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Title	Tempo-spatial patterns of bacterial community composition in the western North Pacific Ocean
Author(s)	Kataoka, Takafumi; Hodoki, Yoshikuni; Suzuki, Koji et al.
Citation	Journal of Marine Systems, 77(1-2), 197-207 https://doi.org/10.1016/j.jmarsys.2008.12.006
Issue Date	2009-04
Doc URL	https://hdl.handle.net/2115/39306
Type	journal article
File Information	JMS77-1-2_p197-207.pdf



**Tempo-spatial patterns of bacterial community composition
in the western North Pacific Ocean**

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ABSTRACT: In the western North Pacific, where subarctic Oyashio waters encounter subtropical Kuroshio waters, phylotype composition of heterotrophic bacteria was estimated by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified bacterial 16S ribosomal DNA. Total bacterial abundance was also measured by flow cytometry. The study area was divided into four water masses: coastal, Oyashio, Kuroshio, and the Kuroshio-Oyashio transition. Abundances of heterotrophic bacteria in the Oyashio, Kuroshio, and Kuroshio-Oyashio transition regions ranged from 0.2×10^5 cells mL⁻¹ to 1.4×10^5 cells mL⁻¹ and were not significantly different, except in the Oyashio region during September. Bacterial compositions were distinct in each water mass. Furthermore, phylotype distributions differed between surface and subsurface waters in the Kuroshio-Oyashio transition region. Out of 61 DGGE bands obtained, 41 were successfully identified as 31 phylotypes: 22 *Gammaproteobacteria*, 4 *Alphaproteobacteria*, 2 unknown bacteria, 2 cyanobacteria, and 1 plastid. Although the *Gammaproteobacteria* OM60 clade was eurytopic in the study area, *Psychrobacter glacincola* and the uncultured *Gammaproteobacteria* SAR92 clade were often observed in the Oyashio region. Overall, our results indicated that *Gammaproteobacteria* were predominant in the bacterial community, which was influenced by the hydrographic properties of each water mass in the study area.

KEY WORDS: Marine bacteria · Phylotype · DGGE · Oyashio · Kuroshio · Pacific Ocean

1. INTRODUCTION

Since the discovery of the microbial food chain in the 1980s (e.g., Azam et al., 1983), the role of heterotrophic bacteria in the carbon dynamics of marine ecosystems has been investigated. Concomitantly, development of genetic fingerprinting techniques has enabled the identification of marine heterotrophic bacteria phylotypes (e.g., Giovannoni et al., 1990; Rappé et al., 2000; Bano and Hollibaugh, 2002). Giovannoni and Rappé (2000) pointed out that marine bacterial communities were probably constructed of cosmopolitan species. However, some studies showed that microbial community compositions differed among sea areas (Suzuki et al., 2001a) or in the vicinity of water mass fronts (Pinhassi et al., 2003). Recently, it has been reported that bacterial distribution was influenced by both abiotic and biotic factors of the marine ecosystems (Fuhrman et al., 2006). These studies indicate that composition of heterotrophic bacteria communities can vary significantly among sea areas.

Bacterial community composition has, however, only been investigated in limited areas, such as the North Sea (Eilers et al., 2000; Eilers et al., 2001), Arctic Ocean (Bano and Hollibaugh, 2002), central Pacific Ocean (Schmidt et al., 1991), eastern Pacific Ocean (Suzuki et al., 2001b), and the Antarctic Ocean (Brinkmeyer et al., 2003). The dominant groups identified in those studies were mainly *Alpha*- and/or *Gammaproteobacteria* (e.g., Rappé et al., 1997; Suzuki et al., 1997). According to the more recent study of Fuhrman et al. (2008), basin-scale geographic patterns of bacterial distribution diversity were primarily regulated by latitude and temperature. On the other hand, on a smaller geographical scale, temperature and phytoplankton productivity in terms of chlorophyll *a* concentration have been reported to control

bacterial phylotype distribution (Pinhassi et al., 2003; Selje et al., 2004), because these two parameters could influence microbial metabolic processes. Different distributions of dominant bacterial phylotypes have been reported among various environments. For example, even in closely related phylotypes in the SAR11 cluster, which is one of the most common phylotypes within *Alphaproteobacteria* (Giovannoni and Rappé, 2000), phylotypes have exhibited different marine habitat distributions (Brown and Fuhrman, 2005). Selje et al. (2004) reported that the RCA cluster of SAR11 generally appeared in cold waters. Such phylotype-specific appearance indicates that each phylotype may possess an ecological niche. The results of these previous reports suggest the need for further studies to understand bacterial ecology and its role in marine biogeochemistry, especially in those regions receiving less emphasis to date.

In the western North Pacific, Kuroshio exists as a western boundary current in the subtropical region and Oyashio is a western boundary current of the Western Subarctic Gyre in the North Pacific. Water masses mixing in the Kuroshio-Oyashio transition region results in complex frontal structures (Yasuda, 2003). In Oyashio waters, diatom blooms occur every spring, resulting in a high production of zooplankton and other organisms in spring and summer (Saito et al., 2002). However, little information is available regarding the microbial loop in the two currents. Nagata et al. (2001) proposed that bacterial growth in the subarctic Pacific was regulated by a combination of temperature and the supply of dissolved organic carbon (DOC). An increase in DOC can be expected during phytoplankton bloom declines because of physiological stress and/or high grazing pressure (Carlson et al., 1994). This indicates that bacteria can play a key role in ecosystems and biogeochemical cycles at the conclusion of diatom blooms. Furthermore, picophytoplankton ($< 2 \mu\text{m}$ in size) was

predominant in the phytoplankton community of Oyashio from summer to winter (Liu et al., 2004), indicating the development of a microbial loop. It has also been suggested that a microbial food chain could develop throughout the year, except during the spring bloom period (Shinada et al., 2001). Recently, Hayakawa et al. (2008) showed that phytoplankton cell death was significant for their loss process in the western North Pacific during late summer and could support microbial food webs by supplying DOC derived from the dead cells. Therefore, in order to examine whether or not the bacterial community structure can change temporally and spatially with hydrographic properties, we compared bacterial community compositions in the Oyashio and Kuroshio-Oyashio transition regions of the western North Pacific over five seasons from 2003 to 2004 using denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified bacterial 16S ribosomal DNA. In addition, tempo-spatial changes in the cell density of heterotrophic bacteria were also investigated by flow cytometry.

2. MATERIALS AND METHODS

2.1 Study area and sampling

Sampling was carried out along a monitoring line, “*A-line*”, during July 14–21 and September 2–21 in 2003 and February 10–23, May 11–25, and July 26 to August 9 in 2004 (Fig. 1), on board the FR/V *Wakataka Maru* and *Hokko Maru* (Fisheries Research Agency of Japan). Seawater samples were collected from 10 m depths with Niskin bottles attached to a conductivity-temperature-depth profiler (CTD). In addition, water sampling was also conducted at 50 and 100 m depths at Station A09 in July 2004 to examine vertical changes in bacterial composition. Water samples

were divided into acid-cleaned polycarbonate bottles, and 1 L of water sample was filtered through 0.2 μm pore size polycarbonate Nuclepore® filters (Whatman) with gentle vacuum pressure (<150 mmHg) within 60 min after sampling. The filters were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Hydrographic data (seawater temperature, salinity, and nutrients) were obtained from the cruise database (FRA, 2007). Station A01 was coastal and the other stations were grouped into three water masses: Oyashio, $<5^{\circ}\text{C}$ and <33.6 salinity at 100 m depth (Kawai, 1972); Kuroshio, $>14^{\circ}\text{C}$ at 200 m depth (Kawai, 1969); and the Kuroshio-Oyashio transition region. Stations in warm-core rings, which were separated from the Kuroshio Extension current and appeared in the Kuroshio-Oyashio transition region (Fig. 1), were classified into the Kuroshio-Oyashio transition region in the present study.

2.2 *Cell density of heterotrophic bacteria*

Water samples (2 mL) were fixed with paraformaldehyde (0.2% (w/v) in final concentration), flash-frozen in liquid nitrogen, and stored at -80°C until analysis. The samples were thawed and filtered through a 35 μm nylon-mesh-capped Falcon cell strainer (Becton-Dickinson) to remove larger cells. Bacterial cells were stained using a working solution of SYBR Green I (Molecular Probes®, diluted 1/1,000 of the commercial solution). Certain subsamples were mixed with Flow-count fluorospheres (Beckman Coulter) and analyzed using an EPICS flow cytometer (XL ADC system, Beckman Coulter). Heterotrophic bacterial cells were selectively counted using EXPO32 software (Beckman Coulter). Cell density of cyanobacteria was not determined in this study, since they were basically excluded from our research target.

The details of the procedure were described in Suzuki et al. (2005).

2.3 *DNA extraction*

DNA extraction was performed following Katano et al. (2001). Briefly, filters were homogenized and incubated with a lysis solution (30 μ L of 10% (v:v) sodium dodecyl sulfate, 500 μ L of Tris-EDTA (TE) buffer and 3 μ L of 10 mg mL⁻¹ proteinase K) at 37°C for 1 h. Cell lysates were frozen and thawed three times. To remove polysaccharides, a mixture of cetyltrimethylammonium bromide (CTAB, Wako) and 0.7 M NaCl solution was added and incubated at 65°C for 10 min. Polysaccharides and proteins were removed with chloroform-isoamyl alcohol (24:1) and then eliminated with phenol-chloroform-isoamyl alcohol (25:24:1). DNA precipitated with isopropanol was washed with 70% ethanol, and resuspended in 30 μ L TE buffer.

2.4 *PCR for DGGE*

The 550 bp of bacterial 16S rDNA genes were PCR-amplified using a universal primer complementary to position 907–926 of *Escherichia coli* numbering (5'-CCGTCAATTCMTTTGAGTTT-3') as a reverse primer and primer complementary to position 341–357 (5'-CCTACGGGAGGCAGCAG-3') as a forward primer. A GC clamp (5'-CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCGCCCCG-3') was attached to the 5' end of the forward primer (Muyzer et al., 1993; Muyzer and Smalla, 1998). PCR mixtures were prepared in a total volume of 100 μ L containing 1 $\times$ Ex TaqTM Buffer (Takara Bio Inc.), MgCl₂ (1.8 mM in September 2003 samples and February 2004; 2.5 mM in July 2003 and May and July 2004 samples), 0.5 mM dNTP,

0.5 μ M of each primer, 2.5 units of Taq DNA polymerase (Ex TaqTM, Takara Bio), and 1 μ L of template DNA. PCR conditions followed those of Muyzer et al. (1993). PCR amplification was performed with 35 cycles using an Eppendorf Mastercycler Personal. The concentrations of PCR products were estimated by agarose gel electrophoresis.

PCR products were loaded on 16 $\times$ 16 cm, 1 mm thick, 8% polyacrylamide (acrylamide:bis = 37.5:1) gels containing a denaturing gradient of 10–50% from top to bottom (7 M urea and 40% formamide were considered to be 100% denaturant). In order to obtain an appropriate resolution of the DGGE bands, the denaturing gradient range was modified as follows: 10–60% for September 2003 and 20–60% for February and May 2004. Electrophoresis was performed with a hot bath DGGE unit (Bio-Rad Laboratories) using 0.5 $\times$ TAE running buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8.0) at 60°C for 4 h at 200 V. Gels were stained for 30 min in ethidium bromide (0.5 μ g mL⁻¹, Bio-Rad Laboratories), destained for 20 min in distilled water, and photographed with a UV transilluminator (UVP, LLC Upland).

2.5.1 *Analysis of DGGE patterns*

DGGE patterns were compared between stations using a binary matrix where 1 and 0 were given when a band was present and absent, respectively (Schäfer and Muyzer, 2001). Band-pattern dissimilarity between pairwise stations was calculated by the Jaccard index as

$$S_j = N_{AB} / (N_A + N_B - N_{AB})$$

where N_{AB} is the number of bands common to both DGGE lanes A and B, and N_A and N_B are the total numbers of bands in lanes A and B, respectively. Unweighted pairwise grouping with mathematical averages (UPGMA) was employed for clustering stations using software R version 1.9.0.

2.5.2 *Statistical analysis*

Redundancy analysis (RDA) was used to examine the relationship between the binary matrix of DGGE banding profiles and the *in situ* environmental variables, namely, salinity, temperature and concentrations of chlorophyll *a*, nitrite, nitrate and phosphate in the each cruise (software R version 2.1, vegan package: Oksanen et al., 2008). RDA is a direct extension of multiple regression for the modeling of multivariate response data. It combines ordination and regression into one technique and attempts to fit simultaneously the relationship between two or more response variables and several predictor variables (Legendre and Legendre, 1998). Prior to RDA, data at Station A01 in July 2003 was removed, because environmental conditions at the station differed from the other stations. In addition, since a preliminary RDA showed high multicollinearity among variables, as indicated by variance inflation factors (VIF) > 20, nitrite and phosphate were eliminated from subsequent analyses because of their high VIF. Similarly, nitrate concentration was removed from the datasets of September 2003 and February 2004, and salinity was eliminated from those of July and September 2003 and February 2004. To test the significance of constraints among the constructed RDA models, a non-parametric multivariate ANOVA test (Ramette, 2007) was conducted.

2.6 *Sequencing and phylogenetic analysis*

To obtain sequence information from the DGGE bands, all visible bands were excised using a clean razor blade. The excised bands were washed twice using 2 mL of distilled water and were frozen and held at -20°C until re-amplification with the PCR system. About 1 mm^3 of each band was directly re-amplified as a template. Again, PCR conditions followed Muyzer et al. (1993) with some modifications including initial denaturation of the template at 95°C for 5 min, followed by 20 cycles of denaturation (1 min at 94°C), annealing [1 min at $65\text{--}55^{\circ}\text{C}$ (touch down $-1^{\circ}\text{C cycle}^{-1}$)], and extension (3 min at 72°C), followed by 7 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), extension (3 min 72°C), and a final extension (7 min at 72°C). After re-amplification, bands were examined with DGGE to confirm band position and non-contamination. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN). Clean amplified products were sequenced on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) with both 907–926 and 341–357 primers, but without the GC-clamp (Muyzer et al., 1993) using a BigDye® v1.0 Terminator cycle sequencing kit (Applied Biosystems). Total volume of the reaction mixture was 10 μL . Sequences were compared to known sequences using a basic local alignment search tool, BLAST (<http://www.ddbj.nig.ac.jp/search/blast-j.html>). Obtained sequences were assigned to each phylogenetic group based on the most similar sequence in the DNA Data Bank of Japan (DDBJ) database (<http://www.ddbj.nig.ac.jp>). The nucleotide sequences of the 16S rDNA gene obtained in this study have been deposited in the DDBJ nucleotide sequence database under accession numbers AB305050–AB305058, AB364983–AB365003, and AB366609.

3. RESULTS

Sampling stations used during each cruise were classified into the coastal, Oyashio, Kuroshio-Oyashio transition, and Kuroshio regions (Table 1). The *in situ* temperatures tended to be lower in Oyashio than those in Kuroshio in each season. In July 2003 and 2004, the seasonal thermocline existed clearly at approximately 20 m depth (data not shown). In May, the highest chlorophyll *a* concentration was found in the Oyashio region ($6.57 \mu\text{g L}^{-1}$ at Station A05) and a high chlorophyll *a* concentration was also observed in the transition area (concentrations were $3.40 \mu\text{g L}^{-1}$ and $1.48 \mu\text{g L}^{-1}$ at Stations A15 and A17, respectively). High concentrations of nutrients [phosphate (PO_4^{3-}) and nitrite plus nitrate ($\text{NO}_2^- + \text{NO}_3^-$)] were detected in the Oyashio region during each cruise (Table 1). Cell densities of heterotrophic bacteria in surface waters ranged from $0.2 \times 10^5 \text{ cells mL}^{-1}$ to $1.4 \times 10^5 \text{ cells mL}^{-1}$ in three of the water masses (Fig. 2). The highest values of heterotrophic bacteria abundance were observed in the Oyashio samples in September 2003 and the lowest in July 2003, close to that of February 2004. However, abundances of heterotrophic bacteria were not significantly different among the three water masses ($p > 0.05$; Kruskal-Wallis test) except between the Oyashio and transition regions during September.

The PCR-DGGE analyses were conducted on samples collected from 10 m depth at 10 stations in July 2003, 6 stations in September 2003, 4 stations in February 2004, 6 stations in May 2004, and 9 stations in July 2004 (Fig. 3 i–v), and also for 50 and 100 m water samples collected at Station A09 in July 2004 (Fig. 3 vi). Fifteen visible bands were obtained in July 2003, 11 in September 2003, 6 in February 2004, 17 in May 2004, and 12 in July 2004. Out of the 61 bands, phylotype identification was

successful for 41 bands (67.2% of the total). DGGE-bands for which sequences were successfully obtained were named “ALN_xx,” referring to a specific 16S rDNA sequence obtained from the “A-line.” Overall, the phylotypes identified composed of 4 *Alphaproteobacteria*, 22 *Gammaproteobacteria*, 2 unknown bacteria, 2 cyanobacteria and 1 organelle plastid of an eukaryote (Table 2). Out of these bacteria, 8 were 100% homologous with phylotypes deposited at DDBJ, while, ALN_gm 04, ALN_gm 08, ALN_gm 13, ALN_gm 14, ALN_gm 23, and ALN_gm 24 exhibited relatively low homologies with their closest phylotypes deposited at DDBJ. Four sequences (ALN_al 04, ALN_cy 01, ALN_cy 02 and ALN_pl 01) exhibited homologies of 100%, 100%, 100% and 99.8% with *Pelagibacter ubique* in *Alphaproteobacteria*, cyanobacteria *Prochlorococcus* and *Synechococcus*, and plastid, respectively, although the sequence lengths were shorter than those expected by the primer set used.

UPGMA clustering of sampling stations using our DGGE data revealed that bacterial compositions in Oyashio stations were basically similar, especially in September 2003 (Fig. 4B), February 2004 (Fig. 4C) and July 2004 (Fig. 4E). However, in July 2003, bacterial compositions at the Oyashio Stations A07 and A14 showed high similarities with the transition-area Station A09 and Kuroshio Station A21 (Fig. 4A), respectively. Bacterial communities at the Oyashio stations were characterized by the occurrence of bands 09, 11, 12, 13 and 15 in September 2003 and then by bands 23 and 27 in May 2004. Alternatively, the Kuroshio communities were characterized by the appearance of band 35 in July 2004. A distant cluster including Station A07 in September 2003 (Fig. 4B) was characterized by bands 09 and 13. Another distant cluster including Station A13 in May 2004 (Fig. 4D) was characterized by an intense band 25 and the disappearance of band 28. At Station A09 in July 2004, the following

bands were found in the deep water samples (Fig. 3vi): band 39 at 50 m, and bands 40 and 41 at both 50 and 100 m depths. However, the following bands were absent from deep waters: bands 37 at 100 m and band 38 at both 50 and 100 m.

The RDA of the DGGE profiles scaled distances of the sampling stations with the environmental parameters (Fig. 5). The arrow vectors for the environmental variables in each plot represent their significance in the distribution of bacteria at the sampling stations. The first and second RDA axes explained from 57.6% to 86.8% of the total variance in each RDA. A non-parametric multivariate ANOVA permutation test showed statistical significance ($p < 0.05$) between bacterial compositions and hydrographic parameters in both September 2003 and July 2004, periods when their correlation coefficients obtained from RDA were also high (> 0.96). Bacterial communities at the Kuroshio stations (A21 in July 2003, A17 in September 2003, A21 in February 2004, and A19 and A21 in July 2004) were related to higher temperature and/or salinity, respectively. On the other hand, those at the Oyashio stations (A04 and A05 in July 2003, A02, A07 and A09 in September 2003, and A03, A04 and A05 in July 2004) were influenced by higher chlorophyll *a* and/or nitrate concentrations, respectively.

4. DISCUSSION

At the Oyashio stations, bacterial abundance was highest in September 2003 and lowest in February 2004 (Fig. 2). In February 2004, *in situ* water temperatures in Oyashio waters were $< 7.1^{\circ}\text{C}$ and such low temperatures have been reported to decrease bacterial growth (Kirchman et al., 1993; Nagata et al., 2001). Although DOC was not

measured in the present study, the DOC level in February was expected to be low based on the observed low chlorophyll *a* concentrations (Table 1), since DOC production is mainly controlled by phytoplankton (Carlson et al., 2002). Therefore, low bacterial abundance in February may be a result of low DOC and low temperature. Meanwhile, the highest abundance of heterotrophic bacteria observed in September 2003 could be the result of the higher water temperatures (9.4–20.8°C) and chlorophyll *a* concentrations (0.34–1.68 $\mu\text{g L}^{-1}$). On the other hands, it has been reported that top-down control by bacteriovorous heterotrophic nanoflagellates (HNF) was higher in summer (Shinada et al., 2001; Saito et al., 2002), suggesting that a significant portion of the bacteria in this study may have been grazed down by HNF.

In our cluster analysis, bacterial community structures were different among water masses (Fig. 4). Furthermore, RDA revealed that environmental conditions (temperature, salinity, and concentrations of chlorophyll *a* and nitrate) controlled the bacterial community structure in September 2003 and July 2004. The bacterial communities in Kuroshio waters were clearly affected by higher temperature and/or salinity (Fig. 5). The band positions of 10, 14, and 35 in DGGE (Fig. 3, Table 2) were specifically appeared in Kuroshio waters, suggesting that genotypes ALN_gm 04, ALN_gm 09, ALN_al 03 adapted to higher temperature and salinity. On the other hand, bacterial communities in Oyashio waters were generally influenced by higher chlorophyll *a* and/or nitrate concentrations (Fig. 5). The bacterial phylotypes detected preferentially in Oyashio waters were discussed below. Although Station A07 was classified into Oyashio waters, the bacterial community compositions at the station were rather different from those at the other Oyashio stations in September 2003 due to the appearance of the

DGGE bands 09 and 13 corresponding to ALN_gm 17 and ALN_gm 23, respectively (Fig. 3 and Table 2). The phylotypes at Station A07 could be influenced by the other environmental factors such as salinity and temperature (Fig. 5A). Although the relationships between environmental variables and bacterial community compositions in July 2003, and February and May 2004 were not statistically significant, similar plots between bacteria and the hydrographic parameters were obtained. The concentrations of chlorophyll *a* and nitrate also affected the bacterial community structures, suggesting that phytoplankton productivity influenced the bacterial community compositions (Pinhassi et al., 2003).

In order to examine whether sampling period can distinguish bacterial community compositions in the study area, a one-gel-comparison was made using the samples collected in February and May 2004. As a result, bacterial communities in the February were distinguished from those in the May using a cluster analysis. The results also indicate that differences in hydrographic conditions between the two periods induced the changes in bacterial community composition significantly (Fig. 6). The bacterial communities in February were affected by nitrate concentrations, while those in May were not significantly influenced by the environmental factors used in this study (Fig. 7). However, similar comparisons were not executed using all the results of our DGGE, because denaturant conditions of each gel were slightly different and the distortion of bands also sometimes occurred in our DGGE gels. Ferrari and Hollibaugh (1999) pointed out such difficulties in comparisons among different DGGE gels.

Our PCR-DGGE analyses revealed that *Gammaproteobacteria* occurred

frequently in the study area (Fig. 3, Table 2). This result was similar to the observations reported by Bano and Hollibaugh (2002), Malmstrom et al. (2007), and Pommier et al. (2007). For example, Pommier et al. (2007) showed a high diversity of *Gammaproteobacteria* genotypes in the Arctic Ocean. Regardless, the reasons why *Gammaproteobacteria* occurred frequently, compared to the frequency of occurrence of other bacterial groups (Table 2) in this study area were unknown.

For the *Gammaproteobacteria* observed in the present study, the 16S rDNA sequences of ALN_gm 04, ALN_gm 08, ALN_gm 13, ALN_gm 14, ALN_gm 23 and ALN_gm 24 showed low similarity (<97%) to those reported previously, indicating that these were probably new genotypes. Alternatively, the sequences ALN_gm 17 and 18 showed 100% similarity to psychrophilic bacteria *Psychrobacter glacincola* sp. nov. (Brinkmeyer et al., 2003) and *P. pacificensis* sp. nov. (Maruyama et al., 2000), respectively. The *Psychrobacter*-related bands often appeared at stations located in cold Oyashio waters. *Psychrobacter* has ever been reported in both the Arctic and Antarctic Oceans (Brinkmeyer et al., 2003) and was reported to be predominant in the bacterial community in the sub-Antarctic Ocean (Prabakaran et al., 2007). These results indicate that ALN_gm 17 (*P. glacincola*) and ALN_gm 18 (*P. pacificensis*) possess low-temperature tolerances. Simon et al. (1999) also showed that temperature controlled the growth rate of heterotrophic bacteria and that psychrophilic bacterial distributions changed along a transect across the Polar Front because of changes in temperature. The DGGE bands 11 and 27 (ALN_gm 11 and 12, respectively) were detected only in the samples from Oyashio waters and were highly homologous to the clone MB11B11 and HTCC2121, which is affiliated with the SAR92 clade of *Gammaproteobacteria* (Cho and Giovannoni, 2004). Recently, Stingl et al. (2007)

found that the SAR92 clade was the dominant genotype in surface waters off the Oregon coast, and that its abundance was correlated with nutrient levels. These observations were consistent with our results in the nutrient-rich Oyashio waters. Other *Gammaproteobacteria*, ALN_gm 06 and 07, were also highly homologous with the OM60 clade, which has often been observed in coastal regions within ca. 25 km from shore (Rappé et al., 1997; Cho and Giovannoni, 2004). However, ALN_gm 06 and 07 were detected not only at neritic stations but also at pelagic stations (A01–11).

In this study, only four phylotypes were *Alphaproteobacteria*. This agrees with studies that reported few *Alphaproteobacteria* sequences from DGGE bands containing 16S rDNA PCR-amplified with 341F/907R primer set (e.g., Bano and Hollibaugh, 2002). However, the SAR11 cluster within *Alphaproteobacteria* is considered to be generally abundant and has high phylogenetic diversity (reviewed by Fuhrman and Hagström, 2008). The reasons for the difference between this study and the review by Fuhrman and Hagström (2008) are uncertain. Out of three *Alphaproteobacteria* identified in the present study, ALN_al 01 and ALN_al 02 were 100% homologous with the SAR11 clade. In particular, ALN_al 01 was homologous to *Pelagibacter ubique*, which was known to be cosmopolitan in surface waters (Morris et al., 2002). In fact, ALN_al 01 was detected at stations from the coastal region (Station A01) offshore to the Kuroshio-Oyashio transition region (Station A09 in July 2004). On the other hand, ALN_al 04 (SAR11) was only detected at 50 and 100 m depths, and not in surface waters. Previous studies (e.g., Riemann et al., 1999; Bano and Hollibaugh, 2002) pointed out that bacterial community structure could vary with depth. Recently, Giovannoni et al. (2005) revealed that *P. ubique* in the SAR11 clade possesses the pigment proteorhodopsin, which can act as a light-driven proton pump

and is considered to be potentially important in supplying energy to cells, suggesting that light may affect their distribution. DeLong et al. (2006) also found depth-specific phylotypes in the SAR11 clade, and suggested that not only light adaptation (Béjà et al., 2001) but also depth-related environmental differences, such as nutrients and oxygen, could be influential factors. However, little is known about the phylotypes of *Alphaproteobacteria* found in subsurface waters. Further phylogenetic studies of the subsurface SAR11 clade are needed.

No *Cytophaga-Flavobacterium* (hereafter, CF) was found in the present study, indicating that the CF group was absent or consistently low in abundance in the study area. In the Arctic Ocean, this group showed low abundance and low diversity, compared to *Alpha*- and *Gammaproteobacteria* (Bano and Hollibaugh, 2002; Malmstrom et al., 2007; Pommier et al., 2007). However, Kisand and Winkner (2003a; 2003b) pointed out that CF had multiple melting domains and were rarely detected as clear DGGE bands when the 16S rDNA fragments with 550 bp were amplified using the primer set 341F-907R, which was used here. Therefore, failure to detect CF might be related to the primer set used and not truly indicates the absence of the group.

In summary, we found that *Gammaproteobacteria* were a dominant group and the bacterial community compositions generally differed among water masses in the study area (Fig. 4). Although exceptions were found, e.g., July 2003, our results indicate that bacterial community compositions were generally distinct among the water masses studied. However, because of the observed exceptions, the effects of other factors such as DOC (Pinhassi et al., 1999; Fandino et al., 2001; Kirchman et al., 2004), ultraviolet radiation (Winter et al., 2001) and iron (Eldridge et al., 2007) on bacterial

community compositions should be examined for better understanding microbial ecology in the study area.

Acknowledgements

We express sincere thanks to Drs. S. Oguma and S. Kakehi for the classifications of water masses. Grateful thanks are also due to the captains and crews of FR/V *Wakataka Maru* and *Hokko Maru* and Dr. T. Ono for their helpful support during sampling cruises. Dr. D. Munroe is also acknowledged for her valuable comments and useful suggestions.

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Legends

Fig. 1. Water currents east of Japan and location of stations along the sampling *A-line*. Pale arrows represent the Oyashio current, which flows southwestward offshore of Hokkaido. The dark arrow shows the Kuroshio current flowing eastward. During May 2004, a warm-core ring (WCR) existed in the study area.

Fig. 2. Temporal changes in bacterial abundance in the Oyashio, Kuroshio and Oyashio-Kuroshio transition regions. Error bars indicate standard deviation. N.D. represents no data.

Fig. 3. DGGE profiles at different stations in each cruise. In (vi), samples were obtained at 10, 50 and 100 m depths at Station A09. In (i) – (v), samples were collected at 10 m. Arrow numbers indicate band positions (Table 2). The gradient range is shown on the left side of each DGGE profile.

Fig. 4. Cluster analysis of similarities among sampling stations based on DGGE profiles in Fig. 3. The labels C, O, K, and T represent coastal, Oyashio, Kuroshio, and Oyashio-Kuroshio transition regions, respectively, and D represents Depth (m).

Fig. 5. RDA ordination biplots of DGGE bands (samples indicated using sample site location code) and environmental variables (represented by arrows; Tem, Sal, Chl *a* and NO₃ represent temperature, salinity and chlorophyll *a* concentration, and nitrate concentration, respectively).

Fig. 6. Cluster analysis of DGGE data on bacterial community compositions in the study area in February and May 2004. Abbreviations are as per Fig. 4.

Fig. 7. RDA ordination biplot of DGGE bands using the samples collected in February and May 2004. Environmental variables were the same as Fig. 5. The symbols located before station name, "F-" and "M-", mean samples collected in February and May, respectively.

Table 1. Maximum and minimum temperature (Tem; °C), salinity, chlorophyll *a* (Chl *a*; µg L⁻¹), phosphate (PO₄³⁻; µM) and nitrite (NO₂⁻, NO₃⁻; µM) at the depth in which samples were collected during each cruise. ND represents not determined.

Date	Station	Water condition	Depth [m]	Tem [°C]	Salinity	Chl <i>a</i> [µg L ⁻¹]	PO ₄ [µM]	NO ₂ [µM]	NO ₃ [µM]
2003									
July	A01	Coastal	0	5.3	32.7	5.87	0.22	0.06	0.48
	A02-07, A11-15	Oyashio	0	8.3 - 15.4	32.7 - 33.3	0.18 - 0.81	0.20 - 0.80	0.02 - 0.17	0.02 - 7.34
	A08-10	Transition	0	14.4 - 15.1	33.5 - 33.6	0.53	0.28	0.04	1.32
	A17-21	Kuroshio	0	19.3 - 21.0	34.4 - 34.5	0.17 - 0.55	0.27	0.02 - 0.07	0 - 1.15
September	A01	Coastal	10	12.3	33.1	ND	ND	ND	ND
	A02-11	Oyashio	10	9.4 - 20.8	32.7 - 33.9	0.34 - 1.68	0.06 - 0.98	0.08 - 0.20	0.43 - 8.73
	A13-15, A20	Transition	10	20.6 - 22.3	33.5 - 33.8	0.32 - 0.47	0.05 - 0.05	0.03 - 0.06	0.04 - 0.42
	A12, A16-19, A21	Kuroshio	10	20.0 - 24.1	33.5 - 34.1	0.27 - 0.34	0.03 - 0.07	0.02 - 0.07	0.01 - 0.44
2004									
February	A01	Coastal	10	0.7	32.7	ND	ND	ND	ND
	A02-15	Oyashio	10	0.6 - 7.1	30.2 - 33.9	0.38 - 0.69	1.05 - 1.70	0.13 - 0.35	13.85 - 23.14
	A16-20	Transition	10	6.7 - 8.4	33.7 - 33.8	0.66 - 0.83	0.71 - 0.96	0.21 - 0.25	9.29 - 11.52
	A21	Kuroshio	10	10.8	34.1	0.20	0.57	0.36	7.49
May	A01	Coastal	0	4.3	31.8	ND	ND	ND	ND
	A02-05, A12-13	Oyashio	0	1.8 - 10.1	32.1 - 34.7	0.66 - 6.57	0.91 - 1.89	0.19 - 0.32	7.72 - 21.80
	A06-11, A14-21	Transition	0	7.9 - 14.6	32.2 - 34.2	0.69 - 3.40	0.35 - 0.90	0.17 - 0.39	1.83 - 7.66
July	A01	Coastal	10	12.2	33.1	ND	ND	ND	ND
	A02-08, A10, A14	Oyashio	10	12.5 - 23.2	32.8 - 33.4	0.33 - 1.55	0.23 - 0.36	0.06 - 0.09	0.11 - 0.65
	A09, A11-13, A15-17	Transition	10	22.5 - 25.6	30.0 - 33.6	0.05 - 0.17	0.06 - 0.10	0.05 - 0.08	0.03 - 0.09
	A18-21	Kuroshio	10	25.6 - 26.7	33.5 - 34.3	0.16 - 0.18	0.04 - 0.05	0.07 - 0.07	0.04 - 0.05

Table 1 Kataoka et al.

Table 2. Obtained 16S rDNA sequence name, closest relatives from DDBJ, present similarity based on aligned base pairs, and band position in DGGE (Fig. 3).

Obtained sequences		Closest relatives		Matched length	Similarity (%)	Band position**				
Name	Accession No.	Group*	Form name (Accession No.)			3J	3S	4F	4M	4J
ALN_al01	AB364983	α , SAR11	<i>Pelagibacter ubique</i> HTCC1062 (AF510191)	521	100.0	02	17	20	32	
ALN_al02	AB364984	α , SAR11	clone F291 (DQ289540)	521	100.0	01				
ALN_al03	AB305050	α	clone S2-62 (EF491378)	525	98.3					35
ALN_al04	AB364985	α , SAR11	<i>Pelagibacter ubique</i> HTCC1062 (AF510191)	424	100.0					39
ALN_gm01	AB305054	γ	<i>Pseudoalteromonas</i> sp. (DQ642819)	548	99.8				24,25	
ALN_gm02	AB364986	γ	<i>Alteromonas</i> sp. KT0903 (AF235119)	548	100.0	04				
ALN_gm03	AB364987	γ	<i>Alteromonas</i> sp. KT0903 (AF235119)	545	99.8	05				
ALN_gm04	AB305058	γ	Uncultured <i>Pseudoalteromonas</i> sp. clone JL-ESNP-I21 (AY664206)	549	96.4		10			
ALN_gm05	AB364988	γ	Uncultured <i>Pseudoalteromonas</i> sp. clone JL-ESNP-I21 (AY664206)	549	98.2		12			
ALN_gm06	AB364989	γ , OM60	clone KTe1119 (AF235120)	549	99.8	08	15		29	37
ALN_gm07	AB364990	γ , OM60	clone KTe1119 (AF235120)	549	99.6			18	28	36
ALN_gm08	AB305051	γ , OM60	clone KTe1119 (AF235120)	549	96.4	03			26	
ALN_gm09	AB364991	γ	clone C22 (EU010181)	549	99.6		14			
ALN_gm10	AB364992	γ	clone AntCL1D1 (DQ906720)	549	99.6	07				
ALN_gm11	AB364993	γ , SAR92	clone MB11B11 (AY033301)	549	100.0		11			
ALN_gm12	AB305052	γ , SAR92	Marine gamma proteobacterium HTCC2121 (AY386341)	549	99.5				27	
ALN_gm13	AB305053	γ , SAR92	clone MB11B11 (AY033301)	549	96.5					34
ALN_gm14	AB364994	γ	clone F141 (DQ289521)	550	95.1	06				
ALN_gm15	AB364995	γ	<i>Halomonas meridiana</i> DSM 5425 (AJ306891)	549	100.0				30	
ALN_gm16	AB364996	γ	<i>Halomonas</i> sp. ARD M36 (AB085657)	549	100.0				31	
ALN_gm17	AB364997	γ	<i>Psychrobacter glacincola</i> ANT9276b (AY167310)	549	100.0		09			
ALN_gm18	AB364998	γ	<i>Psychrobacter pacificensis</i> WP02-1-40 -1 (EF179615)	549	100.0				22	
ALN_gm19	AB305057	γ	<i>Psychrobacter submarinus</i> (AJ309940)	549	99.3				23	33
ALN_gm20	AB364999	γ , SAR86	clone Arctic97A-18 (AF354613)	549	99.3					40
ALN_gm21	AB365000	γ	Uncultured marine bacterium Ant4D3 (DQ295237)	550	98.9					41
ALN_gm22	AB305056	γ	clone SPOTSAUG01_5m49 (DQ009139)	525	98.5					30
ALN_gm23	AB305055	Bac	clone CTD005-80B-02 (DQ513062)	547	94.1		13			
ALN_gm24	AB365001	Bac	clone IG-1-13 (AB262376)	547	95.1			19		
ALN_cy01	AB366609	Cya	<i>Prochlorococcus marinus</i> TAK9803-2 (AF311220)	527	100.0		16			
ALN_cy02	AB365003	Cya	<i>Synechococcus</i> sp. CC9902 (CP000097)	416	100.0					38
ALN_pl01	AB365002	plastid	DGGE gel band LUR8 (AY960283)	507	99.8				21	

* α , γ and Bac indicate *Alpha*-, *Gammaproteobacteria*, and unknown group of bacteria, respectively.

** 3J, 3S, 4F, 4M, and 4J represent July 2003, September 2003, February 2004, May 2004, and July 2004, respectively.

Table 2 Kataoka et al.

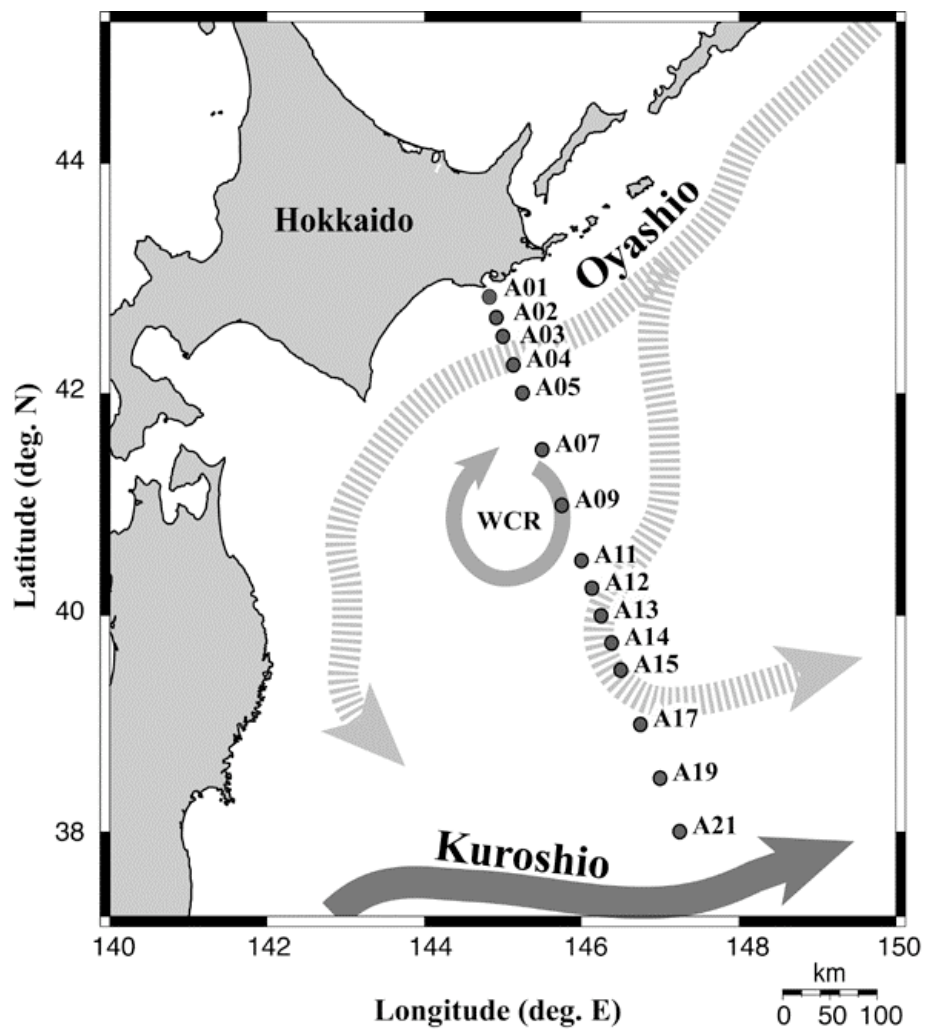


Fig. 1 Kataoka et al.

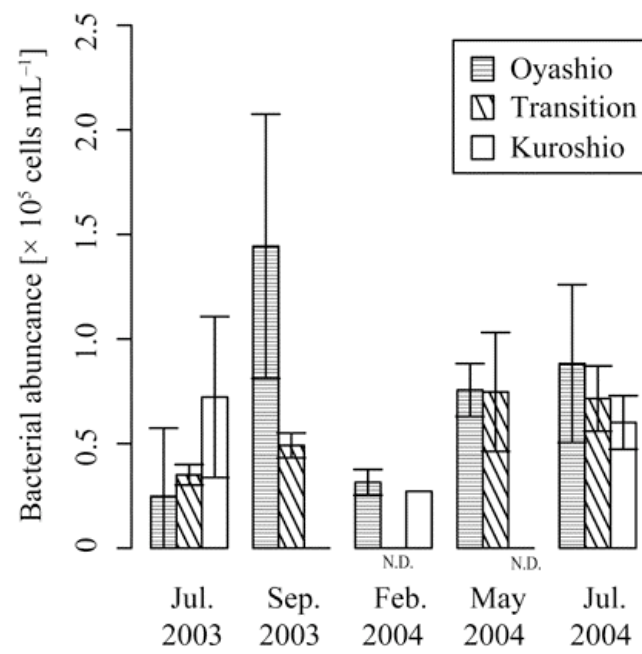
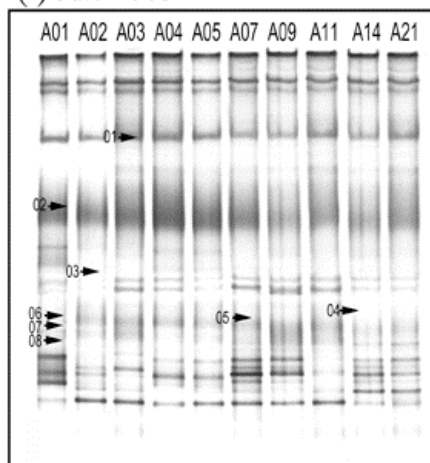
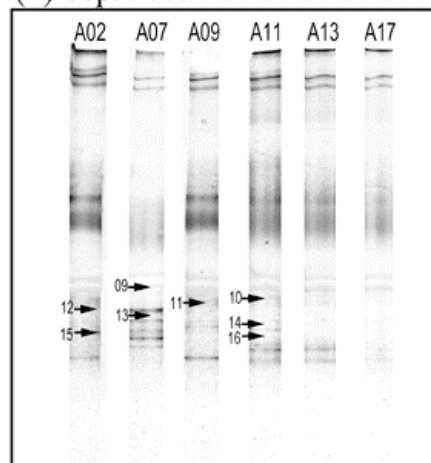


Fig. 2 Kataoka et al.

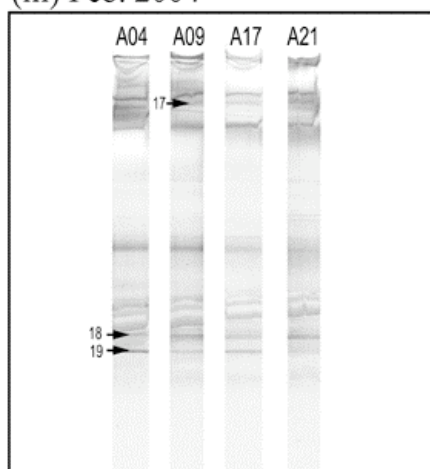
(i) Jul. 2003



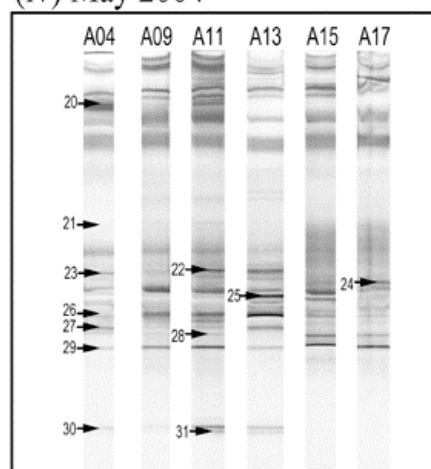
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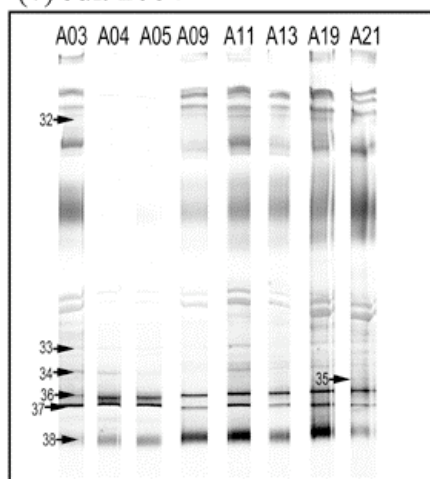
(iii) Feb. 2004



(iv) May 2004



(v) Jul. 2004



(vi) Jul. 2004 A09 vertical

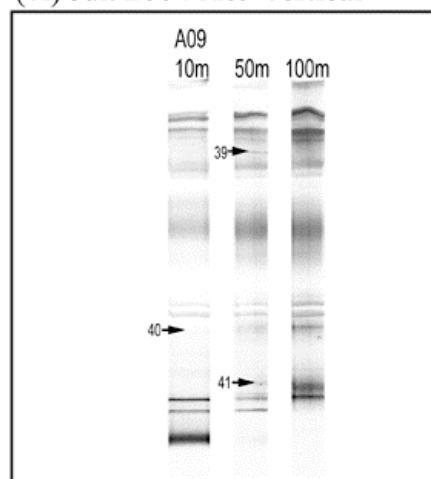


Fig. 3 Kataoka et al.

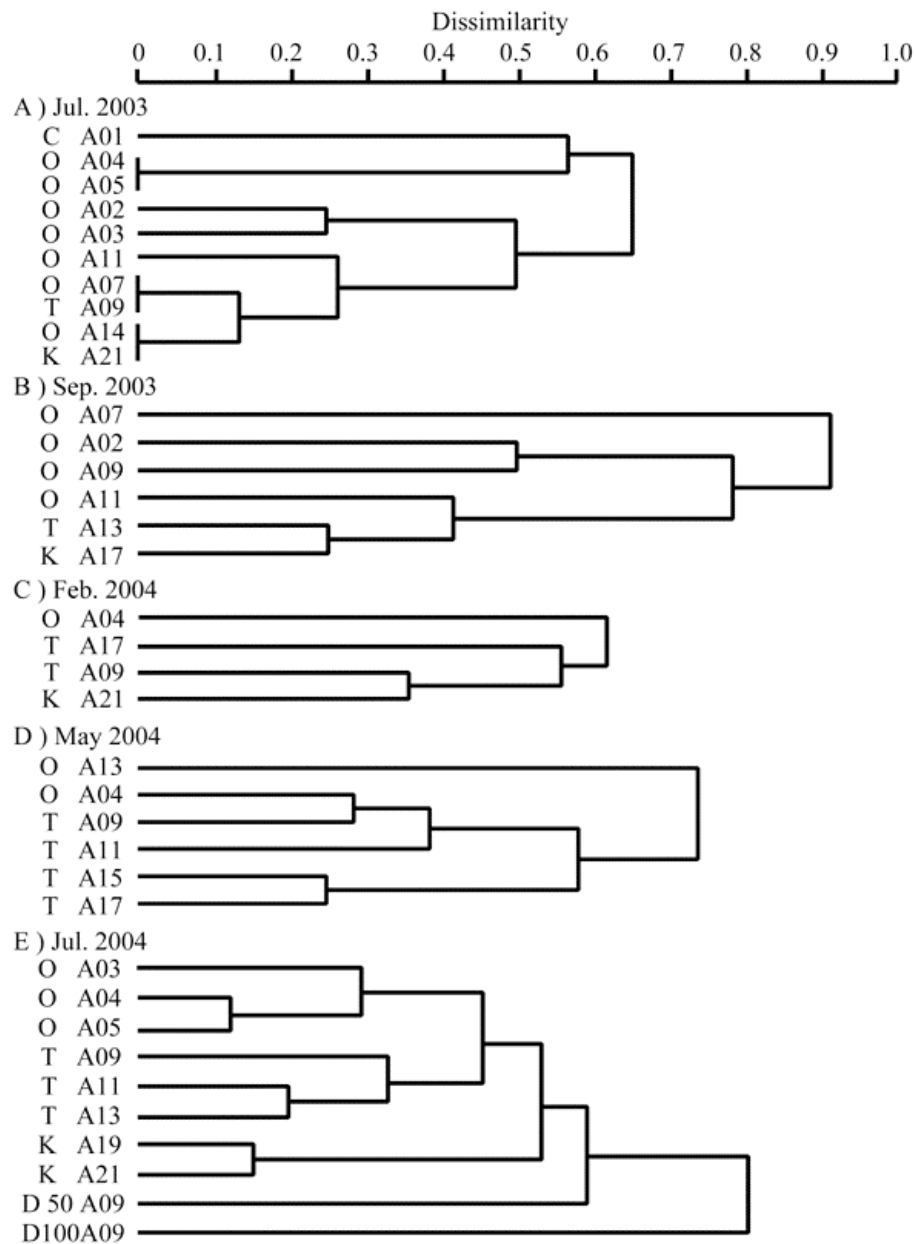


Fig. 4 Kataoka et al.

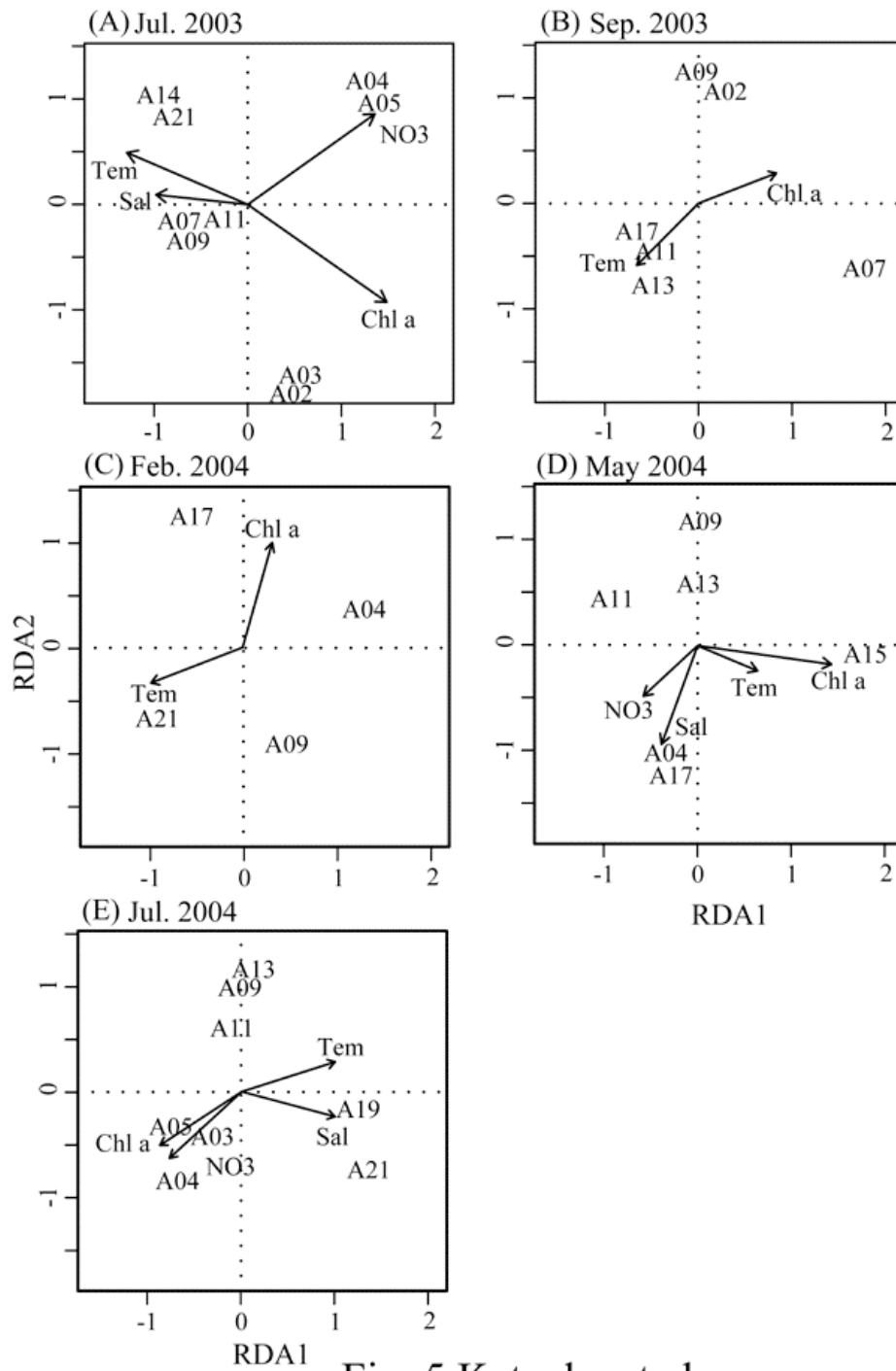


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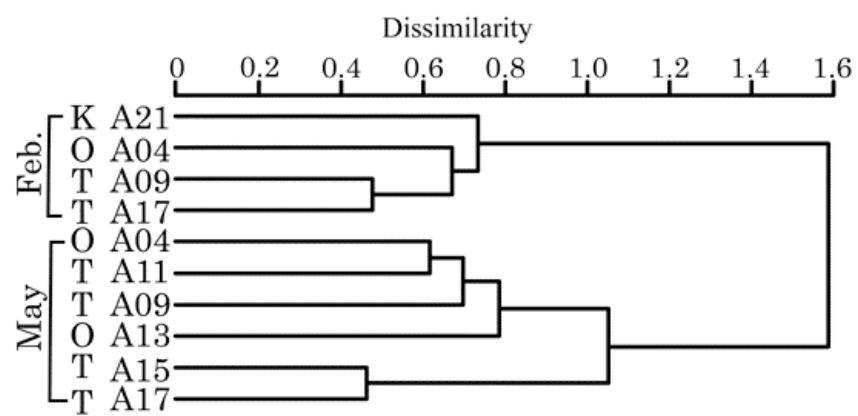


Fig.6 Kataoka et al.

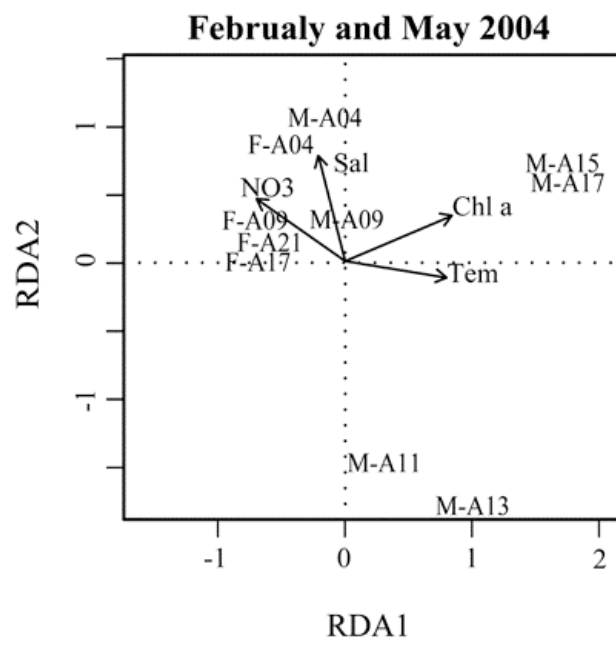


Fig.7 Kataoka et al.