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# Bacterial Diversity in Sea Ice from the Southern Ocean and the Sea of Okhotsk

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**Abstract** In order to reveal the diversity of sea ice bacterial communities in polar and sub-polar regions, we investigated 2 drifting ice floes, one from the Australian side of the Southern Ocean and the other from the Sea of Okhotsk. We extracted bacterial DNA from sea ice and constructed 221 16S rDNA clone libraries including 109 clones from the Antarctic sea ice and 112 from the Okhotsk sea ice. The phylogenetic analysis of 16S rDNA sequences showed that *Roseobacter* and *Sulfitobacter* (*Alphaproteobacteria*), *Psychrobacter*, *Halomonas*, and *Pseudoalteromonas* (*Gammaproteobacteria*) were frequent in the Antarctic sea ice; *Colwellia*, *Psychromonas*, and *Glaciecola* (*Gammaproteobacteria*) and *Polaribacter* (*Bacteroidetes*) were major genera in the Okhotsk sea ice. While *Alphaproteobacteria* and *Gammaproteobacteria* were abundant in both samples, *Bacteroidetes* were detected only in the Okhotsk sea ice. Comparing the bacterial diversity of our samples with that of other studies, bacterial communities in sea ice were similar to one another at the phylum level, whereas their populations were quite different at the genus level. We also tried to detect antimicrobial and heavy metal resistance genes in our samples but didn't identified. Our results provide additional information about the bacterial communities in sea ice.

***Keywords:*** *16S rDNA-based analysis, bacterial diversity, sea ice, Sea of Okhotsk, Southern Ocean*

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## 1. Introduction

Sea ice are the distinctive features of oceans in polar and sub-polar regions. Seawater in these regions becomes cold enough to freeze (below -1.9°C) during winter season. Despite severe conditions caused by low temperature, reduced water activity, and transience, sea ice has been reported to contain many types of microorganisms, such as diatoms, protozoa, bacteria, archaea, and viruses [1,2,3]. Among them, bacteria in sea ice are reported to reside in concentrated brine pockets and remain active in sub-zero temperature [4]. Therefore, they are considered to be a good model for studying adaptation mechanisms of microorganisms to extreme conditions [5].

Microorganisms in sea ice were also reported to be indicators of environmental conditions and marine pollution [6,7,8]. Especially, detection of antimicrobial resistance or heavy metal tolerance among bacteria on polar areas can be utilized to assess anthropogenic influences in natural environments [9,10]. Genes encoding drug resistance are thought to be potent indicator since it has been suggested that human activity in the Antarctica is responsible for the dissemination of bacteria possessing antimicrobial resistance genes [11].

Several studies have reported on a rich diversity of bacteria in sea ice and noted a predominance of Gram-negative and psychrophic or psychrotolerant bacteria in these habitats [12,13,14]. Although some of sea ice bacteria are cultivatable under artificial conditions, culture-independent methods such as 16S rDNA-based analysis have been utilized for comprehensive identification of bacterial diversity in sea ice [12]. So far, many researchers have surveyed bacterial communities in sea ice samples using 16S rDNA-based culture-independent methods [3,12,13,14,15]. These studies reported an abundance of the phylum *Proteobacteria* (classes *Alphaproteobacteria* and *Gammaproteobacteria*) and *Bacteroidetes* (reported as *Cytophaga-Flavobacterium-Bacteroidetes* group) in sea ice bacterial communities in the Arctic and Antarctic regions. At the genus level, *Roseobacter* (*Alphaproteobacteria*), *Colwellia*, *Glaciecola*, *Marinobacter* (*Gammaproteobacteria*) and *Polaribacter* (*Bacteroidetes*) were reported as dominant [14,16].

To date, the majority of studies in sea ice have utilized their samples which were collected from the Indian side of the Southern Ocean [12,14], the Arctic Ocean [2,14], and

the Baltic Sea [17]. The information about sea ice microbiota in other frozen seas, such as the Australian side of the Southern Ocean and the Sea of Okhotsk, is limited. Although Monfort *et al.* [18] studied bacterial dynamics in first-year sea ice around the Sea of Okhotsk, 16S rDNA-based studies in the area have not been reported. Our objective was to reveal bacterial biodiversity in sea ice derived from these unstudied areas and to compare it with previous reports to improve understanding of bacterial communities in sea ice. In addition, we also tried to detect antimicrobial and heavy metal resistance genes in our samples to assume the distribution of these genes in environments under the less artificial influences.

# 2. Materials and Methods

## 2.1. Sample Preparation

Drifting ice floes of the Southern Ocean was collected in Dumont d'Urville Sea during the 24$^{th}$ cruise of the training ship "Umitaka-maru" (Tokyo University of Marine Science and Technology, Tokyo, Japan) in February, 2008. Three blocks of clear ice (each size was approximately 10.0 × 5.0 × 4.0 cm) were used in this study. An ice floe of the Sea of Okhotsk was collected on the shore of Monbetsu City, Hokkaido, Japan (44° 31′ N, 143° 39′ E) in March, 2008. Clear ice block (size was approximately 10.0 × 10.0 × 15.0 cm) was cut off from the entire ice floe was used in this study. Both ice samples were maintained at -20°C for about 2 years until melting. We could not date these ice floes, but considering the environments of the sampling areas, both appeared to be first-year ice. Prior to the analyses, the ice samples were carefully rinsed with cold (-20°C) 99% ethanol for 2 min and then rinsed 3 times with deionized distilled water to remove contaminants from the surface layer of the ice [19]. There are diffenrent types of biota in sea ice, such as bottom community, surface community, and internal community [20], but we used only the clear core part of the ice sample in further examination to study internal community. After cleaning the surface, the ice cores were placed in sterilized metal trays and allowed to thaw at room temperture. As a control, distilled water (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) was frozen and sampled using the same procedure described above.

## 2.2. DNA Extraction

The melted ice was filtered through 3.0-μm pore-size nylon membrane filters (Japan Millipore Inc., Tokyo, Japan) to remove coarse particles. The remaining liquid was sequentially filtered through 0.22-μm pore-size filters (Japan Millipore) to collect small particles, including bacterial cells. Filtered 0.22-μm membranes were washed by shaking with 2 mL of phosphate-buffered saline with 0.05% Tween 80 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 min. The suspension liquids were centrifuged at 22,000 × *g* for 5 min and the supernatants were removed. The pellets were used for DNA extraction.

Bacterial DNA in melted ice samples was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the instructions for extraction of DNA from general bacteria. To extract DNA thoroughly from bacteria with strong cell walls, such as *Mycobacteria*, the residual pellets were resuspended in 25 μL of bacteriolysis buffer from EXTRAGEN MB mycobacterial DNA extraction kit (Tosoh Corporation, Tokyo, Japan). DNA extracted from the same ice floe was combined and the DNA concentration was measured using a Gene Quant pro RNA/DNA calculator (Amersham Pharmacia Biotech, Cambridge, UK), with absorbance at A260.

## 2.3. Cloning and Sequencing of 16S rDNA

Bacterial 16S rRNA genes were amplified by PCR with primers for universal bacterial 16S rRNA gene forward 5′-GAGAGTTTGATCMTGGCTCAG-3′ [21] and reverse 5′-ATTACCGCGGCTGCTGGCAC-3′ [22], yielding 519 bp PCR product. After electrophoresis of the PCR products, we eluted only amplified 16S rDNA from the agarose using the Wizard SV Gel and PCR Clean-Up System (Promega KK, Tokyo, Japan). Cloning was performed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Transformants were screened on LB agar (Invitrogen) containing 50 μg/mL of kanamycin (Sigma-Aldrich, St. Louis, MO, USA). One-hundred-twenty transformant colonies from each clone of the Antarctic and Okhotsk sea ice and 60 colonies from the clone of control ice were selected and preserved in glycerol stocks at -80°C. We conducted colony-direct PCR for 16S rRNA genes with these 300 transformants using the M13 primers provided in the TOPO TA cloning kit. The amplicons were purified using a High Pure PCR Cleanup Micro Kit (Roche Diagnostics, IN, USA) and directly sequenced at Hokkaido System Science Co. Ltd. (Hokkaido, Japan).

The nucleotide sequences were compared to reported sequences by BLASTN search version 2.2.18 (http://blast.ddbj.nig.ac.jp/). Closest relatives that showed >98% identity with sequences reported in the database were assigned to our clones at the genus level. 16S rDNA sequence-based phylogenetic trees were constructed using MEGA software version 5.2 using evolutionary distances (Jukes-Cantor model) and the neighbor-joining method with 1,000 bootstrap replicates [23]. The 16S rDNA sequences in this study were assigned by GenBank as following accession numbers; LC003653 - LC003873.

## 2.4. PCR for Antimicrobial and Heavy Metal Resistance Genes

Detection of antimicrobial and heavy metal resistance genes was performed by PCR. These genes, if found at all, were expected to be present in very low concentrations in melted ice fluid. To detect low-concentration DNA, whole-genome amplification was conducted using an illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Japan, Tokyo, Japan) according to manufacturer's instruction. We conducted PCR for the following antimicrobial resistance genes: $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX-M}$ $bla_{AmpC}$, *aac(3)-Ia*, *ant(2″)-Ia*, *ant(3″)-Ia*, *aph(3′)-Ia*, *aph(3′)-VIa*, *aph(3″)-Ib*, *aph(6)-Id*, *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*M, *tet*S, *erm*A, *erm*B, *mef*A/E, *cat*I, *cat*II, *cat*III, *cat*IV, *qnr*A, *qnr*B, *qnr*S, *van*A, and *van*B [24-33]. We also screened heavy metal tolerance genes (*mer*A, *mer*B, *tcr*B, and *cad*A) by PCR [34,35,36]. Amplified PCR products were purified and directly sequenced.

## 3. Results and Discussion

We obtained 257 mL of melted ice fluid from the Antarctic sea ice, 400 mL from the Okhotsk sea ice, and 160 mL from the control ice. The combined DNA concentration of each sample was as follows: Antarctic sea ice, 380.86 pg/μL; Okhotsk sea ice, 247.01 pg/μL; control ice, 0.20 pg/μL. Compared to the control, large amounts of DNA were extracted from the 2 sea ice samples; thus, the extraction methods we used appeared to be appropriate.

Bacterial 16S rRNA gene libraries from each sample were constructed successfully. We obtained 280 clones including 120 clones from the Antarctic sea ice, 120 from the Okhotsk sea ice, and 60 from the control.

We identified these clones at the genus level by comparing them with sequences on the BLASTN database. Among genera detected in the control, 57 were found only in this sample and were considered to be contaminants that were originally present in the control. Genus *Sphingomonas*, *Novosphingobium*, and unidentified *Betaproteobacteria* were found in both the Antarctic sea ice and the control. They were suspected to be a contaminant introduced during the handling process, thus we excluded them from further consideration. In addition, 3 clones of the Antarctic sea ice and 8 of the Okhotsk sea ice were also excluded from analysis because they did not amplified sufficiently using M13 primers. The remaining 221 strains, 109 from the Antarctic sea ice and 112 from the Okhotsk sea ice, were used in study.

The distribution of 16S rDNA clones of the 2 sea ice is shown in Table 1 and Figure 1. At the class level, class *Gammaproteobacteria* was the most frequent group (n = 78, 71.6%), followed by *Alphaproteobacteria* (n = 29, 26.6%) in the Antarctic sea ice (Figure 1.). The abundance of *Gammaproteobacteria* and *Alphaproteobacteria* in our sea ice was consistent with previous studies of sea ice derived from other frozen oceans [8,13,14]. At the genus level, genus *Psychrobacter* was dominant (n = 41), followed by *Halomonas* (n = 10) and *Pseudoalteromonas* (n = 5) in class *Gammaproteobacteria* (Table 1). This result is distinct from those of previous studies that reported *Colwellia*, *Glaciecola*, and *Marinobacter* were the major genera of *Gammaproteobacteria* in bacterial communities of sea ice in the Southern Ocean [8,14]. Among *Alphaproteobacteria*, *Roseobacter* (n = 8) and *Sulfitobacter* (n = 13) of family *Rhodobacteraceae* were common, consistent with a previous report (Table 1) [8]. The *Cytophaga-Flavobacterium-Bacteroidetes* group, which was reported by others to be one of the major bacterial groups in sea ice [14,16], was not detected from our Antarctic sea ice. The reason for such different observations among studies were unclear, but different sampling sites (Dumont d'Urville Sea, the Australian side of Antarctica) may provide an explanation. Most previous reports analyzed bacterial communities in Antarctic sea ice or seawater which was derived from the Indian or American side of Antarctica [8,10,12,14]. Delille *et al.* [7] reported a human influence on the marine bacterial community of the Dumont d'Urville Sea, revealed by enteric bacteria in seawater, but no comparative study on biodiversity of microorganisms in this area has been reported. An abundance of *Gammaproteobacteria* species which were differed from those of previous reports and the absence of *Bacteroidetes* were distinctive features in our Antarctic sample. These data provide new information about the bacterial community in this area that can further elucidate the characteristics of bacterial ecosystems in the Antarctic region.

In the Okhotsk sea ice, class *Gammaproteobacteria* was also dominant (n = 51, 45.5%); the second most frequent group was phylum *Bacteroidetes* (n = 43, 38.4%) (Figure 1). Classification at the genus level revealed that *Polaribacter* (*Bacteroidetes*) was the most frequent genus in Okhotsk sea ice (n = 43) (Table 1). Among *Gammaproteobacteria*, genus *Colwellia* was the most common (n = 27), followed by *Psychromonas* (n = 12) and *Glaciecola* (n = 10). These results are in agreement with those of previous reports on the bacterial communities in Arctic sea ice [3,14,15]. Given that the Sea of Okhotsk is located in the sub-Arctic rather than the Arctic region, these results suggest that sea ice bacterial communities in cold seas of the Northern Hemisphere may share similar characteristics. To the best of our knowledge, this is the first study to analyze bacterial taxa in ice floes of the Sea of Okhotsk area.

Comparing the 2 sea ice samples, *Gammaproteobacteria* and *Alphaproteobacteria* were more frequently detected in the Antarctic sea ice than in the Okhotsk sea ice (71.6% vs. 26.6% and 45.5% vs. 38.4%, respectively) (Figure 1). Phylum *Bacteroidetes* was the second-largest group in the Okhotsk sea ice (n = 42, 39.3%), whereas no *Bacteroidetes* strains were detected in the Antarctic sea ice. Class *Epsilonproteobacteria* and *Planctomycetacia* were only sparsely detected. Other bacterial groups, especially Gram-positive groups such as phyla *Firmicutes* and *Actinobacteria*, were not detected in both samples. At the phylum or class level, our results are consistent with those of other researchers who analyzed sea ice bacterial communities using culture-dependent or culture-independent assays, except for the absence of *Bacteroidetes* in our Antarctic sea ice [8,13,14]. These results suggest that bacterial communities in sea ice are similar to each other as a whole. However, at the genus level, only *Sulfitobacter* (n = 13 in the Antarctic sea ice and n = 1 in the Okhotsk sea ice) was detected in both ice samples (Table 1). The other genera were distinctly different between 2 sea ice samples as shown in phylogenetic trees (Supporting information). Previous review article on bacterial diversity in sea ice described that there were no cosmopolitan bacterial species which could be isolated from both the Arctic and the Antarctic sea ice [16]. Our results were consistent with this idea and confirmed again that microbial population in sea ice are endemic.

Although we attempted to detect antimicrobial and heavy metal resistance genes, we did not detect any genes at all. Okhotsk ice sample was positive for Hg-resistance gene *merA* but its band was very faint by electrophoresis of PCR product, thus we considered that *merA* gene was not present or, if existed, was very low copy number in the sample. Segawa *et al.* [11] reported a low but detectable presence of antimicrobial resistance genes in Antarctic surface snow and suggested that anthropogenic factors may have led to bacteria possessing these genes. Given that the ice samples used in our study were also derived from remote areas in which human activity is limited, the absence of antimicrobial or heavy metal resistance genes was plausible.

**Table 1. Identified bacteria in sea ice by 16S rRNA gene sequencing of transformants.**

| Phylum | Class | Order | Family | Genus | Antarctic sea ice | Okhotsk sea ice | Total |
|---|---|---|---|---|---|---|---|
| *Proteobacteria* | *Alphaproteobacteria* | *Rhodobacterales* | *Rhodobacteraceae* | *Roseobacter* | 8 [a] | 0 | 8 |
| | | | | *Sulfitobacter* | 13 | 1 | 14 |
| | | | | *Thioclava* | 1 | 0 | 1 |
| | | | | *Octadecabacter* | 0 | 5 | 5 |
| | | *Rhodospirillales* | *Rhodospirillaceae* | *Thalassospira* | 4 | 0 | 4 |
| | | | *Acetobacteraceae* | *Roseomonas* | 0 | 1 | 1 |
| | | | *Erythrobacteraceae* | *Alterierythrobacter* | 2 | 0 | 2 |
| | | | | *Erythrobacter* | 1 | 0 | 1 |
| | | Unclassified *Alphaproteobacteria* | | *Pelagibacter ubique* | 0 | 1 | 1 |
| | | | | *Alphaproteobacterium* SCGC AAA298-C11 | 0 | 1 | 1 |
| | | Unidentified | | | 0 | 3 | 3 |
| | *Gammaproteobacteria* | *Alteromonadales* | *Alteromonadaceae* | *Marinobacter* | 1 | 0 | 1 |
| | | | | *Glaciecola* | 0 | 10 | 10 |
| | | | *Pseudoalteromonadaceae* | *Pseudoalteromonas* | 5 | 0 | 5 |
| | | | *Colwelliaceae* | *Colwellia* | 0 | 27 | 27 |
| | | | *Psychromonadaceae* | *Psychromonas* | 0 | 12 | 12 |
| | | *Enterobacteriales* | *Enterobacteriaceae* | *Klebsiella* | 1 | 0 | 1 |
| | | | | *Pantoea* | 1 | 0 | 1 |
| | | *Pseudomonadales* | *Moraxellaceae* | *Acinetobacter* | 2 | 0 | 2 |
| | | | | *Psychrobacter* | 41 | 0 | 41 |
| | | | *Pseudomonadaceae* | *Pseudomonas* | 2 | 0 | 2 |
| | | *Oceanospirillales* | *Halomonadaceae* | *Halomonas* | 10 | 0 | 10 |
| | | | | *Chromohalobacter* | 2 | 0 | 2 |
| | | *Thiotrichales* | *Piscirickettsiaceae* | *Methylophaga* | 4 | 0 | 4 |
| | | Unclassified *Gammaproteobacteria* | | | 0 | 1 | 1 |
| | | Unidentified | | | 9 | 4 | 13 |
| | *Deltaproteobacteria* | Unidentified | | | 2 | 0 | 2 |
| | *Epsilonproteobacteria* | Unidentified | | | 0 | 1 | 1 |
| *Bacteroidetes* | *Flavobacteria* | *Flavobacteriales* | *Flavobacteriaceae* | *Polaribacter* | 0 | 43 | 43 |
| *Planctomycetes* | *Planctomycetacia* | *Planctomycetaceae* | *Planctomycetaceae* | Unidentified | 0 | 2 | 2 |
| Total | | | | | 109 | 112 | 221 |

[a] The numbers indicate acquired 16S rRNA gene clone strain.

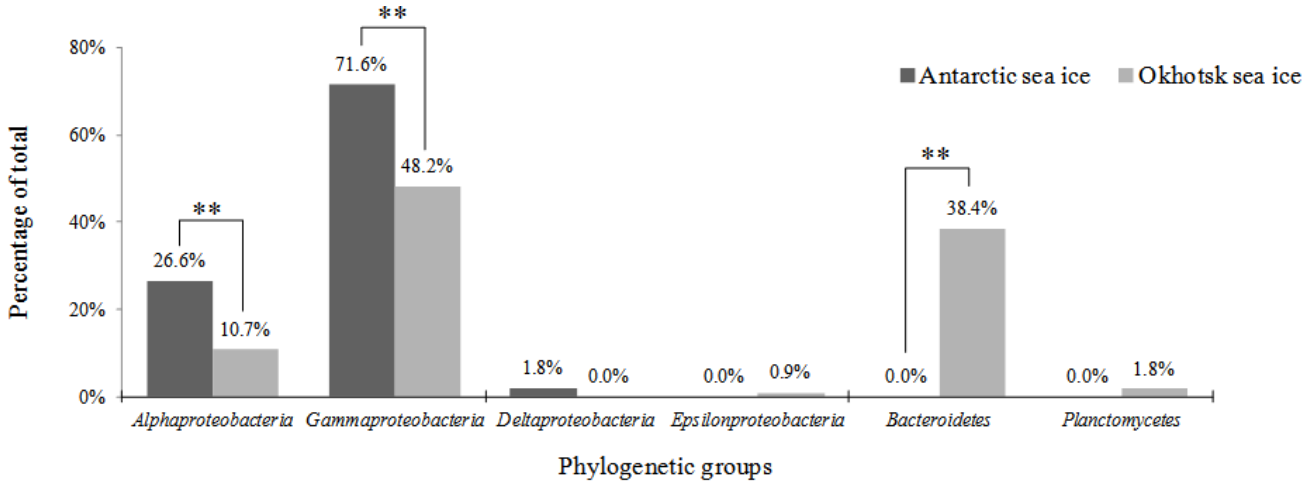


**Figure 1. Phylogenetic distribution of 16S rDNA clones of Antarctic sea and Okhotsk sea ice floe sample. Two asterisks indicate $p < 0.01$ by chi-squared test**

# 4. Conclusion

We extracted bacterial DNA from sea ice samples of the Southern Ocean and the Sea of Okhotsk for analysis and comparison of the bacterial diversity. Bacterial communities in both sea ice samples resembled one another at the phylum level but differed at the genus level. Although our sample size was not sufficient for us to draw broad conclusions, our findings indicated that characteristics of bacterial communities in sea ice are intrinsic to the sampling area. We did not find cosmopolitan bacterial species which could be detected in either of our samples, but at the genetic level, our results suggested that specific genes can distribute globally. Further study using a greater number of samples is needed to determine whether the global spread of bacteria or bacterial genes can occur in the environments.

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# Supporting Information

Phylogenetic trees showing relationship of 16S rDNA sequences from ice floes. Antarctic clones were written as "Ant" and Okhotsk clones were as "Okh". Numbers at branch nodes are bootstrap values (only values of more than 60% are shown). Scale bars indicate Jukes-Cantor distances. (A) *Alphaproteobacteria*; (B) *Gammaproteobacteria*

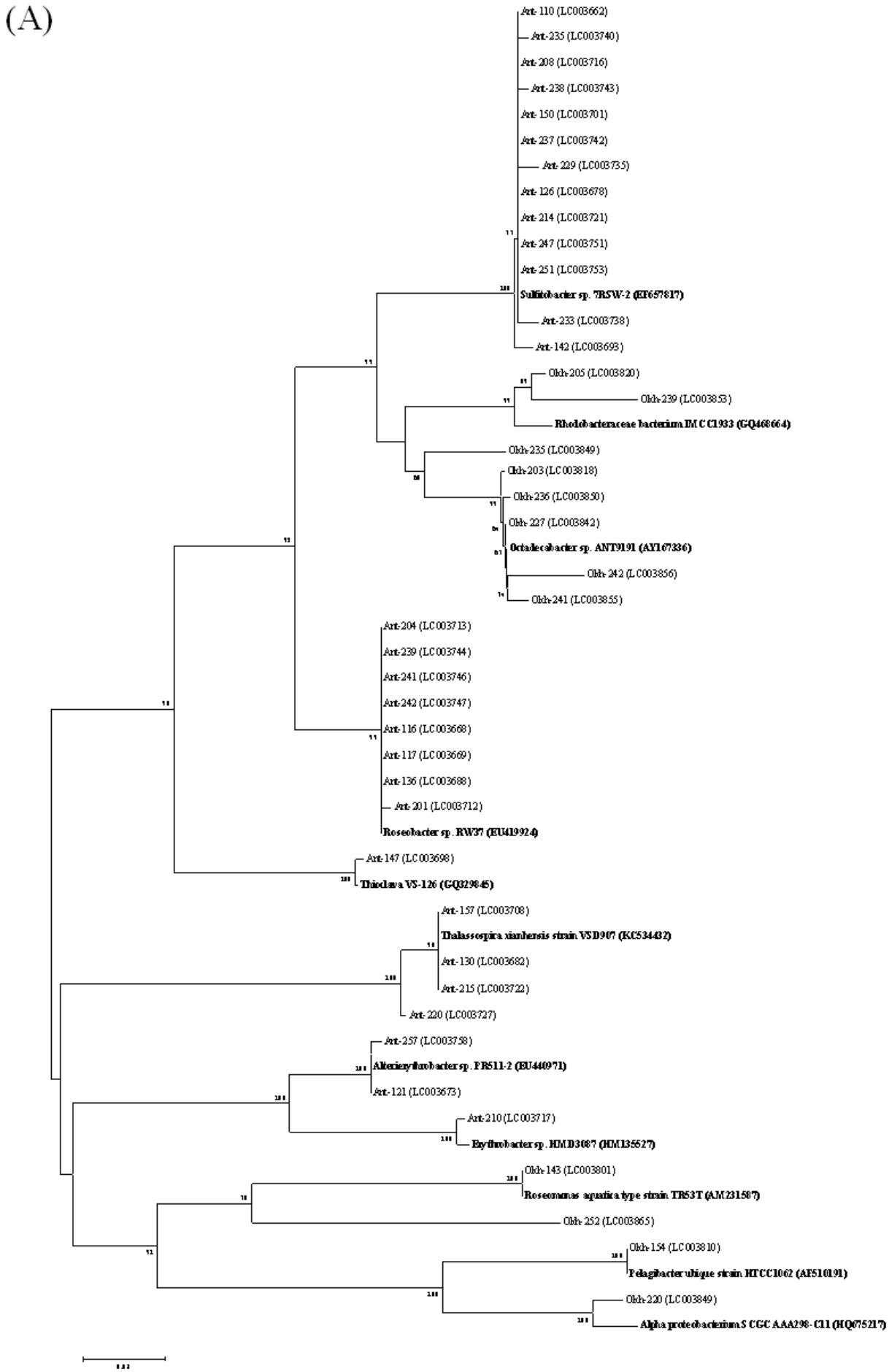
(A)
Art-110 (LC003662)
Art-235 (LC003740)
Art-208 (LC003716)
Art-238 (LC003743)
Art-150 (LC003701)
Art-237 (LC003742)
Art-229 (LC003735)
Art-126 (LC003678)
Art-214 (LC003721)
Art-247 (LC003751)
Art-251 (LC003753)
Sulfitobacter sp. 7RSW-2 (EF657817)
Art-233 (LC003738)
Art-142 (LC003693)
Okh-205 (LC003820)
Okh-239 (LC003853)
Rhodobacteraceae bacterium IMCC1933 (GQ468664)
Okh-235 (LC003849)
Okh-203 (LC003818)
Okh-236 (LC003850)
Okh-227 (LC003842)
Octadecabacter sp. ANT9191 (AY167336)
Okh-242 (LC003856)
Okh-241 (LC003855)
Art-204 (LC003713)
Art-239 (LC003744)
Art-241 (LC003746)
Art-242 (LC003747)
Art-116 (LC003668)
Art-117 (LC003669)
Art-136 (LC003688)
Art-201 (LC003712)
Roseobacter sp. RW37 (EU419924)
Art-147 (LC003698)
Thioclava VS-126 (GQ329845)
Art-157 (LC003708)
Thalassospira xianhensis strain VSD907 (KC534432)
Art-130 (LC003682)
Art-215 (LC003722)
Art-220 (LC003727)
Art-257 (LC003758)
Alterierythrobacter sp. PR511-2 (EU440971)
Art-121 (LC003673)
Art-210 (LC003717)
Erythrobacter sp. HMD3087 (HM135527)
Okh-143 (LC003801)
Roseomonas aquatica type strain TR53T (AM231587)
Okh-252 (LC003865)
Okh-154 (LC003810)
Pelagibacter ubique strain HTCC1062 (AF510191)
Okh-220 (LC003849)
Alpha proteobacterium SCGC AAA298-C11 (HQ675217)

**Figure (A).**

(B)

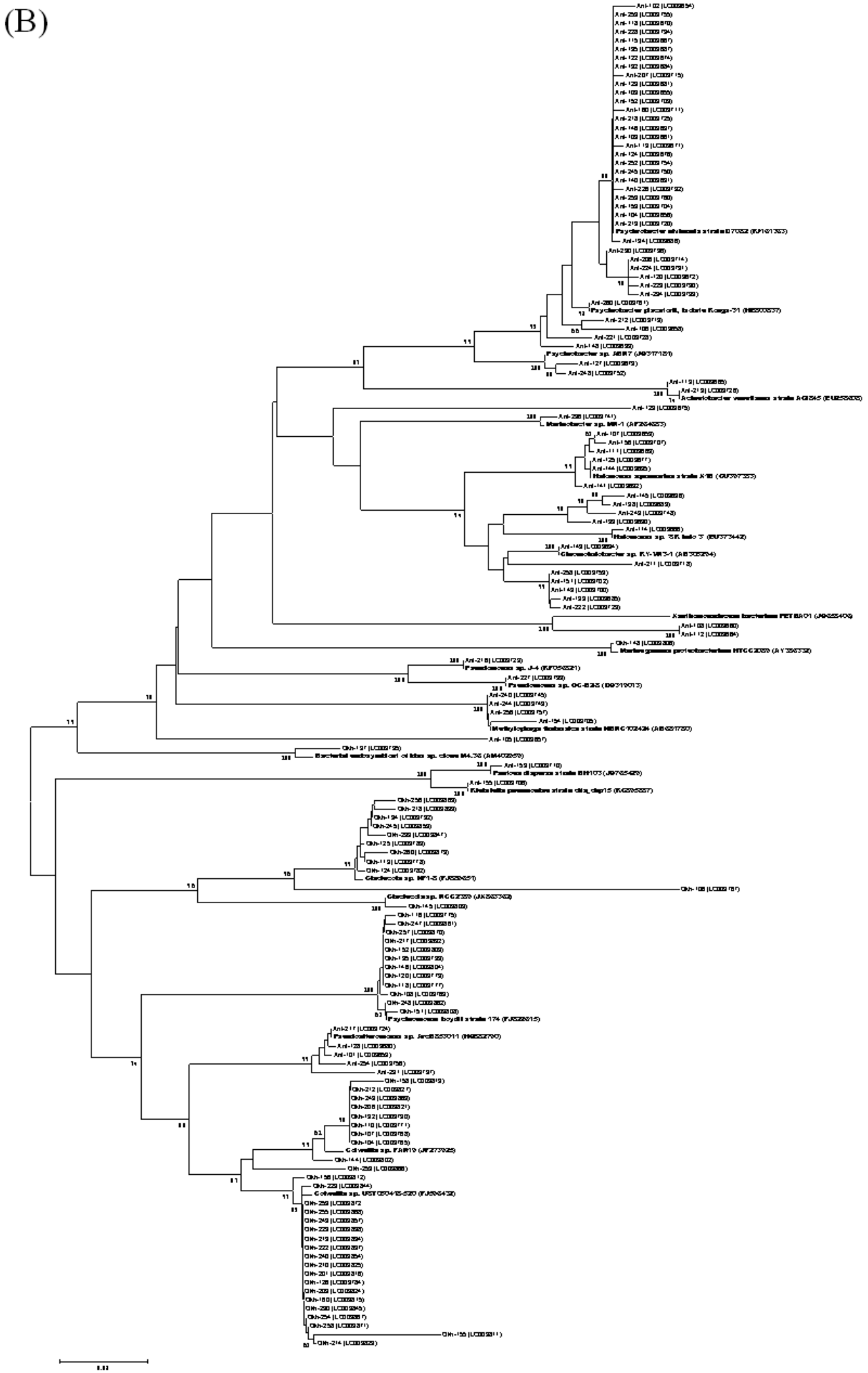

**Figure (B).**