contigs, and other major dsRNA viruses included Picornaviridae, Reoviridae, and Totiviridae. In these major taxonomic groups of viruses, the phylogenetic trees showed viruses detected in this study were divergent from the previously known viruses in each phylogenetic group (Figure 2). RNA viruses detected in this study were also phylogenetically diverse in

each taxonomic group. These results showed zooplankton community work as a reservoir of diverse RNA viruses with high genetic diversity. Viral community detected in this study is unique, suggesting previously unknown virus-host relationships in metazoan zooplankton community. The presence of diverse RNA viroshere was successfully shown using the FLDS method in zooplankton; however, further studies are necessary to reveal diversity, evolution, virus-host relationships and ecological roles of RNA viruses associated with zooplankton community.

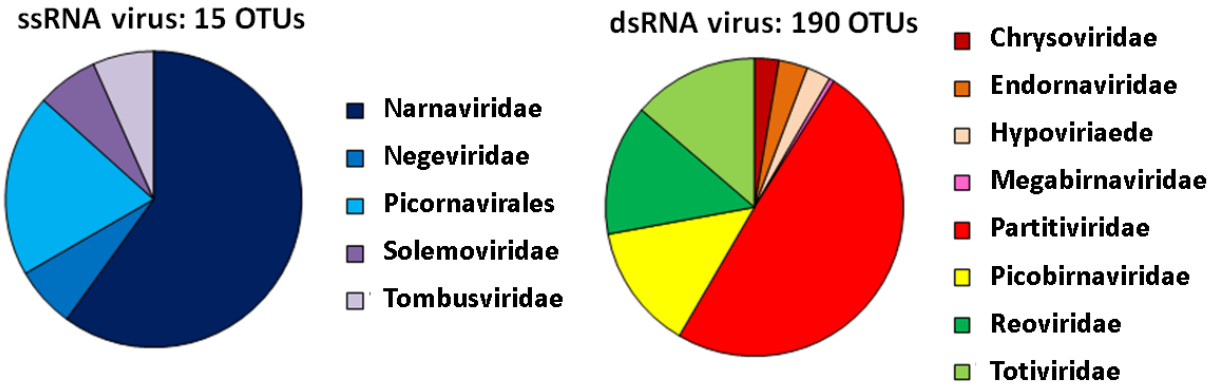

Figure 1. Proportions of taxonomic groups of contings in ssRNA and dsRNA viruses.

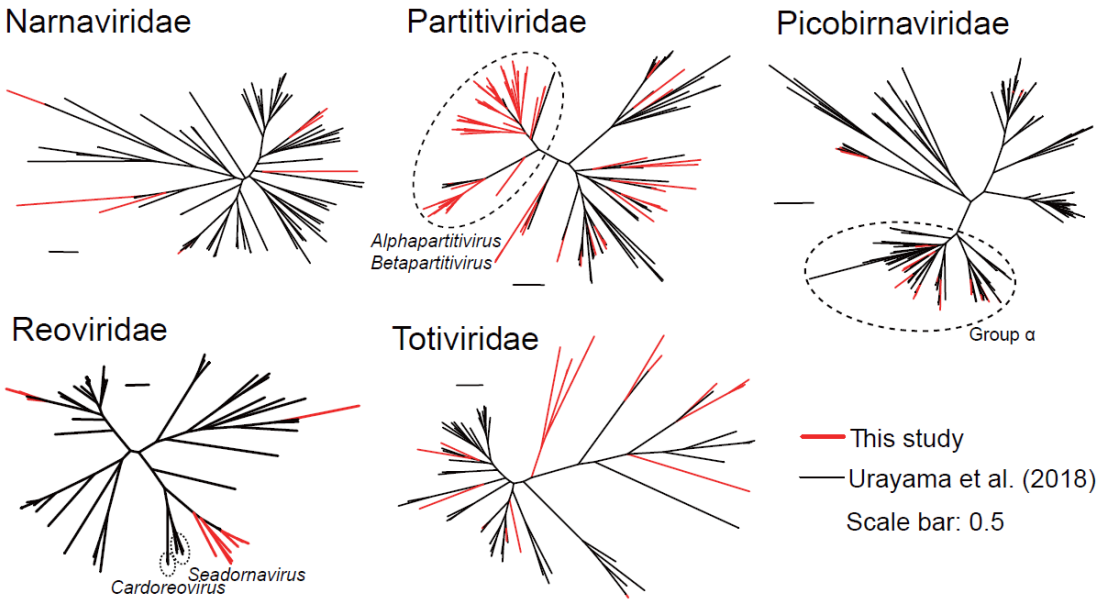

Figure 2. Phylogenetic analyses in major taxonomic groups of RNA viruses. RNA viruses detected in this study, represented by red color, are added to a phylogenetic tree in each taxonomic group in Urayama et al. (2018).

2. Transcriptomics analysis of zooplankton to detect viruses infecting zooplankton

The FLDS method successfully revealed diversity of RNA viruses associated with zooplankton community; however, detailed virus-host relationships are still poorly understood. Although transcriptome analysis using high-throughput sequencer is cost intensive, there are transcriptome data available for zooplankton species on public database. These data mainly focused on gene expression of zooplankton species; however, transcriptomic data are also useful to detect viruses infecting zooplankton. In order to obtain insights on diversity and ecology of viruses infecting marine zooplankton, transcriptome data of marine zooplankton species were used for viral discovery.

In addition to transcriptome data on public database, I also collected zooplankton samples and obtained transcriptome data in the Strait of Georgia in 2018, including four species of *Calanus pacificus, Eucalanus bungii, Metridia pacifica, and Neocalanus prumchrus*. The data on public database used poly(A) tail selection for library preparation. In copepod species in the Strait of Georgia, I used rRNA depletion method for library preparation, and poly(A) tail

selection was also used for two species *E. bungii* and *N. prumchrus* for comparisons of library construction methods. Total 46 transcriptome data from 42 marine zooplankton species were analyzed. In each transcriptome data, sequences were assembled to make contigs. Viral contigs were detected based on results of BLASTX. Proportions of viruses in sequence reads were obtained by mapping sequence data to assembled contigs.

In the comparisons of library construction methods, high proportions of high-quality non-rRNA sequence reads were obtained in poly(A) selection than rRNA depletion methods (Table 1). Total numbers of viruses were larger in rRNA depletion (6 for *N. prumchrus* and16 for *E. bungii*) than poly(A) selection methods (1 for *N. prumchrus* and13 for *E. bungii*). More taxonomic groups of viruses were detected in *N. prumchrus*. Numbers of sequence reads of viruses were also higher in rRNA depletion than poly(A) selection methods in both two species. Thus, rRNA depletion method is more sensitively detect viruses in zooplankton. However, poly(A) tail selection, which are used in transcriptome data on public database, method is also valuable to obtain some insights on zooplankton viruses.

Table 1. Comparisons of library construction methods for viral discovery using transciptomics data. Two copepod species collected in the Strait of Georgia were used for high-throughput sequencing using poly (A) selection and rRNA depletions methods.

	Neocalanus prumchrus		*Eucalanus bungii*	
	poly(A) selection	rRNA depletion	poly(A) selection	rRNA depletion
Raw sequencereads	99,787,117	84,433,227	120,706,579	83,419,222
Trimmed sequence reads	56,087,883	44,118,591	61,554,400	33,641,475
Non-rRNA sequence reads	55421957	34789933	60823923	22589835
Total contig number	158572	199550	268894	322915
Virus contig number	1	6	13	16
Virus sequence reads	6	96	3233	11089
Virus taxonomic groups	1	2	3	3

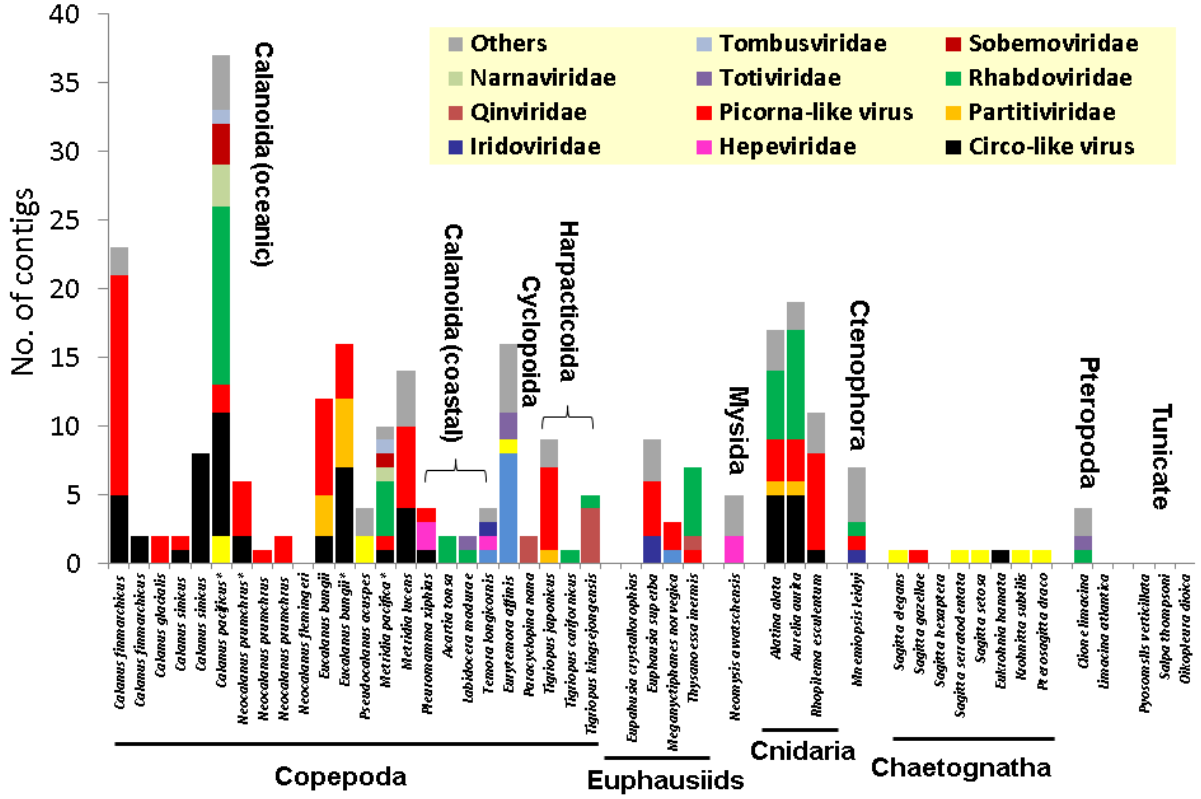

Figure 4. Numbers of OTUs and taxonomic compositions of viruses detected in transcriptomic data of zooplankton. Asterisk indicate rRNA depletion method for library preparation.

Numbers of viral contigs varied from 0 to 37, and both DNA and RNA viruses were detected (Figure 4). Although taxomicNumbers of contigs and taxonomic of viruses were associated with taxonomic groups of zooplankton. For example, no viruses were detected in Tunicate including Salps and Appendicularia. Only one virus was found in most of Chaetoganth species, which mainly belonged to ?. Relatively large numbers of viruses were detected in Cnidaria, and circo-like and picorna-like viruses were commonly observed. Most of transcriptome data were from copepods, and taxonomic compositions were somewhat associated with taxonomic groups of copeods including the order Calanoida (mainly oceanic groups) and Calanoida (mainly coastal groups), Cyclopoida, and Harpacrticoida. In calanoid copepods with oceanic distribution, circo-like and picorna-like viruses were commonly observed. The phylogenetic analysis of circo-like viruses show high genetic diversity, and genetic groups are clustered into each taxonomic group of zooplankton of the genes *Calanus*, other genus of calanoid copepods, Hydrozoa, and Chaetognth. In some species, transcriptome data were available in each developmental stage from egg to adult. I found circo-like virus in each developmental stage, suggesting vertical transmission of viruses. These results indicate coevolution between viruses and zooplankton, though they share same pelagic realms in the ocean.

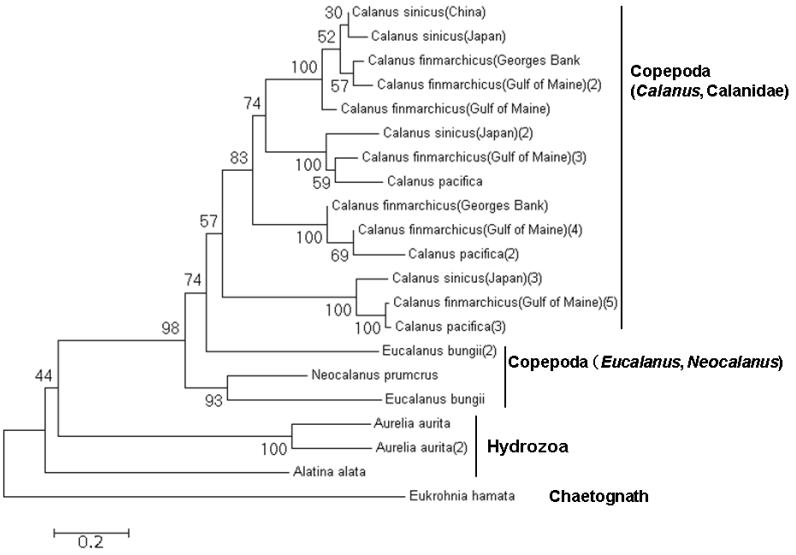

Figure 5. Phylogenetic tree of circo-like virus detected from transcriptome data of zooplankton. Bootstrap value is indicated on each node. Multiple contigs are detected in some transcriptome data, which are indicated by numbers in parentheses.

3. Large-scale population structures and viral infections of pelagic zooplankton

There are no obvious physical barriers in the pelagic ocean, and marine zooplankon have been considered not to have population structures. However, recent molecular analysis revealed basin scale population structure, suggesting adaptation of each population to local environments Although unique viruses were detected from each zooplankton species, it is not clear if these viruses are shared among populations or there are unique viruses in each populations within species. Here I focused on widely distributed copepod *Cosmocalanus darwinii* and collected large-scale samples to reveal biogeography of viruses accociated with zooplankton.

In addition to KH-16-7 and KH-17-4 cruises in the North Pacific Ocean, I joined KH-18-6 and KH-19-6 cruises aboard on RV Hakuho-maru in the Indian Ocean in 2018 and in the South Pacific Ocean in 2019, respectively (Figure 6). Zooplankton samples were collected basically at 0-100 depth. Adult female *C. darwinii* were identified on research ship and incubated to remove gut contents. These samples were preserved in RNAlater. Population structures of *C. darwinii* will be investigated using conventional method of Sager sequencing firstly. The mitochondrial COI and nuclear ITS sequences well be obtained. I also plan to carry out recently developed methods using high-throughput sequencer to obtain genome-wide SNPs data. Planktonic copepods are small; however, MIG-seq was shown to be effective method for population analysis of copepods with small DNA amount (Hirai et al. 2020).

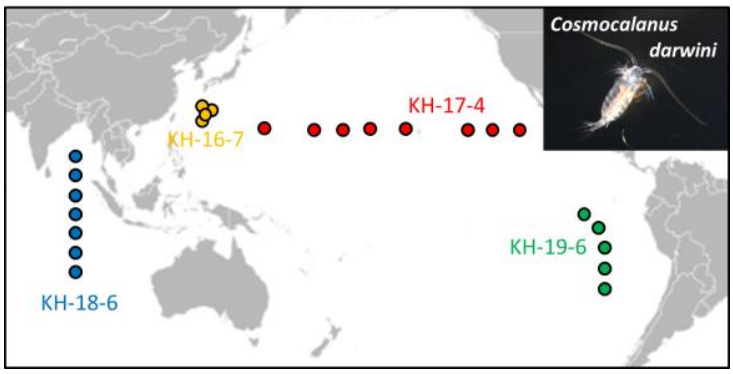

Figure 6. Sampling stations of oceanic coepod *Cosmocalanus darwini* during four research cruieses in the Pacific and Indian Oceans.

Academic paper

Hirai J, Hamamoto Y, Honda D, Hidaka K (2018) Possible aplanochytrid (Labyrinthulea) prey detected using 18S metagenetic diet analysis in the key copepod species *Calanus sinicus* in the coastal waters of the subtropical western North Pacific, *Plankton and Benthos Research*, 13, 75-82.

Hirai J (2020) Insights into reproductive isolation within the pelagic copepod *Pleuromamma abdominalis* with high genetic diversity using genome-wide SNP data, *Marine Biology*, 167, 1.

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Hirai J, Hamamoto Y, Honda D, Hidaka K, Nagai S, Ichikawa T, Metabarcoding diet analysis for revealing predator-prey relationships during the spawning period of Japanese sardine and Pacific round herring in Tosa Bay, PICES Annual Meeting, Victoria, Canada, October (2019).

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