Thesis for the Degree of Master of Science

Metagenomic analysis of bacterial and viral diversity in the intestines of abalone (*Haliotis discus hannai)* and spoon worm *(Urechis unicinctus)*

by

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Metagenomic analysis of bacterial and viral diversity in the intestines of abalone (*Haliotis discus hannai*) and spoon worm (*Urechis unicinctus*)

(환경유전체학을 이용한 전복 (*Haliotis discus hannai*)과 개불 (*Urechis unicinctus*) 장 내 세균 및 바이러스의 다양성 분석)

Advisor : Prof. Kyoung-Ho Kim

by

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ABSTRACT

Abalone is one of typical invertebrate farmed in South Korea and several microorganisms producing novel cellulases and valuable compounds were found in abalone. In case of spoon worms, they inhabit almost all around the world, but they still have difficulty to be cultivated artificially. Moreover, almost studies researching abalones and spoon worms have been focusing on detecting and screening of novel microorganisms and compounds, whereas, few studies have researched microbial diversity in abalones and spoon worms so far.

Therefore, in this study, bacterial and viral diversity in abalones and spoon worms were revealed using the recently revised metagenomic tools.

Results showed that *Mycoplasma* species took over 70 % in the whole bacterial population in abalone intestine, whereas *Vibrio* species occupied about 10 %. In spoon worm samples, Lactic acid bacteria such as *Lactococcus* and *Leuconostoc* species were dominant occupying 63.6 % in the whole bacterial population.

Viral diversity analysis revealed that 5.35 % sequences were matched with known viral sequences in abalone samples. Among known viral sequences, 53 % of known sequences were ds DNA viruses and 45 % were ss DNA viruses and 2 % were unclassified or RNA viruses. 0.1 % of the sequences were known viral sequences in spoon worm samples and 62 %

of known viral sequences were ds DNA virus and 24 % were mostly related to ss DNA viruses and 14 % were unclassified phages. Viral compositions of both abalone and spoon worm samples were mainly occupied by ds DNA viruses such as *Myoviridae*, *Sipoviridae* and *Podoviridae* belonging to *Caudovirales* and ss DNA viruses such as *Chlamydia* phages related to *Microviridae*. Retroviruses and ss RNA viruses were also detected and they occupied less than 0.5 % only in abalone.

Overall, the results from this study, are expected to be applied to the probiotic applications for abalone and spoon worm aquaculture as fundamental informations.

INTRODUCTION

Microbial interactions with various environments occur everywhere on Earth. Also many microorganisms interact with other living organisms and give their hosts advantages or damages as symbionts or pathogens (Bonin *et al.*, 2011). However, many organisms living with microorganisms generally benefit from those microbiological interactions. Also numerous microbial byproducts are generated directly or indirectly through the microbial interactions and the byproducts have influenced on environments or host organisms.

Therefore, many studies have researched about microbial community and interactions with environments. Through the studies about microbial communities, numerous novel microorganisms and their products have been found and used in many fields such as food industry (Caplice *et al.*, 1999), pharmaceutical industry (Thakur *et al.*, 2005), agricultural field (Berg, 2009), brewing industry (Rabotnova, 1954) and many others.

In addition, many current scientific approaches have been focusing on seeking valuable microorganisms and microbial products from the marine environment because the marine environment cover over 70 % of the surface of Earth and indeed a lot of novel microbial products have been isolated from the marine environment (Thakur*et al.*, 2005).

In 2009, over a thousand of novel natural compounds have already been described from the marine environment and especially half of them were known to be originated from marine invertebrates (Sanchez *et al.*, 2012). Moreover, it is estimated that there are still vast unknown marine invertebrates in the marine environment because of a limitation to explore the whole marine environment.

On the other hand, not only seeking valuable microbial products, but microbial diversity analysis in a specific environment has become significant, because it has been revealed that a microbial community takes an important role in a host organism. As an example, the whole

microbes in human (human microbiome) has been known as an additional organ which could promote human health (Petschow *et al.*, 2013). Also this probiotic concept has been studied and tried to be applied in various area like clinical purposes (Alvarez-Calatayud *et al.*, 2013), medical purposes (Heczko *et al.*, 2005) and aquaculture industry especially (Irianto *et al.*, 2002).

Accordingly, for both detecting novel microbial compounds and microbial diversity analysis, various scientific tools have been used. A traditional means, Cultivation method, was one of major way to study diversity of microorganisms in environments. However, the cloning method using 16S rRNA gene sequence revealed that less than 1 % among the whole environmental microorganisms are cultivable in a laboratory condition (Schmidt *et al.*, 1991).

Currently revised metagenomics showed unlimited possibilities to detect and screen a whole microbial genomic resource directly from a specific environment (Handelsman, 2004). This advantage can be applied to medical, industrial, agricultural and ecological fields (Lorenz *et al.*, 2005). For example, a recent study researched fish gut microflora using the metagenomic approaches (Sanchez*et al.*, 2012) introduced that the bacterial diversity analysis can provide important clues to the fish aquaculture industry. In addition to usefulness of metagenomic applications for bacterial diversity analysis, particularly, metagenomic approaches are playing an important role to discover viral diversity (Kristensen *et al.*, 2010), because viruses lack a shared universal phylogenetic marker such as 16S rRNA gene for bacteria and 18S rRNA gene for eukarya.

With the metagenomic tools, in this study, the bacterial and viral diversity analysis in abalones and spoon worms has been conducted. As one typical case of farming edible invertebrates in South Korea, abalone and symbiont microbes, have been studied to find

useful agents and enzymes like alginate lyases (Li *et al.*, 2003) and cellulases (Erasmus *et al.*, 1997, Kim *et al.*, 2011) from microbial community in abalones. Moreover, many useful compounds produced by abalones (Kuanpradit *et al.*, 2012, Nikapitiya *et al.*, 2009) and byproducts produced through the interaction of abalones and microorganisms (De Zoysa *et al.*, 2011, De Zoysa *et al.*, 2010) have been reported. However, with increase of abalone production, mortality rate caused by bacterial pathogens has been increased as well and disease control is mainly relying on antibiotics currently. In contrast with abalones, spoon worms and especially their symbiont microbes have not been much researched so far, even though spoon worms are found all around the world.

Thus, in this study, metagenomic tools were applied to reveal the bacterial and viral diversity in abalones and spoon worms and the results may provide fundamental informations for application of probiotics on abalone and spoon worm farming in Jeollanam-do, South Korea.

MATERIALS AND METHODS

Sample preparation

Abalones (*Haliotis discus hannai*) used in this study were purchased at abalone storing places in Haenam-gun (34°25'26.56"N, 126°30'37.61"E), Jindo-gun (34°28'49.66"N, 126°15'44.13"E) and Shinan-gun (34°34'5.84"N, 126°18'3.39"E), Jeollanam-do, South Korea, right after transport from abalone farms. Spoon worms (*Urechis unicinctus*) were directly collected in a desert island (34°26'5.01"N, 126°44'0.04"E) near Haenam-gun. Samples were kept in an icebox with dry ice during transport and transferred into a deep freezer at -70 ℃ for further experiments. The size and weight of spoon worms and abalones were measured and recorded (Table 1) The samples used for bacterial and viral nucleic acid extraction were isolated from intestinal contents of spoon worms and abalones.

Table 1. Sample sizes and weights were measured for abalone and spoon worms. Abalone shells were included to measure the length and weight of abalones. Sample names were assigned as H : **H**aenam, J : **J**indo, S: **S**hinan and W : spoon **W**orm from Haenam.

Sample	Sample name	Size (cm)	weight (g)
Abalone	H1	7	56.2
	H2	7.5	54.5
	H3	7	63.2
	J1	6.5	38.3
	J2	7	52.3
	J3	6.5	43.1
	S1	8.5	73.9
	S2	8.5	85.7
	S3	8	74.3
Spoon worm	W1	11	52.5
	W2	7	33.7
	W3	9	35.2

Bacterial DNA extraction and 16S rRNA gene amplification

0.5 g of intestinal content was isolated from each specimen and followed by bacterial DNA extraction using the Power Soil DNA Extraction Kit (MOBIO) according to the manufacturer's instruction. After DNA extraction, 1 μl of each baterial DNA extract was added to the Accupower PCR premix (Bioneer) containing Taq DNA polymerase, Taq polymerase buffer and dNTP with barcoded (Table 2) forward 8F (5'-TCA GAG TTT GAT CCT GGC TCA G-3') and reverse 338R (5'-TGC TGC CTC CCG TAG GAG T-3') primers (both primers are 10 pmol in concentration). According to the instruction provided by the Accupower PCR premix manufacturer, the final volume was adjusted to 20 μl containing 1 μl of DNA extract, 1 μl of each primer and 17 μl of distilled water. 16S rRNA gene was amplified under these conditions: pre denaturation at 94 °C for 7 min, 32 cycles of denaturation at 94 °C for 30 sec, primer annealing at 60 °C for 30 sec and extension at 72 °C for 45 sec with one final extension at 72 °C for 10 min. Amplified 16S rRNA gene sequences were then analyzed by the Ion Torrent System.

5'- AAC CTA CG -3'

Table 2. Barcode sequences used in bacterial 16S rRNA gene amplification. The barcode sequences are located at 5' end of each primer.

Sample #		Barcode sequence	Forward primer 8F	Reverse primer 338R
Abalone	H1	5'- AAC CTA CG -3'	5'- TCA GAG TTT GAT CCT GGC TCA G -3'	5'- TGC TGC CTC CCG TAG GAG T -3'
	H2	5'- AAC GGC AA -3'		
	H3	5'- AAG CCG AA -3'		
	J1	5'- AAG CGG TA- 3'		
	J2	5'- AAG GCG TA- 3'		
	J3	5'- AAG GTT GG- 3'		
	S1	5'- AAT AGC GG- 3'		
	S2	5'- AAT AGG CG- 3'		
	S3	5'- ACA CAG AG- 3'		
Spoon worm	W1	5'- AAC CAA GG- 3'		
	W2	5'- AAC CAT CG- 3'		
	W3	5'- AAC CGC TA- 3'		

Analysis of bacterial 16S rRNA gene sequences

For analysis of 16S rRNA, several processes were conducted using the Quantitative Insights Into Microbial Ecology (QIIME) Pipeline (Caporaso *et al.*, 2010). First of all, all 16S rRNA gene sequences with length between 200 and 400 were selected for further analysis. Also, 20 nucleotides of a barcode primer sequence in up and down stream of 16S rRNA gene sequences were discarded after arrangement by barcode sequences. After that, sorted sequences were grouped by clustering with 16S rRNA gene similarity separately, then clustered into Operational Taxonomic Units (OTUs) based on the sequence similarity and then, representative sequences were obtained. Secondly, only bacterial sequences were filtered by discarding chloroplast, mitochondrial and chimera sequences among obtained sequences processed previously. After all, relative bacterial abundance could be seen through the heatmap (OTUs table) based on OTUs and phylogenetic tree of representative sequences of each OTUs could be generated. In this study, some OTUs occupying less than 1% were considered as redundant and discarded for further analysis.

Viral purification and concentration

Intestinal samples collected from spoon worms and abalones were used to isolate viral particles. One gram of each intestinal sample was added in a 15ml conical tube containing 10ml of SM buffer (retaining 0.1 M NaCl, 50 mM Tris-HCl [pH 8.0] and 10 mM $MgSO_4$) and mixed by a vortexer for 20 mins at room temperature. Through this process, viral particles were separated into SM buffer from the intestinal sample. After that, the tubes were centrifuged at 10,000 X g for 10 mins and the supernatant was transferred into a new tube and centrifuged once more to remove sample debris except viral particles. The supernatant containing viral particles was then filtrated to get rid of bacteria and the rest of sample debris using 0.45 μm syringe filter and 0.2 μm syringe filter (Sartorius stedim). The filtrate was subsequently concentrated using the VIVASPIN20(Sartorius stedim), a centrifugal filter which can filtrate small particles and discard liquid by centrifugation at 5,000 rpm until the final volume of the supernatant reached 1 ml. The concentrate was then treated by DNase I (0.1 μg/ml of final concentration) at 37 ℃ for 30 mins and RNase A (0.1 μg/ml of final concentration) at 60 ℃ for 30 mins to degrade DNA and RNA existing out of viral particles. After nuclease treatment, DNase I and RNase A were inactivated by heating at 80℃ for 10 mins and treated by proteinase K (10 μg/ml of final concentration) at 56 ℃ for 15 mins.

Enumeration of viral particles with epi-fluorescence microscopy

Quantitative analysis of viral particles was done by epi-fluorescence microscopy with 100 µl of viral concentrates of both abalone and spoon worm samples using 1X SYBR Gold fluorescence nucleic acid staining dye. First of all, a silicon filter (0.02 µm) was placed on the vacuum flask and 200 µl of virus concentrate was filtrated by vacuum pressure. Filtrates were then stained using 1X SYBR Gold solution in the dark. All viral nucleic acids were visualized under epi-fluorescence microscopy (wavelength 480-495 nm).

Extraction and amplification of viral nucleic acids

Viral RNA and DNA both were extracted from the concentrates containing viral particles using the QIAamp MinElute Virus Spin Kit (QIAGEN) according to the provider's instruction. The viral nucleic acids extracts were used to synthesize cDNA from viral RNA among viral nucleic acids containing both of DNA and RNA using the Power cDNA Synthesis Kit (iNtRON Biotechnology). After cDNA synthesis, Klenow DNA polymerase (Large Fragment *E.coli* DNA Polymerase I, TaKaRa) was applied twice to convert single stranded cDNA and viral DNA into double stranded DNA (Figure 1). In this step, a random octameric primer, K-8N (5'-GAC CAT CTA GCG ACC TCC CAN NNN NNN N-3') was used as a primer with 3 μl of cDNA products to generate double stranded DNA under the condition described by Klenow fragment manufacturer. Amplification of viral cDNA and DNA was then performed twice using the Accupower PCR premix and primer K (5'-GAC CAT CTA GCG ACC TCC AC-3') under the condition : 1 cycle of 95 °C for 5 mins, 5 cycles of denaturing at 95 °C for 1 min, annealing at 59 °C for 1min, extension at 72 °C for 1 min, 33 cycles of denaturing at 95 °C for 20 sec, 59 °C for 20 sec, 72 °C for 1 min + 2 sec each cycle and additional extension cycle at 72 °C for 7 mins (Victoria *et al.*, 2008). PCR products were confirmed using agarose gel electrophoresis and sequenced by the Ion Torrent System.

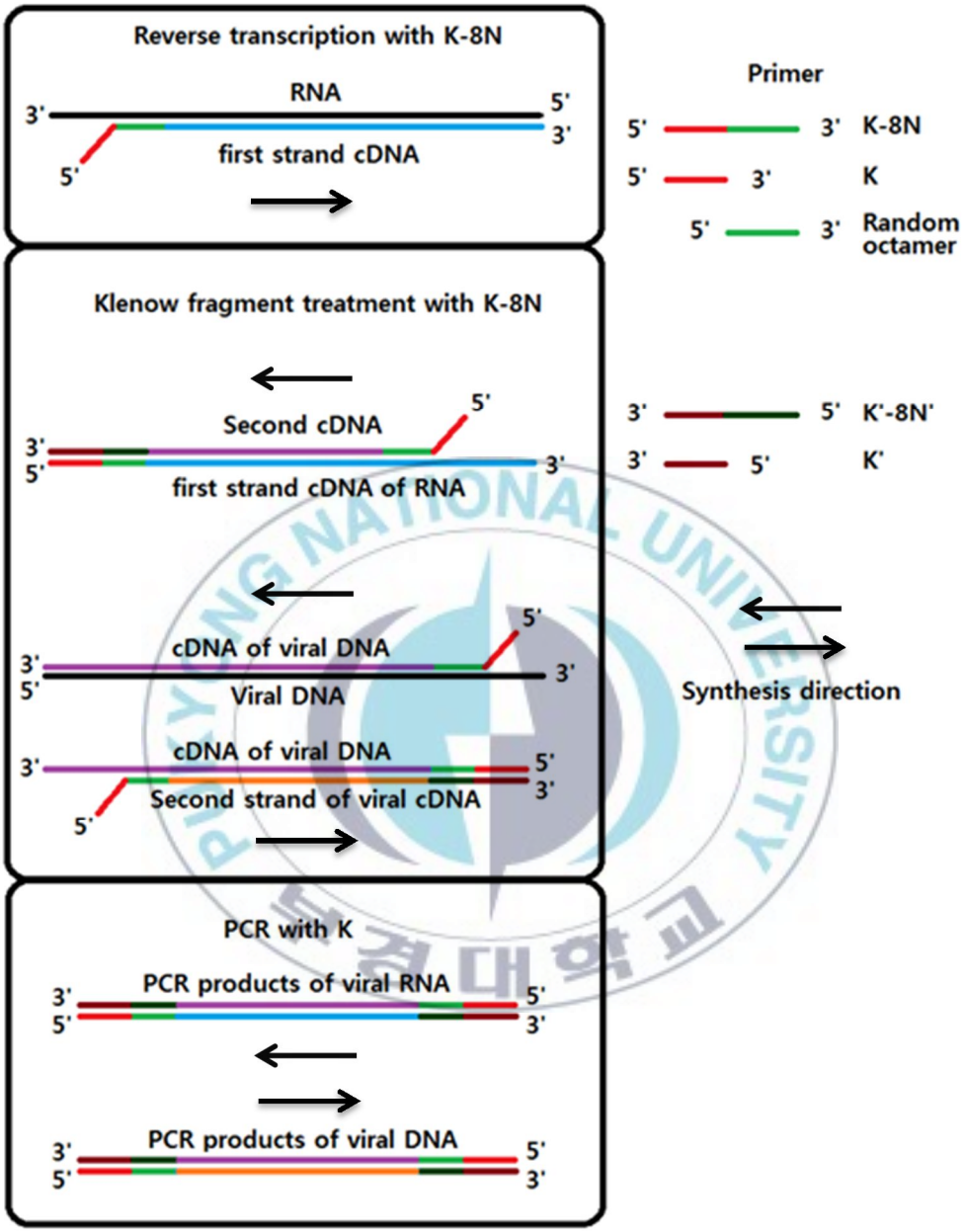

Figure 1. Amplification processes of viral RNA and DNA. PCR products are amplified randomly by primer K-8N containing eight random nucleotides.

Pyrosequencing and bioinformatic analysis of viral metagenomes

For the analysis of the viral diversity, Mothur, a microbial diversity analyzing tool was utilized. In this step, sequences with length between 100 bp and 400 bp were selected. Then, 20 bp in up and down stream of all sequences were discarded to remove primer sequence including random octameric sequences. After that, the CAMERA 2 web server (http://portal.camera.calit2.net) was applied to eliminate duplicate sequences and the processed nucleotide sequences were converted to protein sequences to search viral protein using viral protein database in the CAMERA web server (Sun *et al.*, 2011). The MetaVir web server was used to analyze the processed viral sequences (Roux *et al.*, 2011). All viral sequences were then used to construct graphs illustrating viral composition and phylogenetic relationship in both abalones and spoon worms.

RESULTS

Analysis of bacterial 16S rRNA gene sequences

After the quality check using the QIIME, a total of 508 OTU groups were generated from 26,480 sequences of both abalone and spoon worm samples (Table 3). However, some OTU groups which occupied low percentages and were considered to be redundant, so OTU groups showing less than 1 % among all OTU groups in each sample were eliminated for further analysis. Through those processes above, 52 OTU groups were obtained (Figure 2). The heatmap showed that all samples had slightly different bacterial compositions among abalone and spoon worm samples separately. Bacterial compositions in abalone samples showed many species belonging to the genus *Mycoplasma* were overwhelmingly dominant occupying averagely over 70 % of the whole bacterial composition in all abalone samples, while the family *Vibrionaceae* took approximately 10 %. However, the genus *Lactococcus* mainly consisted the bacterial population of spoon worm samples, occupying averagely over 50 % of the bacterial composition, whereas the genus *Yersinia* and the genus *Leuconostoc* took about 17 % and 14 %, respectively (Figure 3).

Table 3. Bacterial sequence reads obtained after the quality check process using the QIIME.

Sample	Sample name	reads
Abalone	H1	2754
	H2	1118
	H3	975
	J1	795
	J2	1504
	J3	860
	S1	4240
	S2	3153
	S3	5779
Spoon worm	W1	2125
	W2	1648
	W3	1529

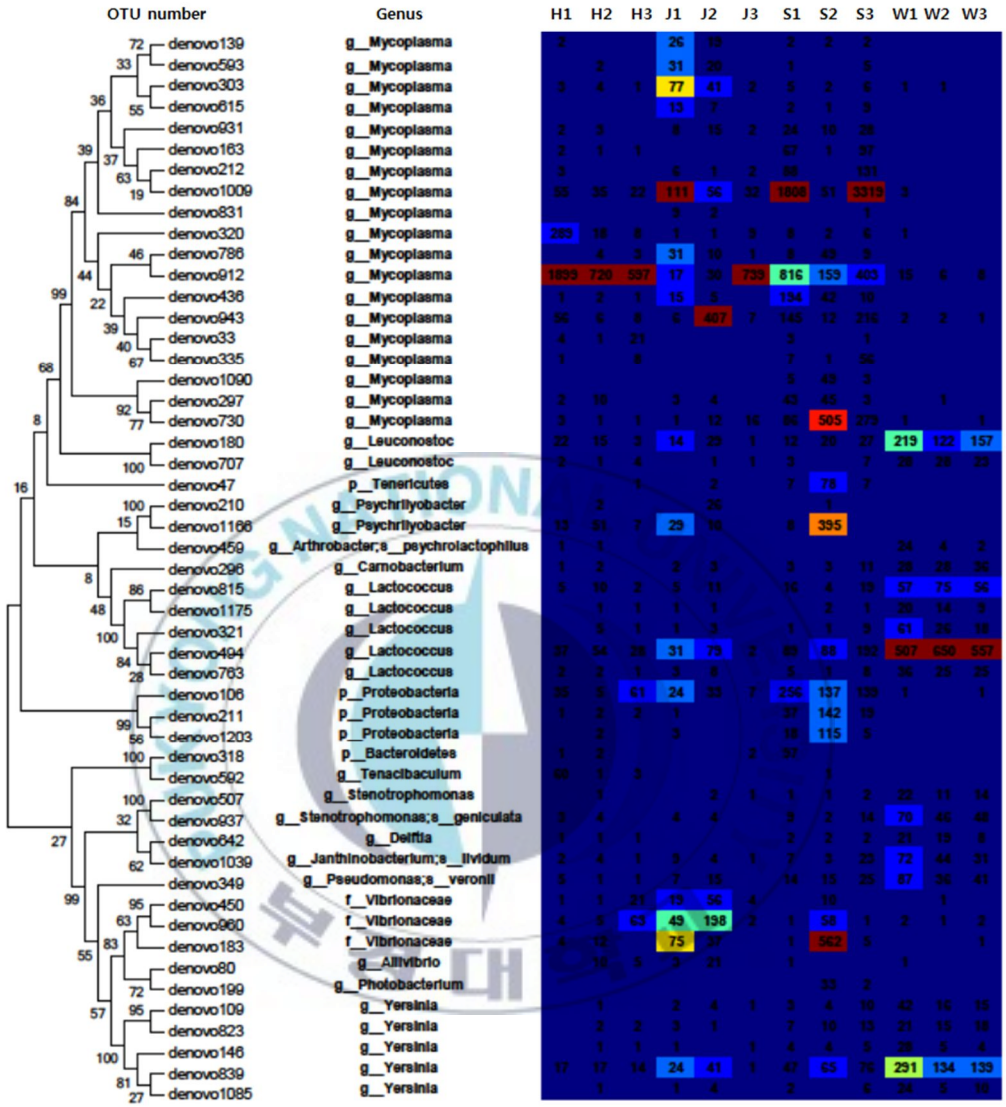

Figure 2. The heatmap shows where representative sequences of OTU groups are located in the phylogenetic tree and which genera are dominant in each sample. The phylogenetic tree was generated using the neighborhood-joining method (1000 replicates)

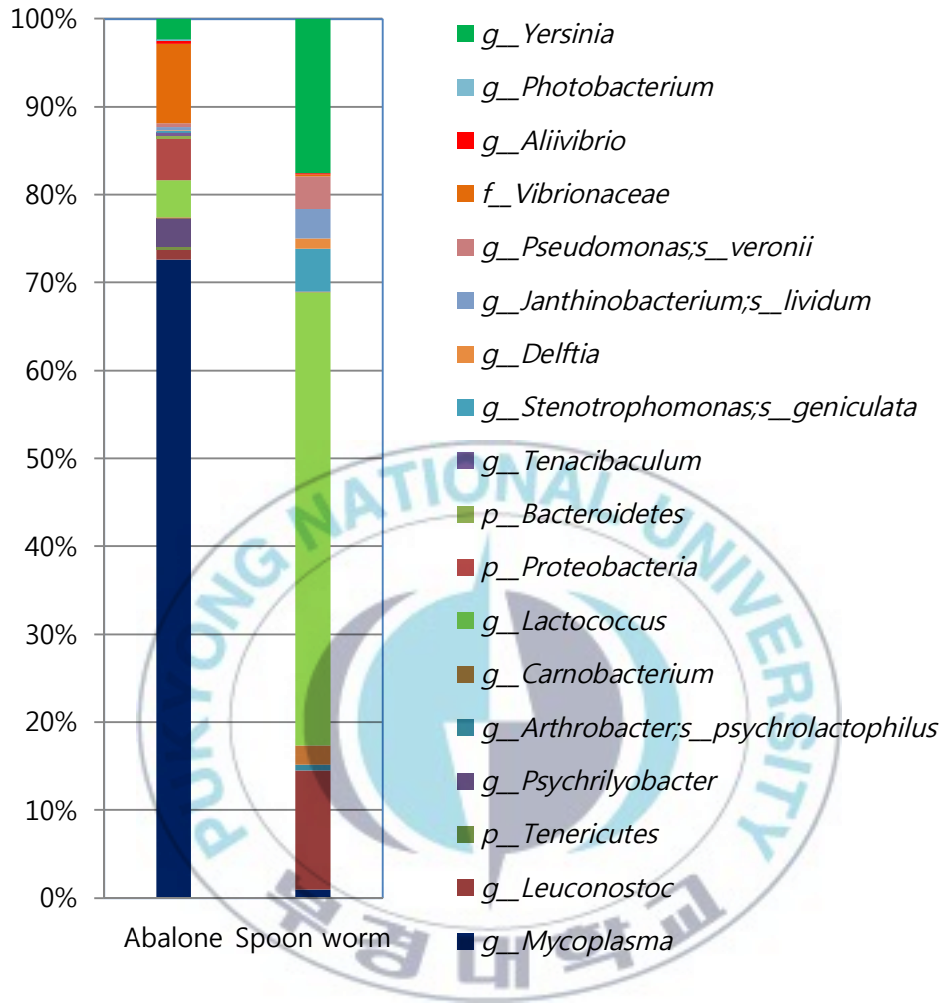

Figure 3. The percentage of each OTU in abalone and spoon worm samples.

Phylogenetic relationship of bacterial samples

The phylogenetic relationship of abalone and spoon worm samples were generated using the QIIME. Both the abundance and diversity of bacteria were illustrated in the weighted unifrac graph and only bacterial diversity was illustrated in the unweighted unifrac graph. On the whole, samples were gathered based on sample type, like abalone and spoon worm (Figure 4).

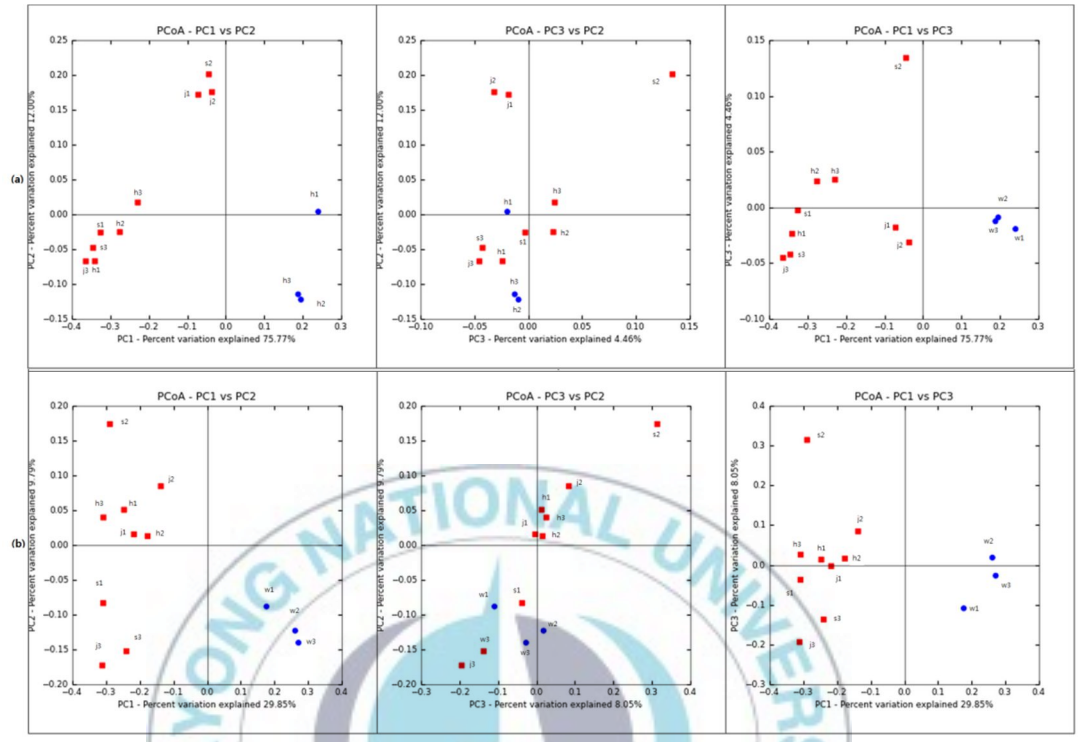

Figure 4. The weighted unifrac showing both of abundance and diversity of samples is illustrated in (a) and the unweighted unifrac showing only diversity is illustrated in (b). Abalone samples are described as red boxes and spoon worm samples are described as blue circles on the graphs.

Abundance of viruses from abalone and spoon worm

The area of a display and sample contact surface are 0.03823 mm^2 and about 78.54 mm^2. One gram of gut sample was used to isolate and concentrate viral particles, then 0.1 ml of virus concentrate was applied for this step. Viral particles were visible under the epi-fluorescence microscope. Viral particles of abalones and spoon worms were then counted in a display and their number was averagely calculated. Averagely, 6.4 x 10^5 viral particles were calculated in abalone samples and 4.9 x 10^5 in spoon worm samples (Figure 5).

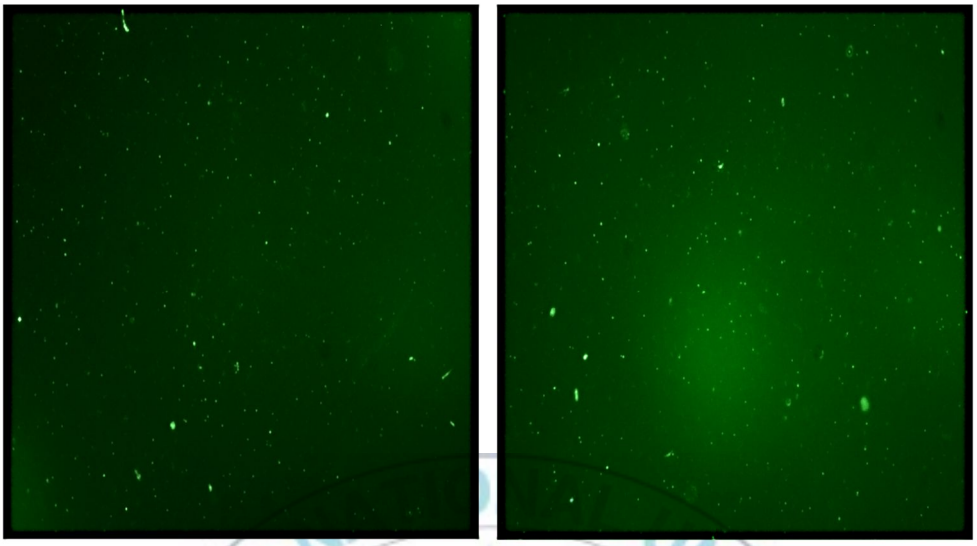

Figure 5. SYBR Gold stained viral particles concentrated from abalone (a) and spoon worm (b) samples counted under epi-fluorescence microscopy (x 1,000).

Pyrosequencing of viral metagenomes

A total of 1,476,112 and 1,551,672 sequences were generated from abalone and spoon worm samples, respectively, through quality check process using Mothur. Through the CAMERA de-duplication process, identical sequences which were unexpectedly amplified during PCR and pyrosequencing processes were discarded. Then, 512,198 and 169,109 sequences of each sample remained. Finally, qualified sequences were then analyzed using the MetaVir web server for viral diversity and phylogenetic relationship. Also, the CAMERA web server was applied for viral protein analysis. 5.35 % and 0.1 % among the whole processed sequences of abalone and spoon worm samples were recognized as known viral sequences on the MetaVir web server. Among known viral sequences of both abalone and spoon worm samples, ds DNA viruses mainly consisted of *Myoviridae*, *Sipoviridae* and *Podoviridae* belonging to *Caudovirales*. ss DNA viruses were mostly made up of *Microviridae*. 2 % were RNA or unclassified viruses in abalone samples, but no RNA viruses were detected in spoon worm samples (Figure 6).

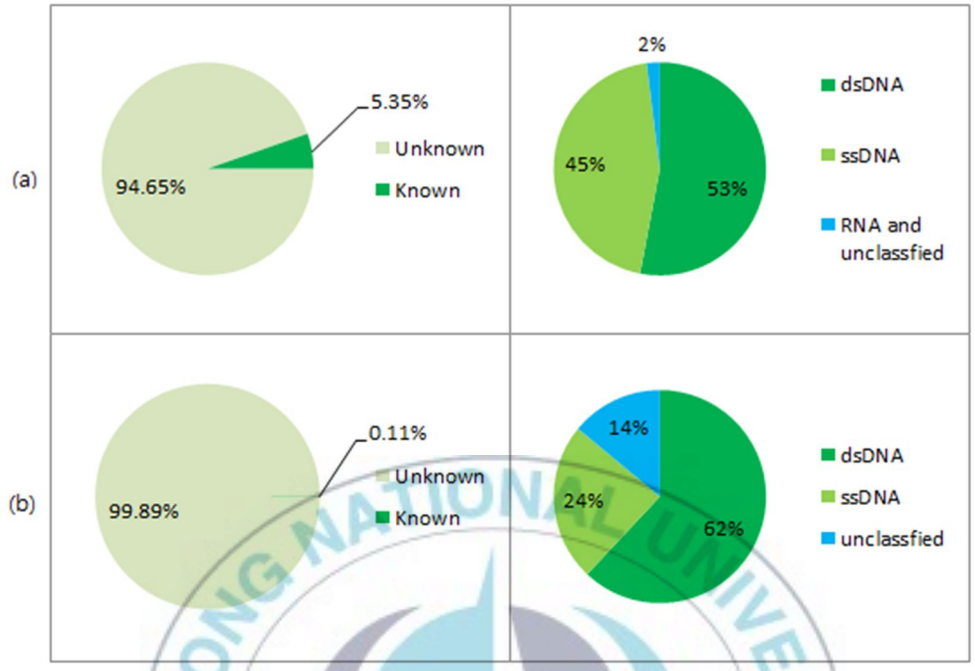

Figure 6. The ratio of known and unknown viral sequences in abalone (a) and spoon worm (b) samples. The viral composition among known viral sequences are illustrated on the right side.

Virome comparison in the environment

The phylogenetic relationship was analyzed by making up the phylogenetic tree using the virome comparison program provided on the MetaVir web server. The viromes of abalones and spoon worms were compared with other viromes previously analyzed from different environments such as deep sea sediment samples, gut samples of humans, freshwater and seawater samples. Though relationships with other viromes were not so highly related to the viromes of abalones and spoon worms, in the phylogenetic tree, abalone and spoon worm viromes were more close to deep sea sediment and freshwater viromes than to seawater viromes (Figure 7).

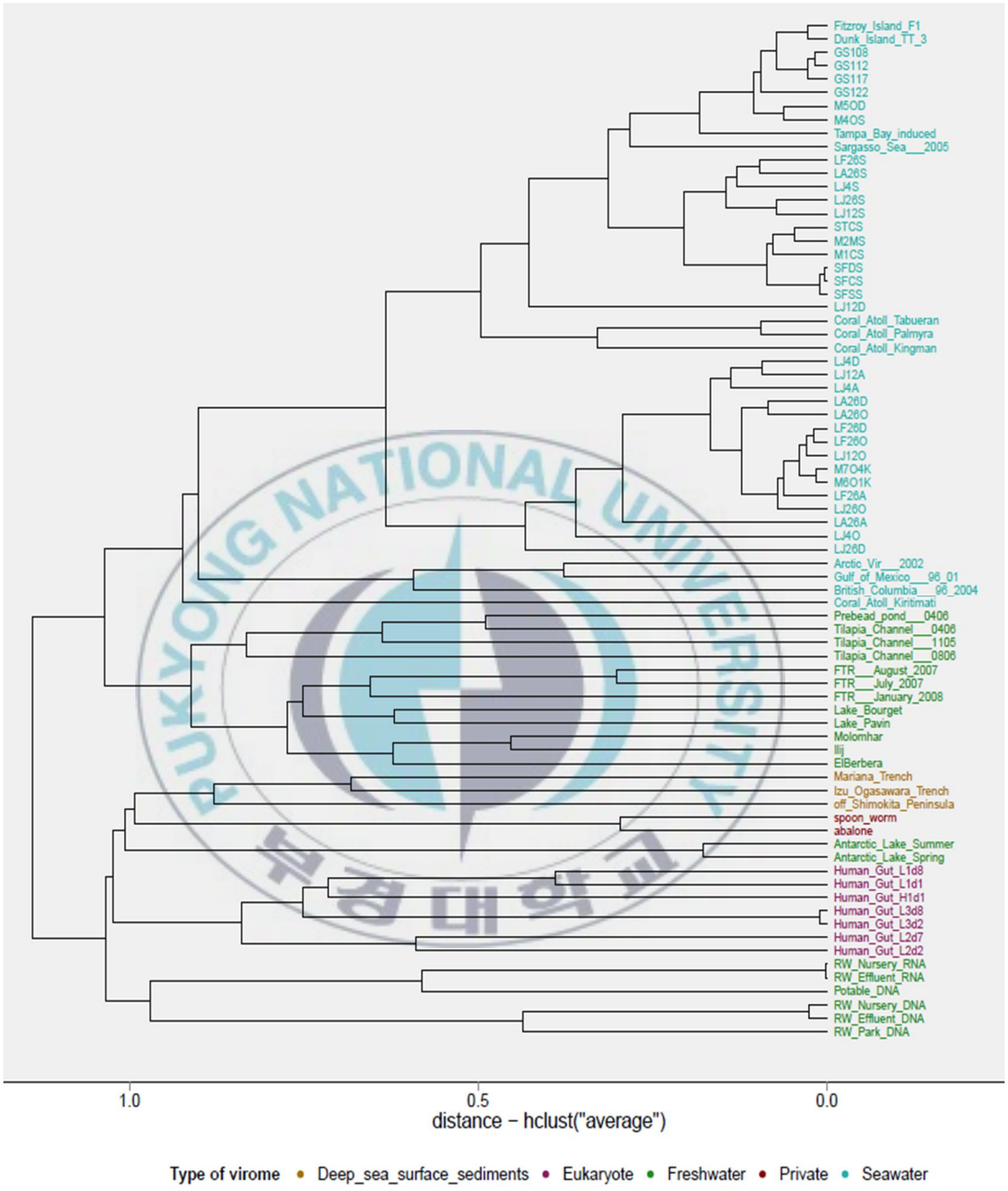

Figure 7. The phylogenetic relationship between abalone and spoon worm viromes, and other environmental viromes.

Virus composition

The whole viral composition, ds DNA and ss DNA viral composition of abalones and spoon worms, were compared with other viral compositions of deep sea sediments, human gut contents, lake water in the Antarctic and seawater (Figure 8, 9, 10).

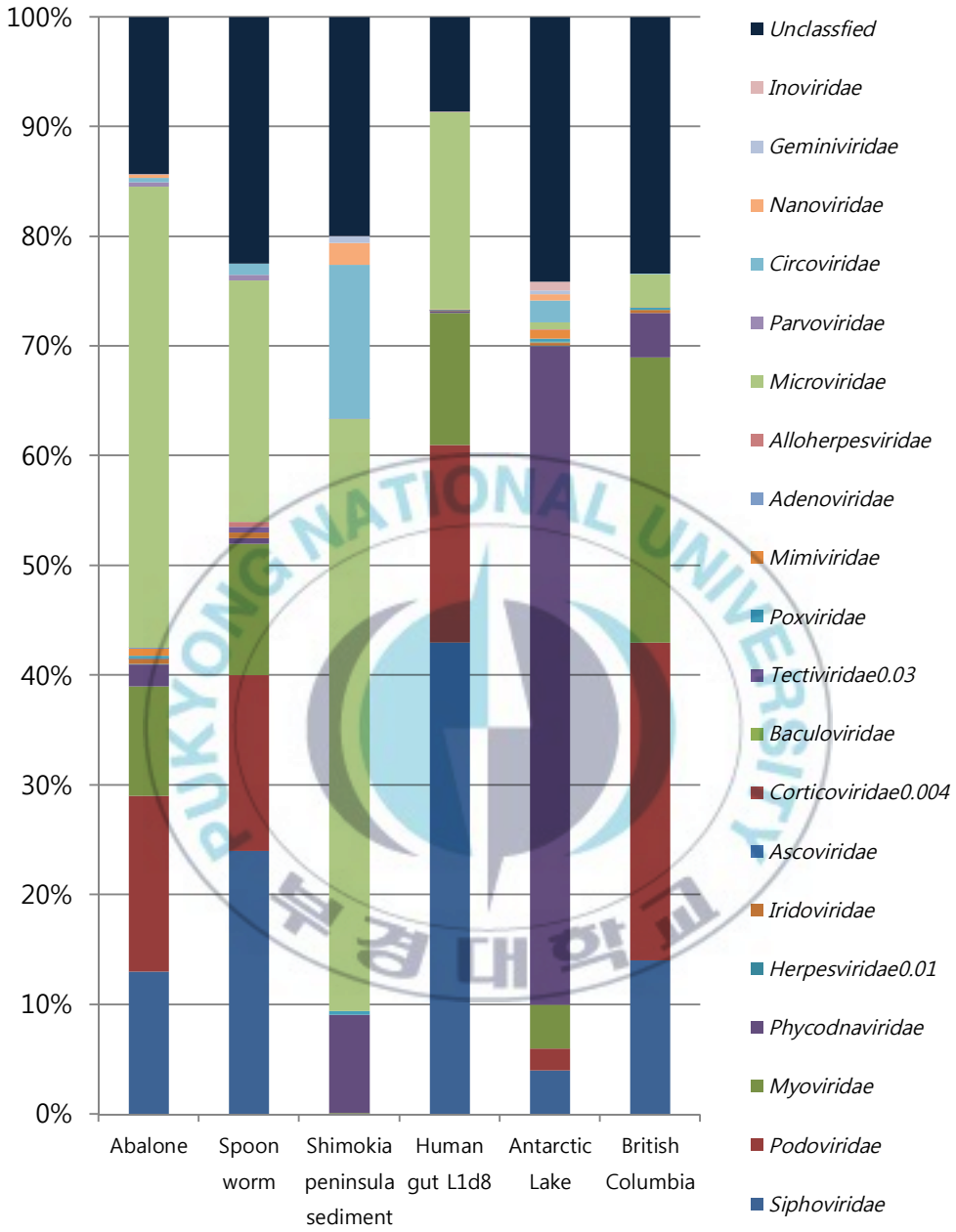

Figure 8. The total viral composition including ds DNA viruses and ss viruses of abalones, spoon worms, deep sea sediments, human gut contents, lake water in the Antarctic and seawater.

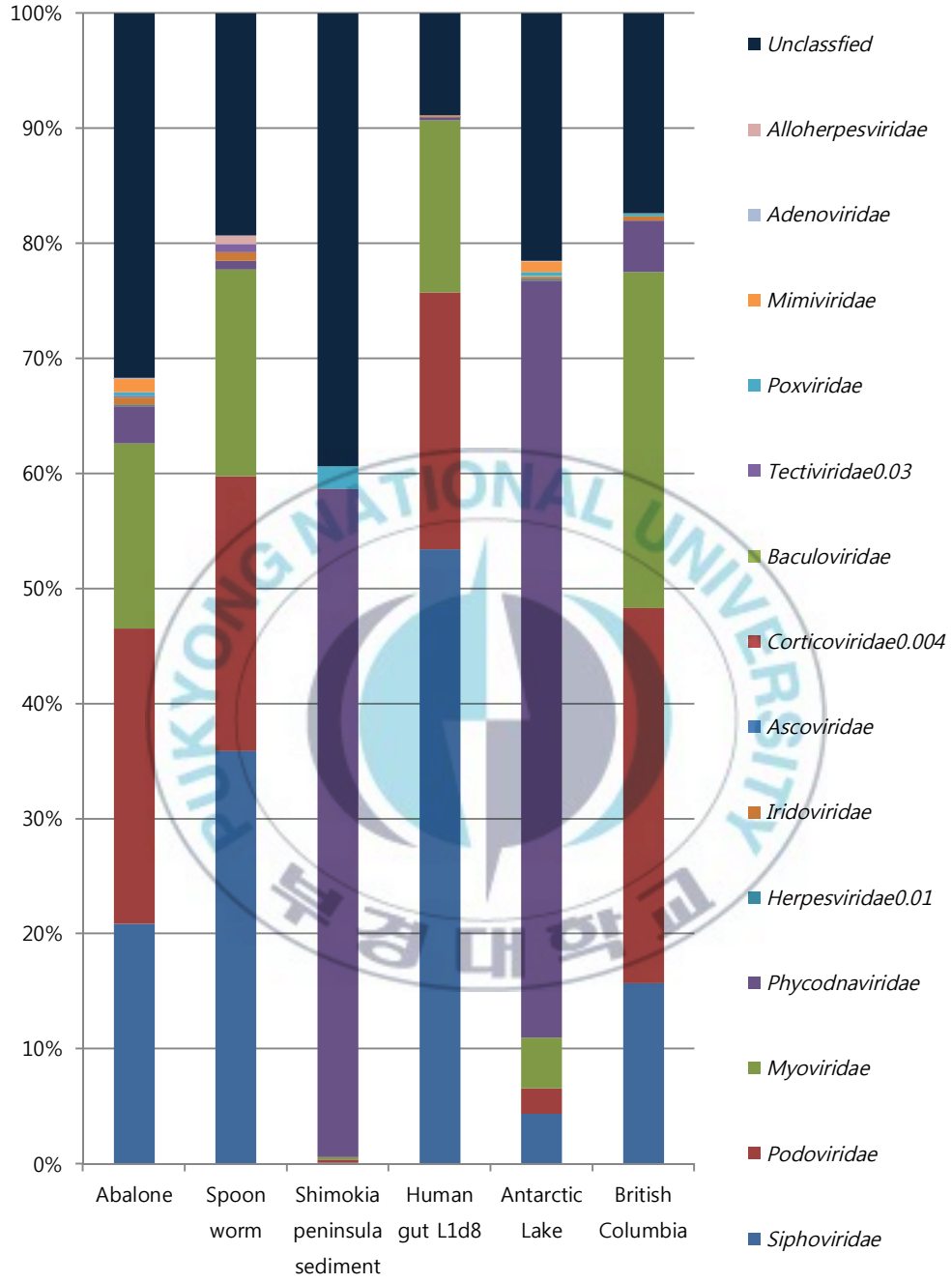

Figure 9. The viral composition including ds DNA viruses of abalones, spoon worms, deep sea sediments, human gut contents, lake water in the Antarctic and seawater.

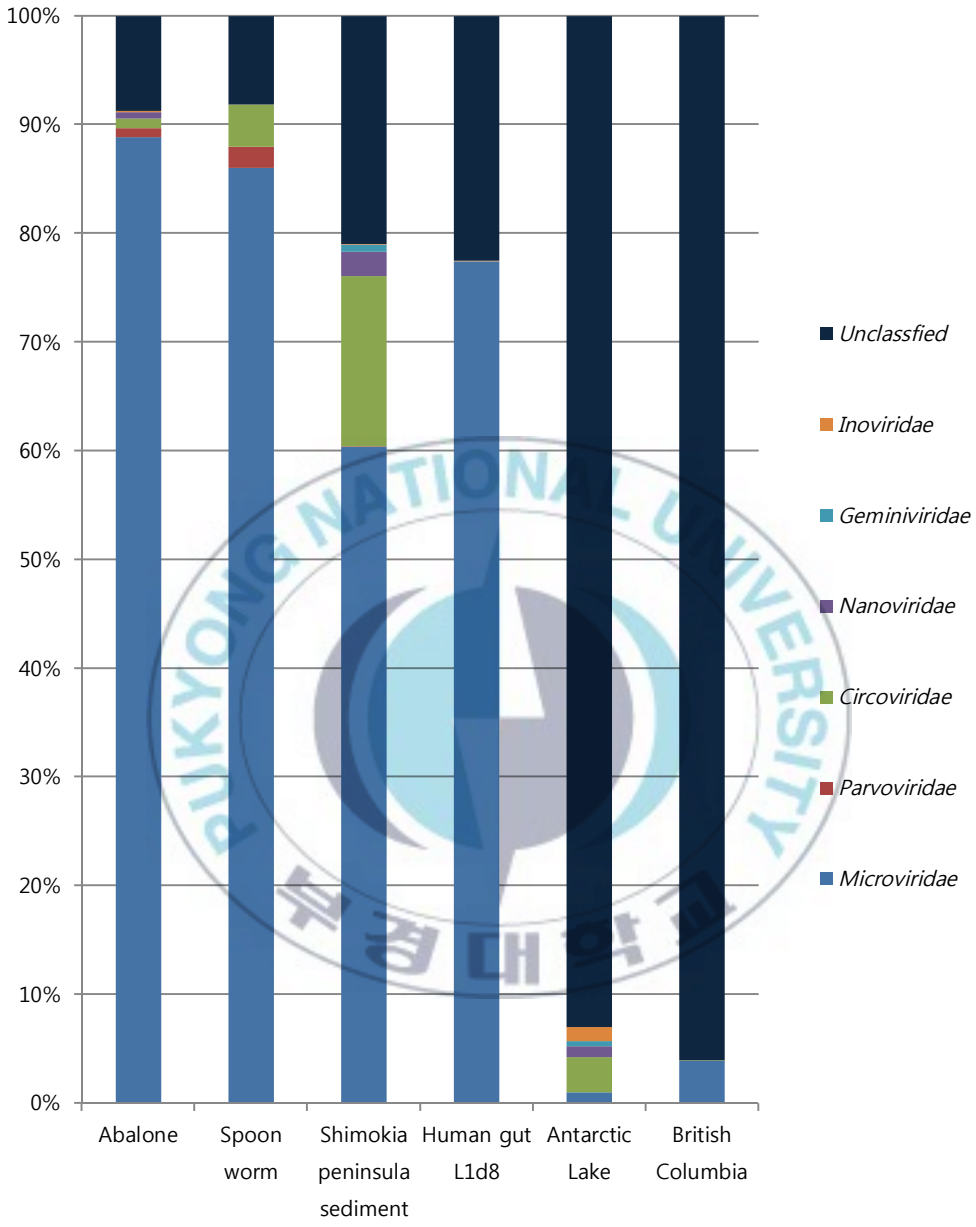

Figure 10. The viral composition including ss DNA viruses of abalones, spoon worms, deep sea sediments, human gut contents, lake water in the Antarctic and seawater.

DISCUSSION

Traditionally, many studies have been relying on incubation methods to detect microbial products. However, metagenomic approaches are becoming a great measure to explore microbial communities. Many metagenomic studies have shown that the microbial community is playing an essential role in a specific environment. For instance, a study showed that a normal microbial group in a fish can prevent diseases caused by *Vibrio* species, so normal microorganisms could reduce the massive abuse of antibiotics in the fish farming industry (Sanchez*et al.*, 2012). Also, the study illustrated that normal microorganisms could replace antibiotics by feeding fishes with appropriate food that could enhance the influence of normal microorganisms in fishes.

In this study, the microbial diversity in abalones and spoon worms collected in Jeollanam-Do was investigated by using metagenomic tools. Bacterial diversity in both abalones and spoon worms was revealed using barcoded primers and pyrosequencing. As a result, the genus *Mycoplasma* was the most dominant bacterial group and occupied averagely over 70 % of the whole bacterial population in all abalone samples.

Many species belonging to the genus *Mycoplasma* are hard to be cultured in laboratory conditions and they are thought as obligate commensals or parasites (Razin *et al.*, 1998) because they do not have an appropriate metabolic system. So, they usually require a host which can provide a metabolic system for them. Some *Mycoplasma* species are known as pathogens, otherwise, they are usually not harmful in most cases (Roediger *et al.*, 2002). Some studies performed recently have shown that *Mycoplasma* species may provide benefits to their hosts as a digestive helper (Bano *et al.*, 2007, Fraune *et al.*, 2008). Another study showed that the presence of *Mycoplasma* species in the digestive bacterial community of their hosts might result from algal related diet (Naidoo *et al.*, 2006). Therefore, *Mycoplasma*

species seemed to be in symbiotic relationship with their hosts, and they may provide and receive nutrients and diet assimilating ability.

The second biggest bacterial population in abalones was composed of the genus *Vibrio*. Also, in other studies, they have been found previously in abalones and some of them showed great cellulose assimilation with *Pseudomonas* species isolated from abalone stomach and intestine (Erasmus*et al.*, 1997). In addition, some studies have found novel *Vibrio* strains producing alginate lyases which are significantly different from other known lyases (Li*et al.*, 2003, WANG *et al.*, 2006). Those *Vibrio* strains were isolated from laminaria that is a staple food for abalone. Moreover, although many bacterial strains producing alginate lyases have been isolated from abalones even before those studies were performed, there must be more bacteria that could produce useful enzymes and natural compounds.

On the other hand, in spoon worms, the genus *Lactococcus* mainly constructed its bacterial population occupying averagely over 50 % of its bacterial composition, whereas the genus *Yersinia* and the genus *Leuconostoc* took about 17 % and 14 %, respectively.

Lactococcus and *Leuconostoc* species are well known lactic acid bacteria. They are found in various environments and isolated from grains, plants, fermented vegetables, mucosal surface of animals, meat products and also from fresh sponges, seaweeds, shellfishes, crabs, fishes and oysters (Chen *et al.*, 2013, Kathiresan *et al.*, 2008). Their applications varies, so they have been applied in many fields, especially in food fermentation industries (Leroy *et al.*, 2004). Moreover, many studies have shown that lactic acid bacteria as probiotics bacteria can be utilized in the aquaculture industry (Gatesoupe, 1999, Iranto*et al.*, 2002) and they are playing crucial roles in aquaculture animals (Balcázar *et al.*, 2006). Therefore, *Lactococcus* and *Leuconostoc* species seemed to have symbiotic relationship with spoon

worms as commensal microbe and they may play important roles, if the probiotic applications are applied to artificial spoon worm culture.

Viral diversity analysis showed that in both abalones and spoon worms, DNA viruses mainly consist of the viral composition and the viral compositions of both abalone and spoon worm samples were mainly occupied by ds DNA viruses such as *Myoviridae*, *Sipoviridae* and *Podoviridae* belonging to *Caudovirales* and ss DNA viruses such as *Chlamydia* phages related to *Microviridae*. Retroviruses and ss RNA viruses were also detected and they occupied less than 0.5 % only in abalone.

All major virus groups, *Myoviridae*, *Sipoviridae*, *Podoviridae* and *Microviridae*, were related to the typical bacteriophage groups which usually infect bacteria and archaea(Ackermann, 1998, Krupovic *et al.*, 2011). Also almost all virus groups were not eukaryote infecting viruses which do not infect abalones and spoon worms as their hosts. Therefore, it is considered that the viral diversity and composition in abalones and spoon worms might be influenced by bacterial or archaeal diversity and also considered as harmless viral groups which do not have a direct relationship with abalones and spoon worms.

Overall, recent studies have revealed that commensal microorganisms play various roles in their hosts and this advantages provided by symbiont microbes can be applied to the probiotic concept for aquaculture industry. However, for application of the probiotics, microbial diversity analysis is essential. Therefore the results from this study may provide the fundamental bio informations to probiotic application for abalone and spoon worm aquaculture.

국문초록

세계적으로 다양한 해양 무척추동물들이 식량 자원으로 생산, 소비되고 또한 한국에서도 많은 종류의 해양 무척추동물들이 양식되고 있다. 현재 한국에서 양식되고 있는 대표적인 해양 무척추동물로써 전복은 타 양식업에 비하여 높은 수익률을 보이고 있으며 2004년부터 매년 약 1,000톤씩 생산량이 증가하여 2011년 생산량은 7,000 톤에 이른다. 반면 개불의 경우 2006년 이후 시범양식에 실패하여 연구를 거듭한 결과 최근 시범양식에 좋은 성과를 보이고 있다. 하지만 여전히 생산량은 낮으며 2009년에는 200톤의 개불이 국내에서 생산되었지만 같은 해 3,600톤이 소비되어 나머지 약 3,400톤을 전량 중국에서 수입되었다.

본 연구에서는 세균의 DNA추출에 Power Soil DNA extraction Kit (MOBIO)를 사용 하였고 특정서열을 포함하고 있는 Barcoded primer (8F, 338R)들을 이용해 세균의 16S ribosomal DNA를 증폭하였고 이를 Pyrosequencing 과정을 통해 서열을 얻었다. 이 과정을 통해 얻어진 세균 서열 정보는 QIIME등 서열 분석 프로그램으로 처리하여 기존의 세균 DNA서열들과 비교, 분석 되었다.

바이러스의 경우 QIAamp MinElute Virus Spin Kit (QIAGEN)을 이용하여 RNA와 DNA를 추출하였으며 추출된 바이러스의 핵산은 Random primer (K-8N)와 함께 cDNA 합성, Klenow fragment를 이용한 double strand DNA합성에 이용되었고 이 후 이들은 Primer (K)를 이용하여 증폭이 이루어졌다. 증폭된 바이러스의 핵산은 Pyrosequencing을 통해 서열화 되어 Mothur, CAMERA 그리고 MetaVir과 같은 프로그램 등을 이용하여 분석되었다.

세균 다양성 분석결과 전복과 개불의 장 내에 존재하는 세균과 바이러스의 다

양성을 확인할 수 있었다. 전복에서는 *Mycoplasma*속이 전체 세균의 다양성의 70% 이상을 차지하였고 다음으로 *Vibrio*속이 약 10% 이상을 차지하였다. 개불의 경우 *Lactococcus*속이 50%, *Yersinia*속과 *Leuconostoc*속이 각각 17.5% 와 13.6%를 차지하고 있었다.

바이러스 다양성 분석결과 전복과 개불 모두의 경우에서 DNA 바이러스가 바이러스 다양성 대부분을 차지했고 ds DNA 바이러스의 경우 *Myoviridae*, *Sipoviridae*, *Podoviridae* 그리고 ss DNA 바이러스의 경우 *Chlamydia* phages 과 *Microviridae* 가 바이러스의 대부분을 차지하였다. Retro 바이러스와 ss RNA 바이러스 또한 전복샘플에서 발견 되었지만 전체 바이러스 다양성에서 극히 일부인 0.35%를 차지하였다.

최근 여러 연구들에 의해 내부 공생 미생물들의 숙주에 대한 영향력이 작지 않다는 결과들이 보고 되었으며 산업적으로 이용될 수 있는 많은 효소와 천연물질들이 무척추동물공생 미생물로부터 발견, 분리되고 있다. 따라서 이 연구는 대표적인 해양 양식 무척추동물인 전복과 양식에 어려움을 겪고 있는 개불의 내부공생 세균과 바이러스의 분포를 조사하여 전복과 개불의 양식에 적용될 수 있는 프로바이오틱스의 적용과 앞으로의 공생미생물자원의 탐색연구에 도움이 될 수 있을 것이라 생각된다.

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