Thesis for the Degree of Master of Science

Microbiome of Two Shrimp Species, *Lebbeus groenlandicus* and *Pandalopsis japonica*, Studied with Bacterial Systematics, Genomics, and Culture-independent Methods

by

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Major in Microbiology

School of Marine and Fishery Life Science

The Graduate School

Pukyong National University

August 2023

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(두 새우 종, *Lebbeus groenlandicus* 및 *Pandalopsis japonica*의 마이크로바이옴의 세균 분류학, 유전체학 및 배양 비의존적 연구)

Advisor: Prof. Kyoung-Ho Kim

by

Hye-Jin Park

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ABSTRACT

The majority of microorganisms coexist and interact within the digestive tracts of both humans and animals. Understanding the microbial community and its characteristics is crucial because microorganisms play various roles in the host body, particularly in relation to health. In this study, we investigated the microbial diversity and conducted genome analysis using culture-dependent methods in the intestines of two shrimp species, *Lebbeus groenlandicus* and *Pandalopsis japonica*. A total of 76 samples were collected from randomly selected individuals, and DNA was extracted from four different organs (stomach, hepatopancreas, anterior intestine, and posterior intestine). Barcode PCR and 16S rRNA gene amplification were performed using the Illumina Miseq platform, and the data were analyzed using QIIME2. The results revealed differences in microbial diversity between *L. groenlandicus* and *P. japonica*.

P. japonica exhibited a dominant presence of class Gammaproteobacteria, particularly the genus *Moritella*, while *L. groenlandicus* showed similar proportions of class Gammaproteobacteria and Alphaproteobacteria. Additionally, the class Mollicutes was notable in several samples. The comparison of diversity between the two species revealed that *L. groenlandicus* had higher richness and evenness compared to *P. japonica*. Additionally, the richness varied among different organs, with *L. groenlandicus* showing lower richness in the hepatopancreas but higher richness in the intestine. In contrast, *P. japonica* displayed low richness across all organs. These findings suggest that the observed microbial diversity structure may be influenced by the characteristics of their deep and cold sea habitat, potentially impacted by the *Moritella* group. In a culture-dependent study conducted on *L groenlandicus*, gram-negative, aerobic bacterial strains M13 (KCTC 92916) and M17 (KCTC 92917) were isolated from the intestine and stomach. Phylogenetic analysis based on the 16S rRNA gene sequences confirmed that both M13 and M17 strains belong to a novel species within the genus *Psychrobacter*. Strain M13 displayed an irregular rod-shaped morphology, non-motility, weak positive oxidase reaction, and positive catalase reaction. This strain exhibited optimal growth at 20°C, pH 6.5, and a NaCl concentration of 1% (w/v). Strain M17 exhibited a diplococci shape, non-motility, and positive reactions in both oxidase and catalase tests. This strain showed optimal growth at 20°C, pH 7.5, and a NaCl concentration of 1% (w/v). The major polar lipids identified in M13 were phosphatidylethanolamine, phosphatidylinositol mannoside, unidentified glycolipid, and unknown phospholipid. In M17, the primary polar lipids observed were phosphatidylglycerol, sphingoglycolipid, and an unidentified

phospholipid. The complete genome size of M13 was 3,207,175 bps, while the genome size of M17 was 3,260,452 bps. The average nucleotide identity (ANI) and average amino acid identity (AAI) values between M13 and M17 were 97.56% and 97.3%, respectively. Based on phylogenetic and genomic analyses, these strains are proposed as novel genera within the family Moraxellaceae. This study highlights the need for further research on the intestinal microorganisms of *L. groenlandicus* and emphasizes the potential for discovering novel strains.

INTRODUCTION

In recent years, starting with human microbiome research, interest in microbiome research has been rapidly increasing as it has expanded to the fields of new energy production, improvement of health care, and production of excellent agricultural, forestry and fisheries foods (Furet *et al.*, 2009). In particular, many studies have shown that most of the microbiome is present in the digestive system and plays an important role in the host's immune response (Kamada *et al.*, 2013). Therefore, many studies related to intestinal microorganisms in humans and animals are being conducted. Furthermore, it is widely acknowledged that there are distinct variations in the intestinal microbiome distribution between humans and animals, with significant differences observed across different animal species. Therefore, investigating the intestinal microbiome in animals is considered crucial, particularly in understanding disease response in animals (Furet *et al.*, 2009)

Shrimps are considered one of the most important marine invertebrates in the food and aquaculture industries. Although the microbiome of shrimp has been extensively studied as a microbial community in marine invertebrates, it remains relatively limited compared to mammals and terrestrial invertebrates. The Pacific white leg shrimp, *Litopenaeus vannamei*, a highly important species in fisheries, has been published several hundred papers focusing on the diversities and roles of its microbiome (Holt *et al.*, 2021). *Lebbeus groenlandicus* and *Pandalopsis japonica*, commonly called Dokdo shrimp, are one of the commercially important and expensive shrimp species used in

various foods in Korea (Kim *et al.*, 2010). However, there are few studies on intestinal microbiome as well as studies on the shrimp. Therefore, this study aims to provide valuable information on the symbiotic relationship between the host and the microbiome by analyzing the microbial diversity of *L. groenlandicus* and *P. japonica*.

A Next Generation Sequencing (NGS) method was used to analyze microbial diversity in the sample. In the case of a general natural environment, it is known that only a small number of microorganisms can be cultured under general laboratory culture conditions (Pace, 1997), which is the same for microorganisms living in the body, and it is estimated that about 20%–60% of microbiome cannot be cultured (Peterson *et al.*, 2009). Therefore, various molecular biological experimental methods have been developed to overcome the limitations of the culture method and decipher microbial communities present in the natural environment at the gene level, among which we used the amplicon sequencing method (Choi *et al.*, 2016), Illumina Miseq platform.

Various bioinformatics analysis platforms have been developed, including QIIME (Caporaso *et al.*, 2010), MOTHUR (Schloss *et al.*, 2009), RDP (Cole *et al.*, 2014) and PlutoF (Abarenkov *et al.*, 2010). These platforms perform separation, identification, and diversity calculations according to barcodes by filtering only sequences suitable for analysis from the raw data (Choi *et al.*, 2016). Among them, we performed alpha, beta, and UniFrac PCoA analyses using QIIME2's pipeline.

After conducting a diversity analysis, since the deep and cold sea is a special environment, it was confirmed using a culture-dependent method, expecting that there would be a special strain in the stomach or intestines of shrimp living there. Various

bacteria were separated using several media, and many colonies were obtained, and colonies were identified and low-similar strains were separated into novel bacteria. As a result, novel candidate bacteria designated as M13 and M17 were found in the intestines and stomachs of *L. groenlandicus*. Through genetic, physiological, phylogenetic, and biochemical comparative analysis, the analysis of these strains will provide insights into the potential roles played by the novel bacteria found in shrimp.

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Chapter 1. Bacterial diversity of *Lebbeus groenlandicus* and *Pandalopsis japonica* by 16S rRNA gene amplicon sequencing

1.1 Abstract

Most of the microorganisms coexist and interact within the digestive systems of human and animals. The microorganisms play various roles in the host body, particularly in relation to health, so, it is crucial to understand the microbial community and its characteristics. Shrimp is one of the important marine products consumed worldwide, but not many studies have been reported on it. Therefore, we investigated the bacterial diversity of the intestinal microbial community of *Lebbeus groenlandicus* and *Pandalopsis japonica*, which are commonly called Dokdo shrimp. We randomly selected nine *L. groenlandicus* and ten *P. japonica* and collected four organs of the stomach, hepatopancreas, anterior intestine and posterior intestine for DNA extraction. A total of 76 samples were performed barcode PCR to proceed with 16S rRNA gene amplicon sequencing. The sequences were obtained from each sample via the Illumina Miseq platform. The raw data were analyzed using QIIME2 (Quantitative Insights Into Microbial Ecology 2). Results showed that the microbial diversity difference between *L. groenlandicus* and *P. japonica*. In case of *P. japonica*, the major class was Gammaproteobacteria, which dominated more than 80%. By contrast, *L. groenlandicus* had similar proportions of Gammaproteobacteria and Alphaproteobacteria. The class Mollicutes also accounted for a significant portion of several samples. At the genus

level, the *P. japonica* showed a large portion of the genus *Moritella* of class Gammaproteobacteria, and family Moritellaceae, but *L. groenlandicus* has more various genera such as *Psychromonas*, *Sphingomonas*, *Photobacterium* et al. Comparing the diversity of *L. groenlandicus* and *P. japonica* by species, richness and evenness of *L. groenlandicus* are much higher than *P. japonica*. In the results by organ, the richness of *L. groenlandicus* was low in the hepatopancreas, but higher in the intestine, and the richness of *P. japonica* was low in all organs. It is assumed that the *Moritella* group influenced it because of the characteristics of living in deep and cold seas.

1.2 Introduction

Microbiome is a compound word of microbiota and genome, and can be called "microbiota genomes" and refers to all microbial communities and genomes that coexist in humans, animals, plants, soil, oceans, lakes, rock walls, and atmosphere (Dao *et al.*, 2016). Recently, it has been spotlighted as the biggest issue in the biological world, and microbiome research, which began in earnest in the late 2000s, mainly focused on the intestinal microbiome, and the intestinal microbiome, which was considered to help digestion, Scabies has been newly recognized as a major factor in determining the host's health status (Kamada *et al.*, 2013). Since then, many studies have revealed that the microbiome plays a role in nutrient absorption, drug metabolism regulation, immune system regulation, brain/behavioral development regulation, and prevention of infectious diseases in the body. In the beginning, human microbiome research was mainly conducted (Chu and Mazmanian, 2013), but as high-speed large-capacity analysis was possible with the development of next generation sequencing (NGS) and metagenomics technologies (Caporaso *et al.*, 2010), it gradually expanded to large-scale research on microbiome in animals and environments. Since 2010, the amount of data has exploded and the scale of research has expanded (Caporaso *et al.*, 2010). However, in comparison with mammals and terrestrial invertebrates, relatively very little is known about the bacteria living in the gut of aquatic invertebrates such as shrimp (Holt *et al.*, 2021).

Shrimp consumption in Korea is about 62 tons per year, and it is one of the most consumed seafood by Koreans. *Litopenaeus vannamei* accounts for most of the shrimp consumption, but *Lebbeus groenlandicus* and *Pandalopsis japonica*, commonly called

Dokdo shrimp, are also commercially important shrimp species in Korea because they are widely consumed as sashimi and sushi for their sweet taste and chewy texture (Kim *et al.*, 2010).

L. groenlandicus is a species in the family Hippolytidae, order Decapoda, and phylum Arthropoda. It has a relatively big size among family Hippolytidae, especially the largest among the shrimps caught in Korea in the family Hippolytidae. Its body is short and thick (maximum 34mm) (Bae and Oh, 2014), covered with rough fur, bright and colorful, with irregular white and orange-pink stripes. This species is mainly caught with traps in the sea near Ulleungdo, East Sea, Korea (Kim *et al.*, 2013b), and lives mainly at depths of 200-400 m (Bae and Oh, 2014). As a species that lives in cold seas, it is distributed in subarctic sea areas. It is widely distributed all over the world, including the East Sea of Korea, Greenland, the Northeast of the United States, the coastal waters of the Arctic Ocean of Canada, the Sea of Okhotsk, and Hokkaido, Japan (Kim *et al.*, 2013b).

P. japonica is a species of family Pandalidae, order Decapoda, and phylum Arthropoda (Kwon *et al.*, 2014). The surface of the body is hairless and smooth, and the horns that extend between the eyes are curved upward, with the ends divided into two. The body color is generally red and there are several irregular white stripes on the side of the body (Kim *et al.*, 2013a). It is distributed on deep reefs in the northern part of the East Sea in Korea and the coast of the Okhotsk Sea, and mainly lives in deep and cold habitats with a depth of 60-650 m and a temperature of 4-6 °C (Kim *et al.*, 2013a).

Next-generation sequencing (NGS) techniques were used to analyze intestinal microorganisms of two shrimp species. NGS technology has the multiplexing ability to

simultaneously perform billions of reactions, and sequencing is possible even with a small amount of sample. A combination of forward and reverse barcodes was used to classify shrimp intestinal samples, and a 16S rRNA gene sequence of each sample was obtained to confirm culture-independent bacterial diversity (Wei *et al.*, 2020). After that, alpha and beta diversity results were obtained for each species and organs through the QIIME2 standard analysis method (Caporaso *et al.*, 2010).

Unlike *Litopenaeus vannamei*, which has been extensively researched and biofloc cultured, there are few studies have been conducted on shrimp species of *L.groenlandicus* and *P. japonica*, and in particular, there are few studies related to microbiomes other than Mitochondrial genome (Kim *et al.*, 2010), Sperm Structure (Kim *et al.*, 2003), and Gonad and androgenic gland development (Kim *et al.*, 2006). Therefore, this study will provide valuable information on the bacterial diversity and symbiotic relationship between the two species of shrimp consumed as expensive seafood in Korea.

1.3 Materials and Methods

1.3.1 Sample collection and DNA extraction

Two types of shrimps, *L. groenlandicus* and *P. japonica*, were caught in the East sea near Ulleung island, East Sea, Korea. The shrimps were promptly flash-frozen aboard the vessel, immediately after being caught. After transferred to the laboratory, the samples were stored in the deep-freezer at -80 ℃ before DNA extraction. Each shrimp was randomly selected, and the weight and size were measured. Shrimp skin was washed clean with 70% ethanol and dissected by sterile tweezers and scissors to prevent contamination. Stomach, hepatopancreas, anterior intestine, and posterior intestine were dissected and used for DNA extraction. A total of 76 samples were obtained from nine *L. groenlandicus* and ten *P. japonica* specimens. DNA was extracted from each sample using an E.Z.N.A. Soil DNA kit (Omega Bio-Tek, Norcross, GA, USA). Gel electrophoresis was performed to visually confirm that DNA was extracted. In addition, nanodrop spectrophotometer (Optizen NANO Q, OPTIZEN) was performed to measure the DNA concentration of each sample. DNA was stored at -80 ℃ until next experiments.

1.3.2 Barcode PCR and NGS sequencing

Extracted DNA was used for barcode PCR and NGS sequencing. To distinguish samples, a dual-indexing primer system was used in which a sample was tagged with the combination of a forward and a reverse barcode. Twelve forward and twelve reverse barcode primers were designed by attaching different twelve bps nucleotide sequences in front of the 16S rRNA gene 319F and 806R primers, respectively. 1 μl of the

template, 1 μl of barcoded forward, and 1 μl of reverse primer were added to the PCR premix (Bioneer, Korea) and DNase/RNase free water was added up to 20 μl of final volume. PCR conditions began with the pre-denaturation at 98 ℃ for 5m followed by 28 cycles of denaturation at 98 ℃ for 30s, annealing at 58 ℃ for 30s, and extension at 72 ℃ for 30s, and ended with the final extension at 72 ℃ for 5m. The amplified DNA was purified (Biotech, Korea), the concentration was measured by the Nanodrop spectrophotometer (Optizen NANO Q, OPTIZEN). The DNA was pooled to contain the sample proportion of each sample. Through Illumina Miseq sequencing, the sequence was obtained as paired-end reads.

Table 1. The list of barcoded PCR primer sequences used in this study

	Index	heterogeneity spacer (0–7 bp)	319F / 806R	Total sequence
319F_1	CCTAAACTACGG		ACTCCTRCGGGAGGCAGCAG	CCTAAACTACGGACTCCTRCGGGAGGCAGCAG
319F_3	GTGGTATGGGAG	T	ACTCCTRCGGGAGGCAGCAG	GTGGTATGGGAGTACTCCTRCGGGAGGCAGCAG
319F_4	TGTTGCGTTTCT	GT	ACTCCTRCGGGAGGCAGCAG	TGTTGCGTTTCTGTACTCCTRCGGGAGGCAGCAG
319F_5	ACAGCCACCCAT	CGA	ACTCCTRCGGGAGGCAGCAG	ACAGCCACCCATCGAACTCCTRCGGGAGGCAGCAG
319F_6	GTTACGTGGTTG	ATGA	ACTCCTRCGGGAGGCAGCAG	GTTACGTGGTTGATGAACTCCTRCGGGAGGCAGCAG
319F_7	TACCGGCTTGCA	TGCGA	ACTCCTRCGGGAGGCAGCAG	TACCGGCTTGCATGCGAACTCCTRCGGGAGGCAGCAG
319F_8	CACCTTACCTTA	GAGTGG	ACTCCTRCGGGAGGCAGCAG	CACCTTACCTTAGAGTGGACTCCTRCGGGAGGCAGCAG
319F_9	TTAACTGGAAGC	CCTGTGG	ACTCCTRCGGGAGGCAGCAG	TTAACTGGAAGCCCTGTGGACTCCTRCGGGAGGCAGCAG
319F_12	GAGGAGTAAAGC	CGA	ACTCCTRCGGGAGGCAGCAG	GAGGAGTAAAGCCGAACTCCTRCGGGAGGCAGCAG
806R_1	CCTAAACTACGG		GGACTACHVGGGTWTCTAAT	CCTAAACTACGGGGACTACHVGGGTWTCTAAT
806R_3	CCATCACATAGG		GGACTACHVGGGTWTCTAAT	CCATCACATAGGGGACTACHVGGGTWTCTAAT
806R_4	GTGGTATGGGAG	A	GGACTACHVGGGTWTCTAAT	GTGGTATGGGAGAGGACTACHVGGGTWTCTAAT
806R_5	ACTTTAAGGGTG	A	GGACTACHVGGGTWTCTAAT	ACTTTAAGGGTGAGGACTACHVGGGTWTCTAAT
806R_6	GAGCAACATCCT	A	GGACTACHVGGGTWTCTAAT	GAGCAACATCCTAGGACTACHVGGGTWTCTAAT
806R_7	TGTTGCGTTTCT	TC	GGACTACHVGGGTWTCTAAT	TGTTGCGTTTCTTCGGACTACHVGGGTWTCTAAT

806R_8	ATGTCCGACCAA	TC	GGACTACHVGGGTWTCTAAT	ATGTCCGACCAATCGGACTACHVGGGTWTCTAAT
806R_9	AGGTACGCAATT	TC	GGACTACHVGGGTWTCTAAT	AGGTACGCAATTTCGGACTACHVGGGTWTCTAAT
806R_10	ACAGCCACCCAT	CTA	GGACTACHVGGGTWTCTAAT	ACAGCCACCCATCTAGGACTACHVGGGTWTCTAAT
806R_11	TGTCTCGCAAGC	CTA	GGACTACHVGGGTWTCTAAT	TGTCTCGCAAGCCTAGGACTACHVGGGTWTCTAAT
806R_12	GAGGAGTAAAGC	CTA	GGACTACHVGGGTWTCTAAT	GAGGAGTAAAGCCTAGGACTACHVGGGTWTCTAAT

1.3.3 Bioinformatic analysis of reads

The resulting raw reads were analyzed using QIIME2 (version 2023.2). QIIME (pronounced as chime and stands for Quantitative Insights into Microbial Ecology) is a pipeline for microbiome analysis that starts from raw DNA sequencing data and ends with visualization and statistical analysis (Caporaso *et al.*, 2010). The barcode and primer sequences were removed, and diversity analysis was performed using only sequences of 200 bp or more. After importing the data into QIIME2, it was classified into amplitude variables (ASV), using dada2 program, and feature tables and representative sequences were obtained using the obtained features. Dada2 calculated the number of good reads remaining after the quality control steps (Mohsen *et al.*, 2019). Taxonomic assignment to each ASV was conducted based on the SILVA database (Koh *et al.*, 2023).

Alpha and beta analysis were performed using the pipeline of QIIME2, and UniFrac PCoA analysis and barplot were obtained. As α-Diversity, Chao1, Simpson, and Shannon indices were represented: Chao1 index estimates the numbers of observed species (richness), and Simpson and Shannon indices are an estimator for both species richness and evenness. PCoA plot is a β-diversity representing the distances between the microbiome of samples in a low-dimensional space (Koh *et al.*, 2023).

1.4 Results and discussion

As a result of performing UniFrac PCoA on samples of *L. groenlandicus* (G) and *P. japonica* (J), it can be seen that the intestinal microbiota of the two species were distinct. Bacterial diversity of the *L. groenlandicus* (G) was observed much higher, and in the case of the *P. japonica* (J), it showed an overall clustered appearance. At the class level, *P. japonica* (J) showed a relatively simple variety, and *L. groenlandicus* (G) showed a much more diverse appearance. *P. japonica* (J) was mainly composed of class Gammaproteobacteria, which accounted for over 80% of the samples, and rarely, class Alphaproteobacteria was observed. *L. groenlandicus* (G) was largely dominated by Gammaproteobacteria and Alphaproteobacteria, with a significant presence of class Mollicutes and the OD1 group. At the genus level, the *P. japonica* (J) was mainly represented by the genus *Moritella* of class Gammaproteobacteria, with a significant proportion. It was also observed a similar portion of family Moritellaceae, which belongs to a similar sequence group. In the case of *L. groenlandicus* (G), a certain portion of the genus *Moritella* and family Moritellaceae was observed, but it was more diverse with genera *Psychromonas*, *Sphingomonas*, *Photobacterium*, and *Aliivibrio*. Overall, it can be seen that *L. groenlandicus* (G) has much higher diversity compared to *P. japonica* (J) at the class and genus levels.

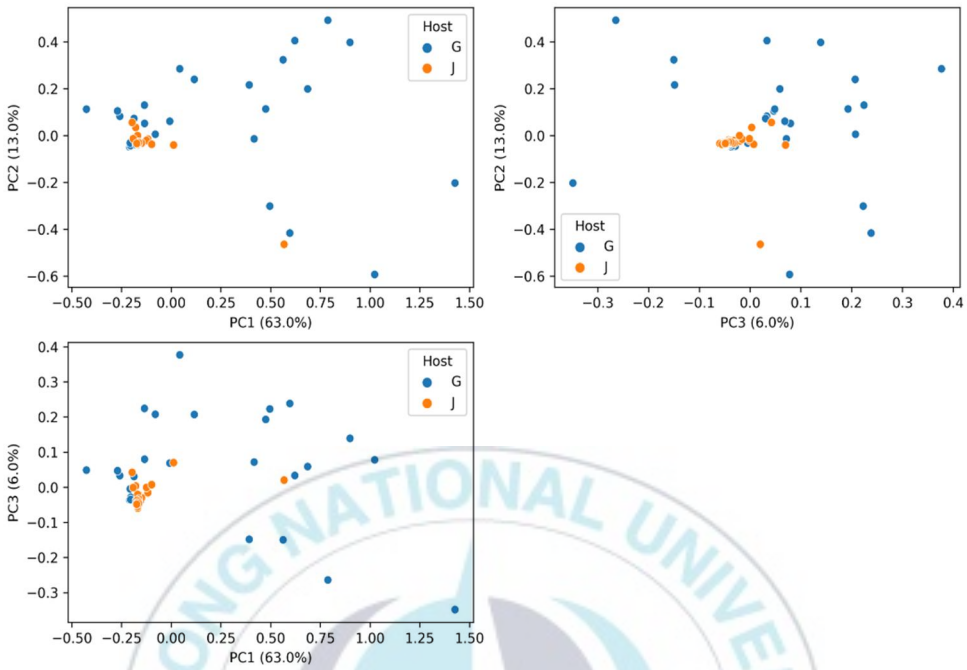

Figure 1. Unweighted UniFrac PCoA plot of shrimp microbiome (G: *Lebbeus groenlandicus*, J: *Pandalopsis japonica*)

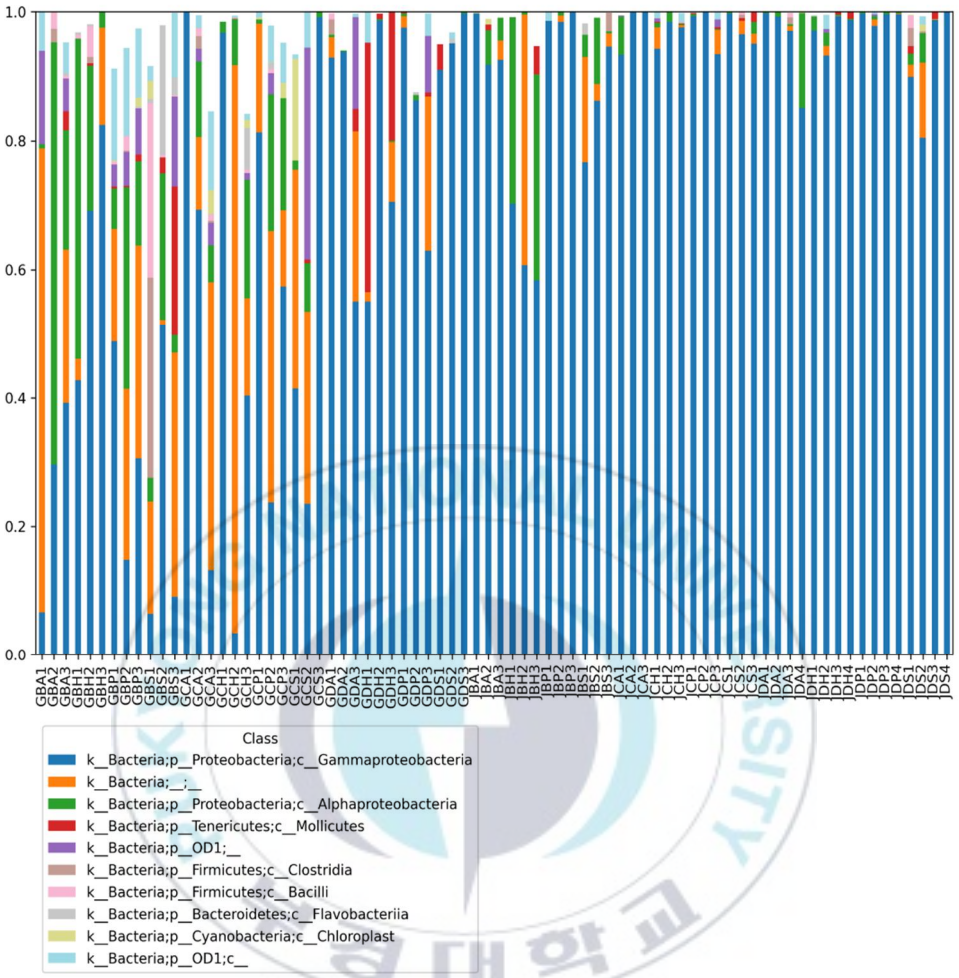

Figure 2. Diversity of shrimp microbiome at class level (G: *Lebbeus groenlandicus*, J: *Pandalopsis japonica*)

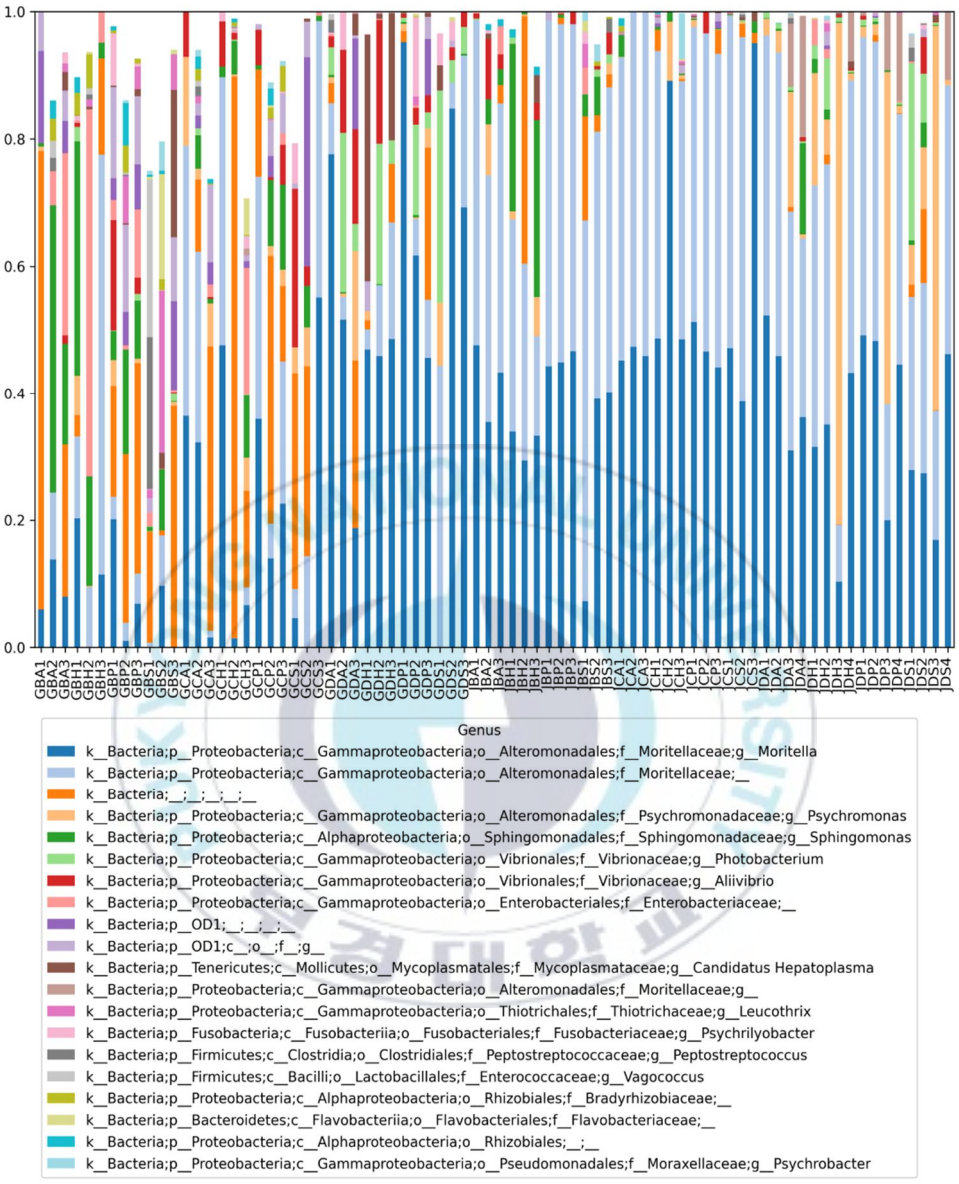

Figure 3. Diversity of shrimp microbiome at genus level (G: *Lebbeus groenlandicus*, J: *Pandalopsis japonica*)

A significant difference in species richness and evenness was observed between the two shrimp species using Chao1 and Shannon indices. *L. groenlandicus* (G) showed high species richness and evenness within the same species, indicating high diversity compared to *P. japonica (J)*. In contrast, *P. japonica (J)* can be seen that diversity appears in a fairly narrow range. As a result of comparing the diversity by species and organs, *L. groenlandicus* (G) showed very low richness and evenness in the hepatopancreas compared to other organs. Hepatopancreas regarded as a organ for secretion of digestive enzymes rather than as a pathway for food passage. It is assumed that the low microbial diversity is due to its function. On the other hand, *P. japonica* (J) showed low richness and evenness in all organs.

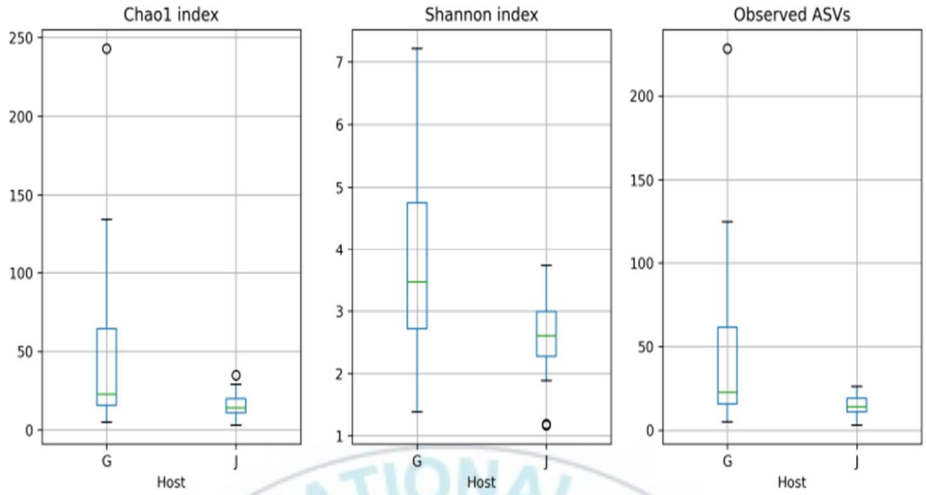

Figure 4. Comparison of diversity index by species

(G: *Lebbeus groenlandicus*, J: *Pandalopsis japonica*)

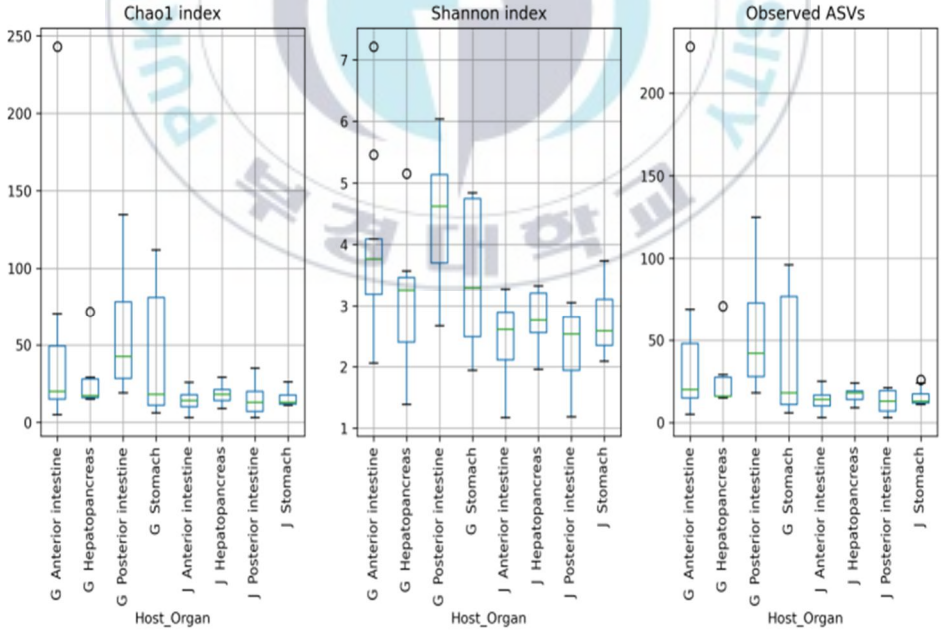

Figure 5. Comparison of diversity index by species and organs

(G: *Lebbeus groenlandicus*, J: *Pandalopsis japonica*)

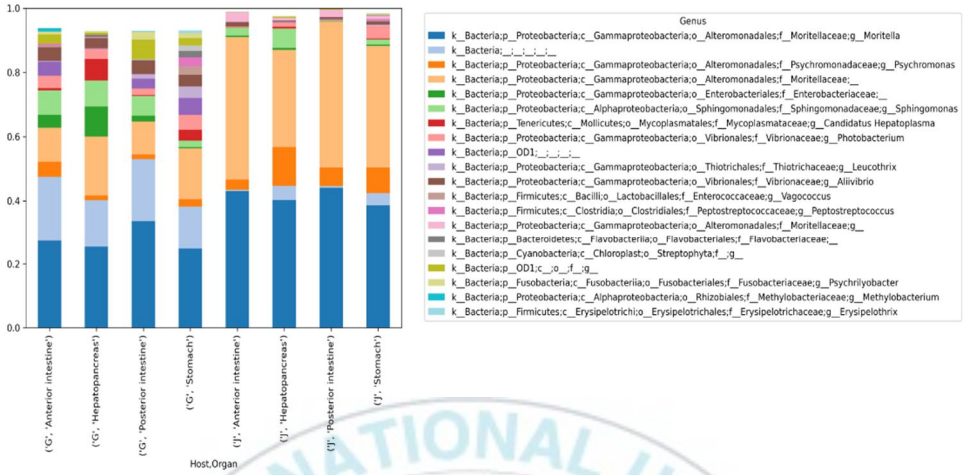

Figure 6. Comparison of mean values of taxa at the genus level by species and organs

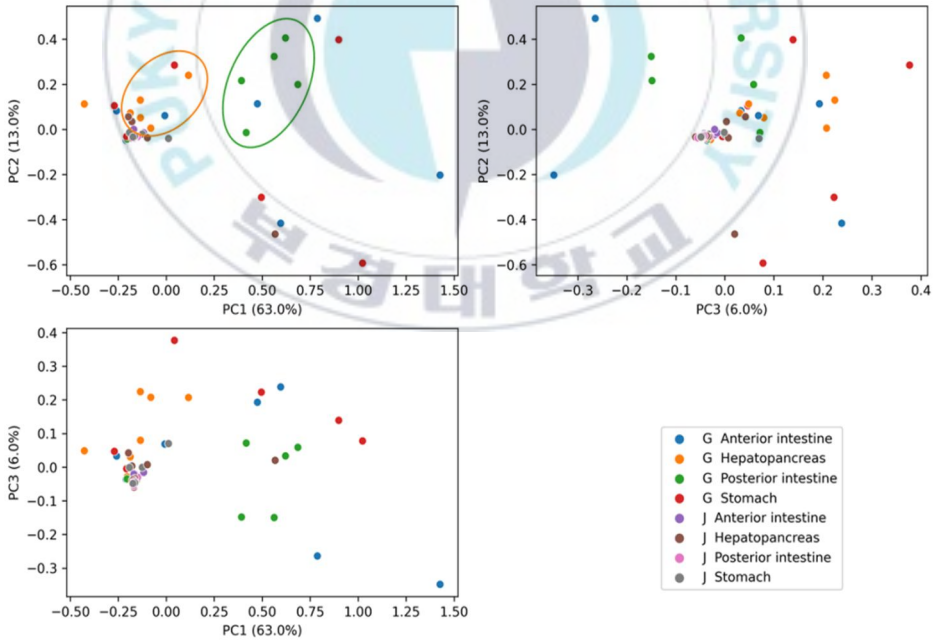

Figure 7. Unweighted UniFrac PCoA plot of shrimp microbiome (by species and organs)

1.5 Conclusion

16S rRNA amplicon sequencing revealed the bacterial diversity of digestive systems of *L. groenlandicus* and *P. japonica*. When comparing the two shrimp species, overall, the *L. groenlandicus* had much higher diversity at the class and genus level than the *P. japonica*. However, it is consistent that both species are dominated by class Gammaproteobacteria and Alphaproteobacteria. Among all organisms, Gammaproteobacteria is a common family, particularly within the digestive systems, but the notable presence of the genus *Moritella* suggests that these shrimps inhabit deep and cold seas. Additionally, the fact that this genus is widely distributed in the shrimp's intestine is expected to have a significant impact on the shrimp. This study may provide information on the microbial diversity of the digestive systems of both *L. groenlandicus* and *P. japonica* through culture-independent methods.

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Chapter 2. Isolation and genomic analysis of M13 and M17, novel psychrophilic bacteria, isolated from *Lebbeus groenlandicus*

2.1 Abstract

A gram-negative, aerobic bacteria, designated M13 (KCTC 92916) and M17 (KCTC 92917), were isolated from a intestine and stomach of *Lebbeus groenlandicus*, caught in the East Sea near Ulleung island. The strain M13 was coccobacilli-shape, non-motile, oxidase-weak reaction and catalase-positive. Growth of strain M13 was observed at 4–25 ℃ (optimum, 20 ℃) and pH 5.0–9.0 (optimum, pH 6.5) and in the presence of 0–6% (w/v) NaCl (optimum, 1%). The major polar lipids were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG), and unidentified glycolipids. The 16s rRNA gene sequences showed low similarity with the *Psychrobacter luti* NF11 (T) (98.55%), a member of Gammaproteobacteria. The strain M13 has a genome of total 3,207,175 bps (sum of contig 1: 3,177,391 bp and contig 2: 29,784 bp). The morphology of strain M17 was coccobacilli-shape, non-motile, and both of oxidase and catalase reactions were positive. As a result of finding out the optimal growth conditions for strain M17, it was revealed that 20 ℃, pH 7.5 and 1% (w/v) of NaCl concentration is the most appropriate conditions. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG), and unidentified glycolipids.constituted the primary cellular polar lipids. The strain17 has the low sequence similarity with its closely

related genus, *Psychrobacter arcticus* 273-4 (T) (98.89%). The genome of the M17 strain was included a total of 3,260,452 bps, comprising contig 1 with a length of: 3,218,680 bp and contig 2 with a length of 41,772 bp. Average nucleotide identity (ANI) and average amino acid identity (AAI) values between strain M13 and M17 were calculated from whole genome sequencing. The values were 97.56%, 97.3% respectively. On the basis of phenotypic, phylogenetic, genomic analysis presented in this study, we suggest that the strain M13 and M17 were the novel genera which belongs to the family Moraxellaceae. In addition, it is assumed that several novel strains could be obtained within the digestive system of *L. groenlandicus*. This study showed the necessity for more research on the intestinal microorganisms that coexist with *L. groenlandicus*, regarded as an unexplored and valuable resource. This study will contribute to understand the characteristic of microbiome of the *L. groenlandicus*.

2.2 Introduction

Psychrobacter species have been identified by incubation and non-culture methods in the respiratory organs of marine mammals (Apprill *et al.*, 2017). They are distributed widely in host environments such as skin (Apprill *et al.*, 2014), intestines (Kudo *et al.*, 2014), the throat and intestines of birds (Kämpfer *et al.*, 2015), and fish (Svanevik and Lunestad, 2011) as well as in host environments such as seawater (Yoon *et al.*, 2005), sea ice (Bowman *et al.*, 1997), marine sediment (Matsuyama *et al.*, 2015), glacial ice (Zeng *et al.*, 2016), and permafrost soil (Bakermans *et al.*, 2006). Based on their distribution in cold seawater and ice, it is suggested that there are *Psychrobacter* that are well adapted to survive in low-temperature environments.

Most *Psychrobacter* strains are resistant to varying temperatures between 4 ℃ and 25 °C, and salt concentrations between 0 ℃ and 5%. More than 90% of the 92 *Psychrobacter* strains, including 38 species of *Psychrobacter* and unclassified strains, have been confirmed to grow under these conditions. 31% of *Psychrobacter* can grow at temperatures as high as 37 ℃ and 54% of *Psychrobacter* can grow at a salt concentration as high as 10%. *Psychrobacter* spp. indicates a wide range of growth temperatures (0-38℃), but some strains, such as *Psychrobacter frigidicola* and *Psychrobacter glacincola*, are psychrophilic (growth restricted below 20 ℃). There is little difference in the maximum growth temperature, except for a few *Psychrobacter* spp. that grow only at low temperatures (Welter *et al.*, 2021).

Strains M13 and M17 were isolated from the intestine and stomach of *Lebbeus groenlandicus*, respectively. The organs were finely homogenized, and then mixed with only 0.1g of sterile phosphate-buffered saline solution (PBS). Dilutions of 10^{0}, 10^{-1},

and 10^{-2} were prepared, and 100 μl of each dilution was dispensed and evenly spread. The media used were tryptic soy agar (TSA), marine agar (MA), nutrient agar (NA), and brain heart infusion agar (BHI). Based on the characteristics of the host inhabiting in cold temperatures (4-6 °C), the plates were incubated at 4 °C for 5 days. A total of 31 colonies were observed, and each colony was individually isolated onto the agar plate it grew on. After performing colony PCR and sequencing on each colony, it was found that 5 strains had low 16S rRNA sequence similarity with closely related species. Based on the analysis of the 16S rRNA gene sequence and phylogenetic analysis, it was confirmed that strains M13 and M17 were closely related in phylogenetic position. Therefore, the aim of this study was to analyze the characteristics of these two strains. Strain M13 showed a 98.55% similarity to *Psychrobacter luti* NF11 (T), and M17 exhibited a 98.89% similarity to *Psychrobacter arcticus* 273-4 (T). They belong to genus *Psychrobacter*, family Moraxellaceae, order Moraxellales, and class Gammaproteobacteria.

Physiological and biochemical tests such as cell morphology, oxidase, catalase test, API kit, etc. were performed for each strain. Genomic analysis of these psychrophilic bacteria will reveal their phylogenetic positions at each taxonomic level and be able to infer the relationship between the intestinal microbiome and the host.

2.3 Materials and Methods

2.3.1 Cultivation and isolation

To investigate the microorganisms in the stomach and intestine of *L. groenlandicus* using a culture-dependent approach, we collected specimens near Ulleung Island. The dissected organs were homogenized in a sterile phosphate-buffered saline solution (PBS), using 0.1 g of the sample. Dilutions of 10^{0}, 10^{-1}, and 10^{-2} were prepared and 100 µl of each dilution was dispensed and spread onto tryptic soy agar (TSA), marine agar (MA), nutrient agar (NA), and brain heart infusion agar (BHI). Since *L. groenlandicus* lives in cold temperatures of 4-6 °C, the plates were incubated at 4 °C for 5 days. A total of 31 colonies were confirmed to be pure isolates for 16S rRNA gene sequencing.

2.3.2 Colony PCR

The 27F (5'-AGAGTTTGATCCTGGCTCAG-3') primer and 1492R (5'-GGYTACCTTGTTACGACTT-3') primer were added to the tube containing PCR premix, each at a volume of 1 µl. After adding DNase/RNase-free water to reach a final volume of 20 µl, each colony was added to the tube. PCR process was performed as follows: The templates were pre-denaturated at the temperature of 95 ℃ for 7 minutes. They were denaturated at 95 ℃ for 30 seconds, annealed at 60 ℃ for 30 seconds, and extended at 72 ℃ for 60 seconds. This process was repeated 30 times. Final extension step was performed at 72℃ for 5 minutes. The colony PCR results were confirmed by electrophoresis on a 1% agarose gel. The amplified PCR products went through for DNA sequencing using the 8F primer.

2.3.3 Phylogenetic analysis

The 16S rRNA gene sequence was compared to type strains of Ezbiocloud database to determine a taxonomic affiliation. The twenty reference sequences and an outgroup, *Moraxella lacunata* NBRC 102154(T) (BCUK01000202), were obtained from the database. To analyze taxonomic positions, the 16S rRNA gene sequences were aligned by ClustalW multiple alignment MEGA11. Phylogenetic trees were reconstructed by the neighbour-joining (NJ) with Kimura's two-parameter model and bootstrap analyses based on 1000 replicates using MEGA 11. Based on the results above, for the candidate novel strains with low similarity, including the outgroup, a tree was constructed using the same method to confirm their positions.

2.3.4 Morphological and biochemical tests

Cells were Gram-stained using a Gram stain kit (Remel, Thermo Scientific) and observed under a 1000x optical microscope and their cell sizes were measured. Motility were observed by using the wet mount and the hanging drop methods. The presence of oxidase activity was determined by oxidase test reagent, 1% (w/v) tetramethyl p-phenylenediamine (bioMérieux). Catalase activity was confirmed by observation of bubble formation in 3% (v/v) hydrogen peroxide solution. Growth on different media such as tryptic soy agar (TSA), marine agar (MA), nutrient agar (NA), and brain heart infusion agar (BHI) were checked. All media incubated at 4 ℃ for 5 days. To investigate the optimal growth conditions of the strains, their growth was observed based on temperature, pH, and NaCl concentration variations. The strains were cultured on marine agar to determine the optimal temperature, and incubated at temperatures -

4, 0, 4, 8, 10, 15, 20, 25, 30, 35 and 40 ℃ for 5 days. pH (pH 4–12 at 1 pH unit) was examined in modified marine broth. It was grown in modified marine broth with pH (pH 4–12 at 1 pH unit) and NaCl (NaCl 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 % (w/v)) 2.5% (w/v) at each optimal temperature for 5 days. Biochemical tests were performed using API kits (API ZYM, API 20NE, and API 50CHB; bioMérieux, Inc), following the manufacturer's instructions.

2.3.5 Polar lipids

Polar lipids were extracted using strains cultured on marine agar at the optimal temperature. Polar lipids were performed by two-dimensional thin-layer chromatography (TLC Silica gel 60 F_{254}; Millipore). A mixture of chloroform and methanol in a 2:1 ratio to make a total volume of 30 ml in a 50 ml glass tube. The 0.3g of bacterial cells were added and thoroughly mixed by shaking overnight. The mixture was centrifuged at 3500 rpm for 15 minutes, and then filtered through filter paper into a 50 ml conical tube. The filtered solution was evaporated at room temperature (<37°C), and then dissolved by adding chloroform : methanol : 0.3% aqueous NaCl (3.8 ml : 3 ml : 2.9 ml) and shaking thoroughly for 15 minutes. The mixture was centrifuged at 3500 rpm for 5 minutes to separate the chloroform layer, which was then transferred to a new glass tube and evaporated at room temperature. A total of 300 μl of a 2:1 mixture of chloroform and methanol was added, and TLC was performed.

Polar lipids were separated on a thin layer chromatography silica plates (10 X 10 cm). After two-dimensional chromatography (Minnikin *et al.*, 1984), dry the TLC plate in the oven for 20 minutes and apply each reagent. Molybdophosphate acid (10% in

EtOH), ninhydrin (0.2% in EtOH), a-naphthol (0.15g in EtOH 40ml, sulfuric acid 6.5ml, UDW 4ml), sodium methaperiodate (1% in UDW), molybdenum blue, dragendorff reagent are applied. Through this, it was possible to observe whole lipids, aminolipids, glycolipids, choline and sphingomyelin groups, phospholipids, and alpha-glycol groups (Komagata and Suzuki, 1988).

2.3.6 Whole genome sequencing

In order to understand functional genes, whole genome sequences were retrieved. To extract DNA, the Genomic DNA Prep Kit (BIOFACT) was used, and the extracted DNA was purified. After measuring the DNA concentration, it was sent to an NGS company for sequencing and sequence assembly. Genome analysis was performed in the order of Pacbio SMRT sequencing and De novo Assembly. Constructing graphical circular genome maps was performed using CGView Comparison Tool (Grant and Stothard, 2008). The gene prediction and annotation. was used RAST (Rapid Annotation using the Subsystem Technology) pipeline, followed by subsystem analysis. The presence and characteristics of prophages were determined using PHASTER (PHAge Search Tool Enhanced Release). Additionally, KEGG (Kyoto Encyclopedia of Genes and Genomes) was employed to identify metabolic pathways of the strains, and antiSMASH was used to identify genes contributing to secondary metabolites. Using the Ezbiocloud platform as a basis, the average nucleotide identity (ANI) and average amino acid identity (AAI) between M13 and M17 were calculated.

2.4 Results and discussion

Comparing the 16S rRNA gene sequences obtained from sequencing results with closely related strains using Ezbiocloud, a total of five strains were identified as potential novel strains. By constructing a neighbor-joining phylogenetic tree, including the five strains and closely related type strains as well as an outgroup strain *Moraxella lacunata* NBRC 102154(T)(BCUK01000202). The phylogenetic analysis indicated that M13 and M17 were closely related and shared a similar taxonomic position within the tree. Additionally, most of the strains that showed close similarity with M13 and M17 exhibited similar patterns. In the phylogenetic tree, M13 and M17 were classified within the *Psychrobacter* genus. The strain with the closest similarity to M13 based on 16S rRNA gene sequence was *Psychrobacter luti* NF11(T), showing a similarity of 98.55%, while M17 showed low sequence similarity of 98.89% with *Psychrobacter arcticus* 273-4(T). Therefore, M13 and M17 should be classified as novel strains within the *Psychrobacter* genus, and further identification experiments were conducted to compare their characteristics.

Table 2. 16S rRNA sequence similarity of strain M13 and other strains showed ten closest strains on the ez-biocloud server

Rank	Name	Strain	Similarity (%)
1	*Psychrobacter luti*	NF11 (T)	98.55
2	*Psychrobacter arcticus*	273-4 (T)	98.41
3	*Psychrobacter fozii*	NF23 (T)	98.34
4	*Psychrobacter cryohalolentis*	K5 (T)	98.26
5	*Psychrobacter aquaticus*	CMS 56 (T)	98.19
6	*Psychrobacter glaciei*	BIc20019 (T)	98.19
7	*Psychrobacter glacincola*	DSM 12194 (T)	98.12
8	*Psychrobacter okhotskensis*	MD17 (T)	98.12
9	*Psychrobacter vallis*	CMS 39 (T)	98.12
10	*Psychrobacter frigidicola*	DSM 12411 (T)	98.05

Table 3. 16S rRNA sequence similarity of strain M17 and other strains showed ten closest strains on the ez-biocloud server

Rank	Name	Strain	Similarity (%)
1	*Psychrobacter arcticus*	273-4 (T)	98.89
2	*Psychrobacter fozii*	NF23 (T)	98.74
3	*Psychrobacter cryohalolentis*	K5 (T)	98.74
4	*Psychrobacter glacincola*	DSM 12194 (T)	98.59
5	*Psychrobacter okhotskensis*	MD17 (T)	98.59
6	*Psychrobacter glaciei*	BIc20019 (T)	98.59
7	*Psychrobacter immobilis*	DSM 7229 (T)	98.44
8	*Psychrobacter luti*	NF11 (T)	98.37
9	*Psychrobacter adeliensis*	DSM 15333 (T)	98.37
10	*Psychrobacter namhaensis*	SW-242 (T)	98.29

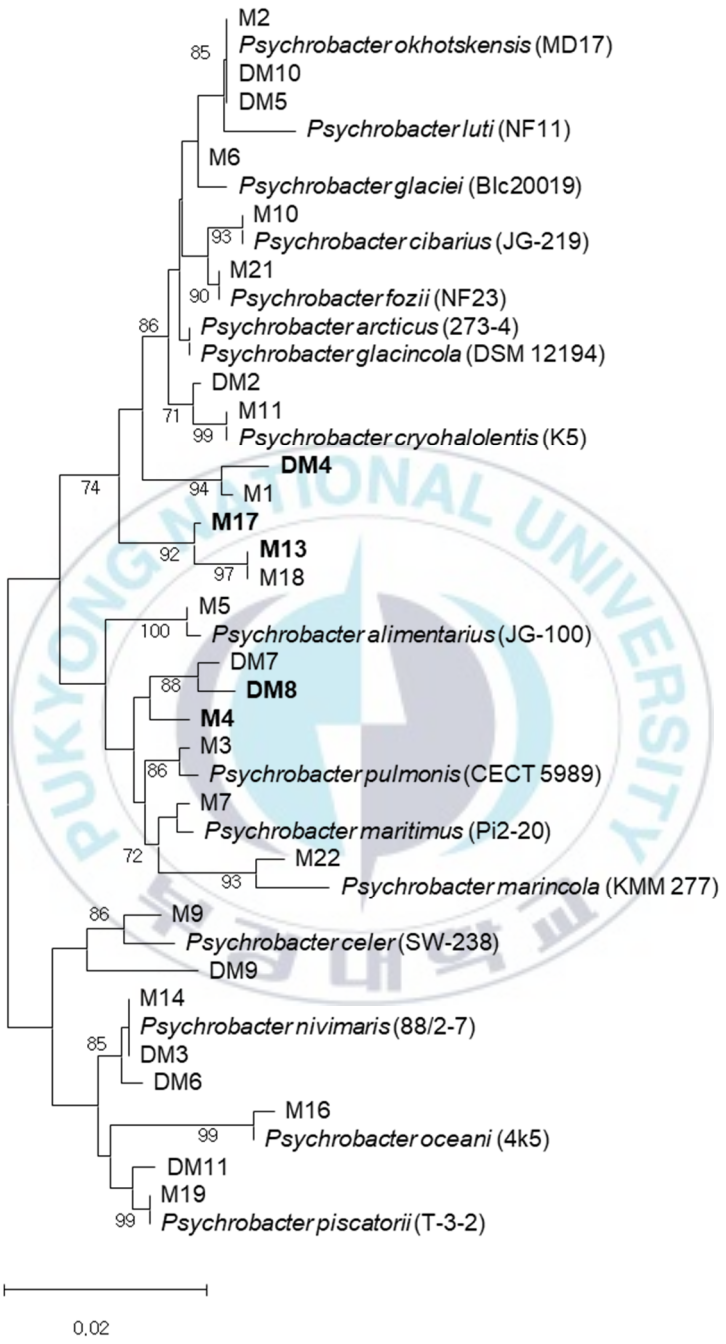


Figure 8. Phylogenetic tree of the candidates of novel species

Numbers indicate bootstrap percentages from neighbour-joining after 1000 replication; values below 70% are not shown

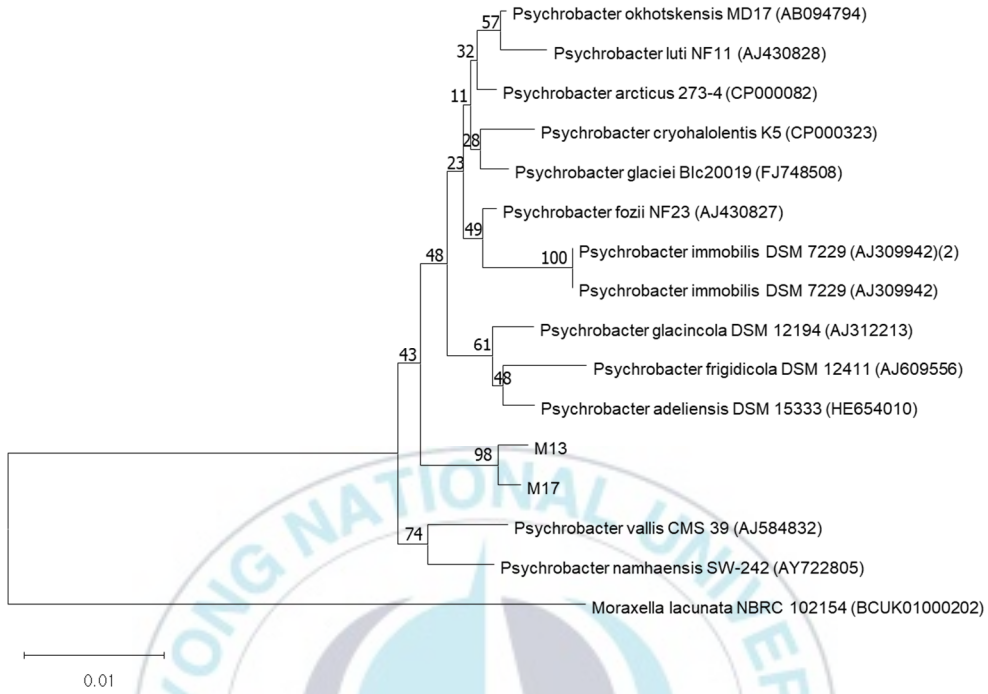


Figure 9. Phylogenetic tree showing the position of strain M13 and M17

Numbers indicate bootstrap percentages from neighbour-joining after 1000 replication; values below 50% are not shown

Both of the strains M13 and M17 were gram-negative (Figure 10) and no motility was observed. The strains showed positive reactions for oxidase and catalase activities, but the oxidase reaction result of M13 was weak. Based on the observation of growth on various types of media, M13 and M17 can grow in TSA, MA, BHI media in common. Both M13 and M17 strains grew within the temperature range of 4°C to 25°C, with an optimal growth temperature of 20 °C. M13 and M17 showed similar growth capabilities within a pH range of 5 to 9. The optimal pH for M13 was determined to be pH 6.5, while M17 showed the highest growth at pH 7.5. M13 and M17 demonstrated distinct NaCl tolerances, with M13 able to grow within a range of 0% to 6% NaCl, while M17 showed in a narrower range of 0% to 2% NaCl. However, both strains exhibited an optimal NaCl concentration of 1%. In the enzyme activity test using API kit (API ZYM, API 20NE, and API 50CHB; bioMérieux, Inc), M13 and M17 showed mostly negative reaction results. Both strains had a weak reaction in the enzyme activity for Esculin, while only M17 showed a weak reaction in the presence of Ferric citrate (Table 4). The major polar lipids of M13 and M17 were found to be phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG), with the presence of unidentified glycolipids. PE is involved in the composition of cell membranes and cell walls and plays a significant role in the immune system (Vance and Tasseva, 2013). PG is a lipid widely distributed in almost all organisms and contributes to the structure of cell membranes (Wada and Murata, 2007). DPG, similarly, is an essential component of cell membranes, contributing to cell stability and functionality (Vik *et al.*, 1981).

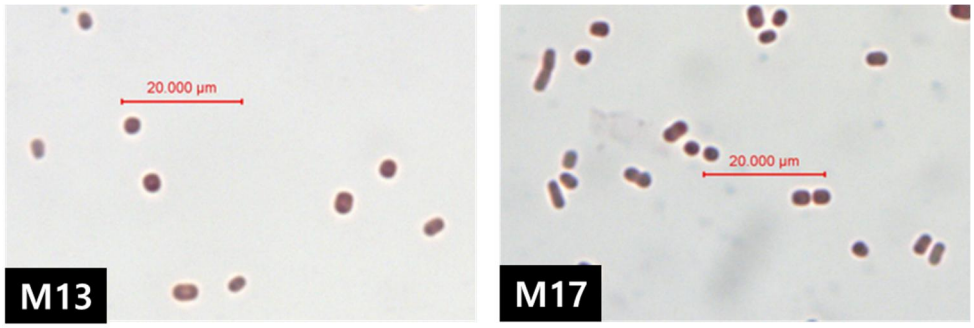


Figure 10. Results of gram staining of strains M13 and M17 under an optical microscope (1,000X)

Table 4. Phenotypic characteristics that differentiate strain

Characteristic	M13	M17
Colony morphology	circular, convex, white	circular, convex, beige
Growth media	TSA, MA, BHI	TSA, MA, BHI
Temperature range (℃)	4-25	4-25
(Optimum Temp.)	(20)	(20)
pH range	5-9	5-9
(Optimum pH)	(6.5)	(7.5)
NaCl range (%)	0-6	0-2
(Optimum NaCl)	(1)	(1)
Cell shape	Coccobacilli	Coccobacilli
Cell size	2.0–2.8 X	2.0–2.7 X
(width X length μm)	2.5–3.0	2.5–3.1
Gram staining	Negative	Negative
Mobility	x	x
Oxidase	W	+
Catalase	++	+
Nitrate reduction	+	+
Enzyme activity		
Esculin	W	W
Ferric citrate	-	W
L-Arabinose	-	-
D-Ribose	-	-
D-Xylose	-	-
D-Galactose	-	-
D-Glucose	-	-
D-Fucose	-	-
potassium Gluconate	-	-
potassium 2-Ketogluconate	-	-
potassium 5-Ketogluconate	-	-

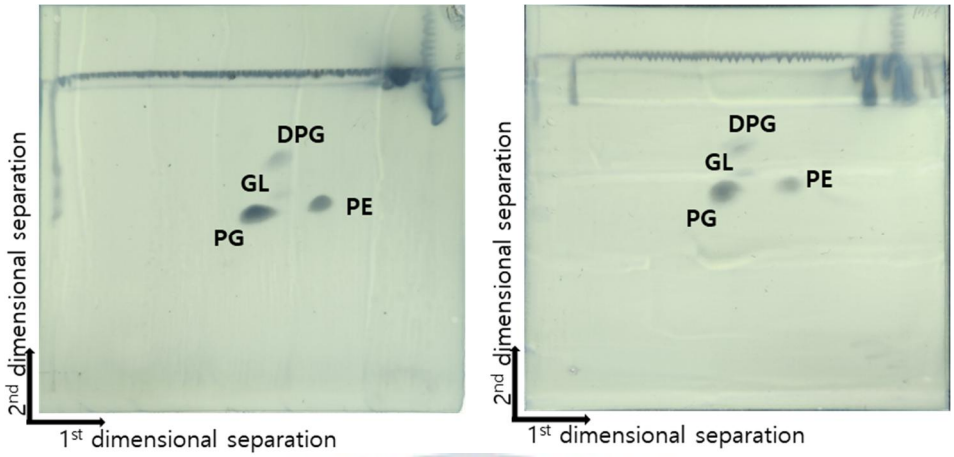


Figure 11. Two-dimensional TLC of the total polar lipids of strain M13 (left) and M17 (right)

In the analysis of the whole genome, both M13 and M17 were found to have one long circular contig and one short circular contig, with a total length of approximately 32 million base pairs (bps) (3,207,175 bps for M13 and 3,260,452 bps for M17). The G+C content of M13 and M17 was similar at approximately 42.6 mol% (Table 5). Using BUSCO, it was confirmed that the genomes were fully analyzed without any missing genes (Table 6). From the center to the outside: genome label, GC skew (green and purple), G + C content (black), CDSs colored by COG categories on the reverse strand, CDSs including DNAs on the reverse strand, CDSs including DNAs on the forward strand, CDSs colored by COG categories on the forward strand (Figure 12). Using RAST, the subsystem analysis revealed that both M13 and M17 were classified into approximately 30% of the total categories, specifically 26 and 24 categories, respectively. The coding sequences accounted for around 2,700 in number, and there were 9 rRNA sequences, 46 and 47 tRNA sequences, and 1 tmRNA sequence. Notably, the category "Metabolism of Aromatic Compounds" within the subsystem exhibited significant differences compared to other categories (Figure 13). To further investigate this, the entire pathway, including the metabolism of aromatic compounds, was traced using KEGG. The pathways shared by both strains are indicated in blue, pathways specific to M13 are shown in green, and pathways unique to M17 are represented in red (Figure 14). Both M13 and M17 exhibited highly similar metabolic pathways. M17 possesses the initial compounds involved in the decomposition of the aromatic compound benzoate, which is an aromatic compound. Subsequently, M13 also contributes to the degradation process. This pathway confirms the differences observed in the Aromatic Compounds category, which were evident in the previous subsystem

analysis (Figure 15). Using PHASTER, analysis of prophage presence revealed that both M13 and M17 exhibited the detection of prophages in a specific one region of contig1 (Figure 16, 17). M13 had an incomplete prophage with only integrase, transposase, and tail. It had a length of 6.8 kb and belonged to six phage types, and contained a total of 10 proteins and had an attachment site (Table 7). In the case of M17, it had an incomplete prophage with a tail. The length of the prophage was 7.8 kb, and it was classified into four phage types. It contained a total of seven proteins, and it did not have an attachment site (Table 8). Using antiSMASH, the genes involved in the biosynthesis of secondary metabolites in M13 and M17 were identified and predicted. The results indicated that both strains have genes contributing to the production of betalactone compounds, and the gene clusters exhibited the highest similarity to plipastatin and Non-Ribosomal Peptide (NRP) genes (Table 9, 10). However, the similarity was very low at 15%, suggesting the possibility of having novel compounds.

Table 5. Assembly results of M13 and M17

	Contig	Form	Contig Length	GC contents
M13	contig1	circular	3,177,391	42.64
	contig2	circular	29,784	40.72
M17	contig1	circular	3,218,680	42.62
	contig2	circular	41,772	40.91

Table 6. BUSCOs results of M13 and M17

Sample Name	Complete BUSCOs	Complete and single-copy BUSCOs	Complete and Duplicated BUSCOs	Fragmented BUSCOs	Missing BUSCOs	Total BUSCOs
M13	123 (99.19%)	123 (99.19%)	0 (0.0%)	1 (0.81%)	0 (0.0%)	124 (100.0%)
M17	123 (99.19%)	123 (99.19%)	0 (0.0%)	1 (0.81%)	0 (0.0%)	124 (100.0%)

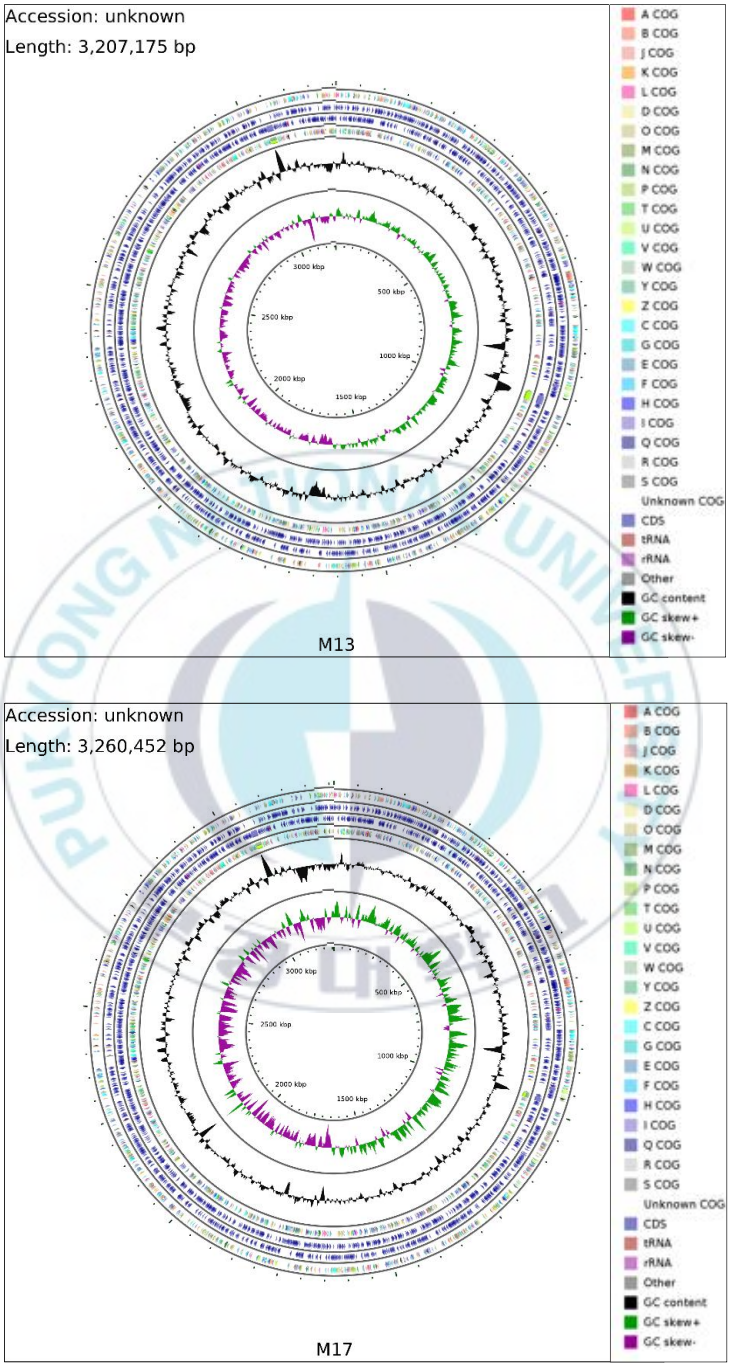
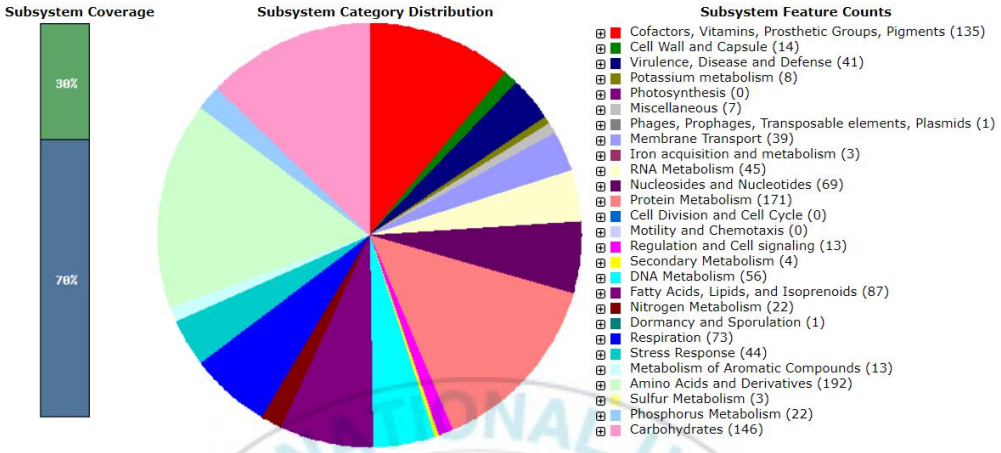


Figure 12. Genome map of M13 and M17 genomic DNA

(A)



(B)

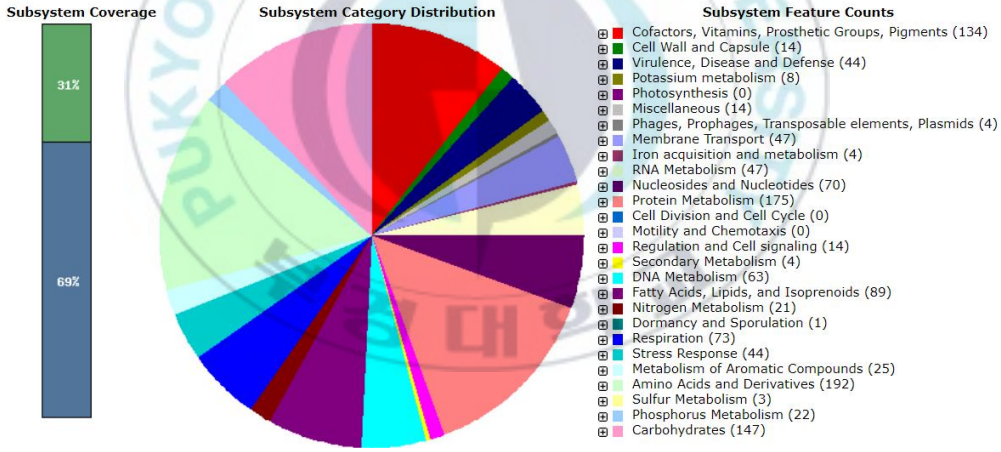


Figure 13. Subsystems of (A) M13 and (B) M17 genomic DNA annotated by Rapid Annotations using Subsystems Technology

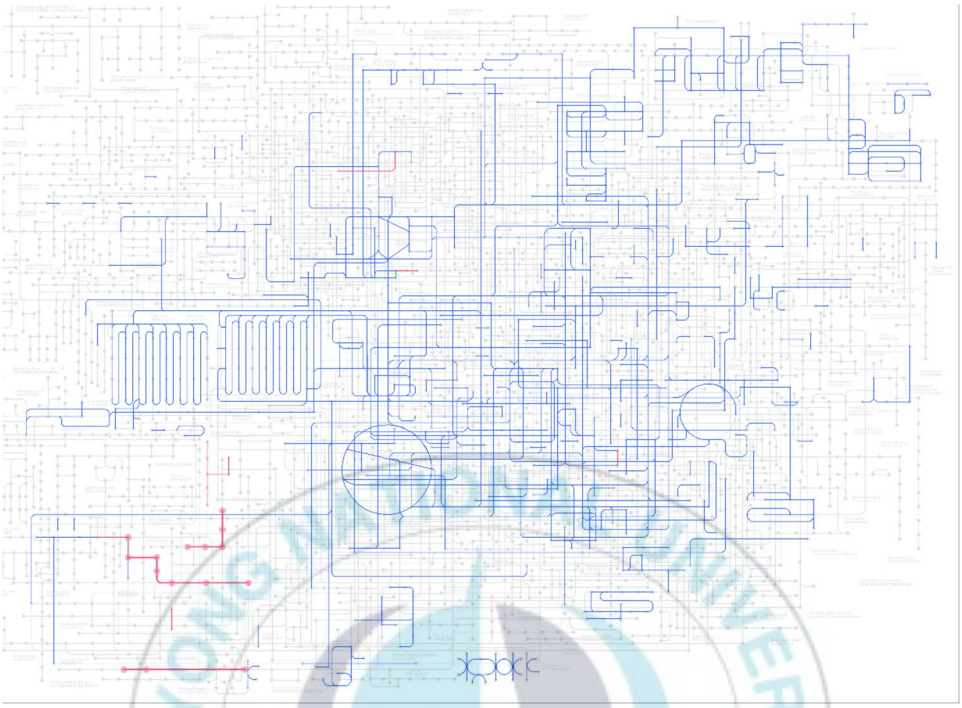


Figure 14. Metabolic pathway of M13 and M17 using KEGG

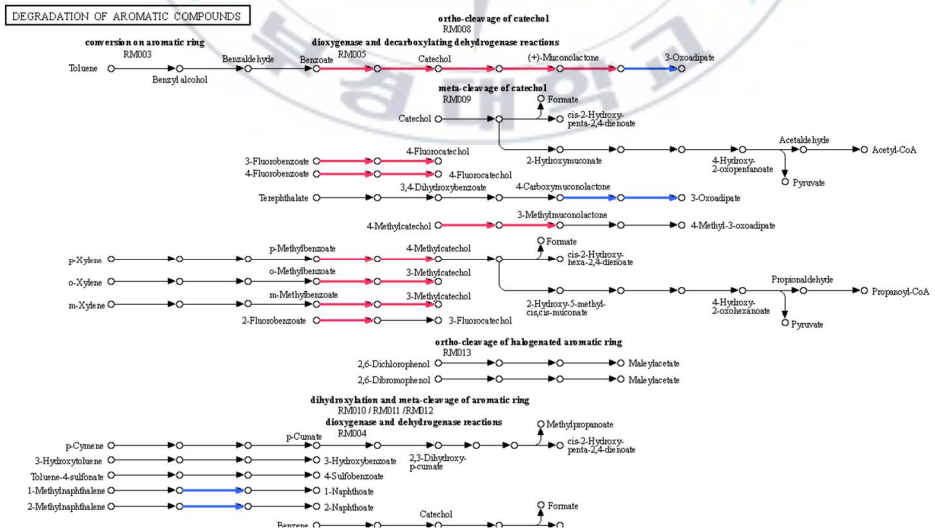


Figure 15. Metabolism aromatic compounds pathway using KEGG

red: the metabolic pathway of M17, blue: the metabolic pathways of M13 and M17

Figure 16. Location of prophage sequences in the M13 genome

Table 7. Detailed information of a prophage detected in the genome of strain M13

Region details of M13 prophage	
Region	1
Region Length	6.8Kb
Completeness(score)	incomplete(50)
Specific Keyword	integrase, transposase, tail
Region Position	contig.1.cir:1680199-1687048
tRNA	0
Total Proteins	10
Phage Hit Proteins	6
Hypothetical Proteins	4
Phage + Hypothetical Protein %	100%
Bacterial Proteins	0
Attachment Site	yes
Phage Species	6
First Most Common Phage #	1
First Most Common Phage %	20%
GC %	41.47%

Figure 17. Location of prophage sequences in the M17 genome

Table 8. Detailed information of a prophage detected in the genome of strain M17

Region details of M17 prophage	
Region	1
Region Length	7.8Kb
Completeness(score)	incomplete(50)
Specific Keyword	tail
Region Position	contig.1.cir:2167806-2175634
tRNA	0
Total Proteins	7
Phage Hit Proteins	6
Hypothetical Proteins	1
Phage + Hypothetical Protein %	100%
Bacterial Proteins	0
Attachment Site	No
Phage Species	4
First Most Common Phage #	3
First Most Common Phage %	28.57%
GC %	42.11%

Figure 18. Secondary metabolite region of M13

Table 9. Identification and prediction of M13 genes involved in the biosynthesis of secondary metabolites using antiSMASH

Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	betalactone	447,924	475,493	plipastatin	NRP	15%

Figure 19. Secondary metabolite region of M17

Table 10. Identification and prediction of M17 genes involved in the biosynthesis of secondary metabolites using antiSMASH

Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	betalactone	460,363	487,932	plipastatin	NRP	15%

The results of ANI and AAI values showed an OrthoANIu value of 97.56%, and a two-way AAI of 97.30% (SD: 7.75%) derived from the comparison of 2,545 proteins. With a similarity of over 95%, it is inferred that the two strains are of the same species.

Table 11. Results of ANI (average nucleotide identity) analysis between the strain M13 and M17

Metric	Value
OrthoANIu value (%)	97.56
Genome A length (bp)	3,206,880
Genome B length (bp)	3,258,900
Average aligned length (bp)	2,140,781
Genome A coverage (%)	66.76
Genome B coverage (%)	65,69

Table 12. Results of AAI (average amino acid identity) analysis between the strain M13 and M17

Method	AAI value
One-way AAI 1	93.01% (SD: 17.53%), from 2,752 proteins
One-way AAI 2	91.92% (SD: 19.08%), from 2,816 proteins
Two-way AAI	97.30% (SD: 7.75%), from 2,545 proteins

2.5 Conclusion

Strain M13 and M17 were obtained from the intestinal and stomach of *Lebbeus groenlandicus*, a species inhabiting the cold and deep sea. The analysis of phylogenetic relationships based on the 16S rRNA gene sequences confirmed that both strain M13 and M17 belong to a novel species within the genus *Psychrobacter*. This study gained knowledge about the properties of the novel bacteria, including the genetic, physical and biochemical properties. Comparative genomics studies have been gained with fundamental knowledge through the availability of the complete genome sequence. Additional tests will be needed to identify these strains. These results will provide more information about genus *Psychrobacter*, and the *L. groenlandicus* is considered a valuable resource that has not been extensively researched yet.

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국문초록

대부분의 미생물은 인간과 동물의 소화기관 내에서 공존하고 상호작용한다. 미생물은 숙주의 체내에서 건강과 관련된 다양한 역할을 하기 때문에 미생물 군집과 그 특성을 이해하는 것이 중요하다. 본 연구에서는 두 종류의 새우, 가시배새우와 물렁가시붉은새우의 소화기관 내 미생물 다양성을 배양 비의존적 방법을 통해 분석하였다. 무작위로 선택된 개체로부터 네 가지 다른 부위(위, 간 췌장, 전장, 후장)로 나누어 총 76개의 샘플을 수집하고, DNA를 추출하였다. 이후 바코드 PCR을 수행하고 Illumina Miseq 플랫폼을 통해 amplicon sequencing을 하였다. Sequencing 결과로 받은 raw 데이터는 QIIME2를 사용하여 분석되었다. 분석 결과, 가시배새우와 물렁가시붉은새우 사이의 미생물 다양성 차이가 나타났다. 전반적으로 종, 부위별 미생물 다양성은 물렁가시붉은새우에 비해 가시배새우가 더 높았다. 물렁가시붉은새우의 대부분은 *Moritella* 속이 차지하고 있었는데, 이러한 결과는 깊고 차가운 바다 환경에서의 생활 특성 때문이며, 미생물 다양성 분포에 영향을 미친 것으로 생각된다. 가시배새우에 대한 배양 의존적 연구가 실시된 결과, 가시배새우의 장과 위에서 그람 음성, 호기성인 M13(KCTC 92916)과 M17(KCTC 92917) 균주가 분리되었다. 16S rRNA 유전자 서열을 기반으로 한 계통 분석 결과에서 M13 균주와 M17 균주 모두 새로운 *Psychrobacter* 속이라는 것이 확인되었고 두 균주의 분류학적 위치가 가까운 것으로 나타났다. M13과 M17은 그람 음성균으로, 운동성이 관찰되지 않았다. 두 균주의

최적 생장 온도, pH, NaCl 농도는 각각 20° C, pH 6.5와 pH 7.5, NaCl 농도 1% (w/v)로 유사했다. API kit를 이용한 효소 활성 시험에서 M13과 M17은 대부분 음성 반응을 보였다. 두 균주 모두 Esculin에 대한 효소활성에서 약한 반응을 보인 반면, M17만이 Ferric citrate에서 약한 반응을 보였다. M13과 M17의 주요 극성 지질은 phosphatidylethanolamine(PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG)로 동일하였고, unidentified glycolipid도 확인되었다. Whole genome 분석 결과, 전체 게놈 사이즈는 약 320만 bps, G+C 함량은 약 42.6%로 유사했고, 물질대사 경로, 2차 대사 물질 생합성에 관여하는 유전자 모두 비슷한 결과를 보였다. M13과 M17 균주 간의 염기 서열 일치도의 평균값(ANI)은 97.56%, 아미노산 일치도의 평균값(AAI)은 97.3%로, 두 균주는 동일한 종인 것으로 추측된다. 계통 분석 및 게놈 분석을 통해 이 균주들은 Moraxellaceae 과의 *Psychrobacter* 속에 속하는 새로운 종으로 제안된다. 이 연구는 가시배새우의 장내 미생물에 대한 추가적인 연구의 필요성과 가시배새우가 새로운 균주 발견의 가능성을 지니는 가치 있는 자원인 것을 강조한다.