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Abstract

Bacterial populations transported from ground environments to the atmosphere get dispersed throughout downwind areas and can influence ecosystem dynamics, human health, and climate change. However, the vertical bacterial distribution in the free troposphere was rarely investigated in detail. We collected aerosols at altitudes of 3,000 m, 1,000 m, and 10 m over the Noto Peninsula, Japan, where the westerly winds carry aerosols from continental and marine areas. During the sampling period on March 10, 2012, the air mass at 3,000 m was transported from the Chinese desert region by the westerly winds, and a boundary layer was formed below 2,000 m. Pyrosequencing targeting 16S rRNA genes (16S rDNA) revealed that the bacterial community at 3,000 m was predominantly composed of terrestrial bacteria, such as *Bacillus* and *Actinobacterium* species. In contrast, those at 1,000 m and 10 m included marine bacteria belonging to the classes *Cyanobacteria* and *Alphaproteobacteria*. The entire 16S rDNA sequences in the clone libraries were identical to those of the terrestrial and marine bacterial species, which originated from the Chinese desert region and the Sea of Japan, respectively. The origins of air masses and meteorological conditions contribute to vertical variations in the bacterial communities in downwind atmosphere.

Key words: airborne bacteria, Asian dust, bioaerosol, free troposphere, phylogenetic analysis

1. Introduction

Bioaerosols, which include bacteria, fungi, and viruses, are transported from ground environments to the free troposphere and account for a substantial proportion of the organic carbon fraction of atmospheric aerosols (Jaenicke, 2005; Iwasaka et al., 2009). Dust events are known to transport airborne microorganisms, thereby supporting the microbial immigration to downwind ecosystems (Griffin et al., 2007; Maki et al., 2010; Yamaguchi et al. 2012). In Asian regions, airborne microorganisms transported over long distances increase allergenic burden, consequently increasing the incidence of asthma (Ichinose et al., 2005) and contributing to the dispersion of diseases such as Kawasaki disease in humans (Rodó et al., 2011) and rust diseases in plants (Brown and Hovmøller, 2002). Some microorganisms isolated at high altitudes have been reported to induce allergy levels similar to that caused by Asian mineral dust particles (Liu et al., 2014). Bioaerosols are thought to influence atmospheric processes in clouds by participating in atmospheric chemical and microphysics process (Delort et al., 2010). Microorganisms in the atmosphere are known to act as ice nuclei and cloud condensation nuclei affecting ice-cloud formation (Möhler et al., 2007, Pratt et al., 2009, Joly et al., 2013). Viable bacteria have been identified in super cooled cloud droplets, and their ability to metabolize organic matters in the environment has been confirmed (Sattler et al., 2001; Vaïtilingom et al., 2013).

Bacterial community structures in the atmosphere should be investigated for understanding the long-range transport processes of airborne bacteria and their influence on ecosystem dynamics, human health, and climate change in downwind areas.

The atmosphere is a heterogeneous environment; meteorological shifts and dust events considerably alter bacterial abundance and composition in the Asian atmosphere (Hara and Zhang, 2012; Maki et al., 2014). In addition, airborne microorganisms on North American mountains (2,700 m above sea level) were found to change in response to Asian dust events that included highly diverse bacterial species (Smith et al., 2013). Pyrosequencing technology that can analyze large numbers of nucleotide sequences has been applied for estimating the variations in the composition of bacterial communities on mountains (Bowers et al., 2012) and in rural and urban areas (Brodie et al., 2007) at the family level. However, there are a few of researches investigating the bacterial communities at high altitudes above ground level using pyrosequencing analysis (Bowers et al., 2009; DeLeon-Rodriguez et al., 2013), and the vertical distribution of airborne bacteria between the ground and high altitudes is unclear.

For more comprehensive understanding of airborne bacterial communities at high altitudes, we collected air samples at altitudes of 3,000 m and 1,000 m using an aircraft and a balloon, and at 10 m from a building, over the edge of the Noto Peninsula, Japan (Fig. 1a). In this region, the air sampling of aerosols moving from continental areas to Japan can be conducted while avoiding aerosol contamination from local areas. We analyzed the meteorological data for estimating the air mass conditions during the sampling periods, and determined the aerosol amounts by using meteorological monitoring techniques. Bacterial community structures in the air samples were analyzed using pyrosequencing targeting bacterial 16S rRNA genes (16S rDNA). However, pyrosequencing generates short-length sequences that can lead to misreads, allowing us to understand bacterial composition at the class or family level, but not at the species

level (Womack et al., 2010). Therefore, the compositions of bacterial species in the air samples were phylogenetically analyzed using the cloning library technique targeting 16S rDNA.

2. Materials and Methods

2.1. Sampling

Aerosol samplings at 3,000 m above ground level were performed over the Noto Peninsula, Japan, from 11:40 JTC to 12:40 JTC on March 10, 2012, by an aircraft. The aircraft traveled 150 km northwest, returned to Suzu and traveled 150 km northeast (Fig. 1a). Air samples at 1,000 m above the ground level were collected over Suzu (37.5°N, 137.4°E) from 15:50 JTC to 16:50 JTC by a tethered balloon. Suzu is located on the northern coast of the Noto Peninsula and is the arrival site for aerosols from continental areas (Fig. 1a). The sampling processes using an aircraft and a tethered balloon were employed in our previous investigation (Maki et al., 2013). In addition, an air sample at 10 m above ground level was collected on the roof of a building from 12:00 JTC to 13:00 JTC. For the sampling procedures at these three altitudes, total 700 L of air samples were collected through a sterilized polycarbonate filter (0.22-μm pore size; Whatman, Tokyo, Japan) for 1 h during the sampling time, and two filtrates were obtained for each altitude. When the aerosol concentrations are below 1.0×10^4 particles/m^3 in atmosphere, bacterial DNA has not been detected from the air samples collected using this sampling device.

2.2. Characteristics and trajectories of air masses

Air quality and atmospheric data were obtained from the Wajima Meteorological Observatory of the Japan Meteorological Agency, which is located 50 km from Suzu (Fig. 1a). Environmental data were collected using a radiosonde at 3:00 JTC and 9:00 JTC. At altitudes from 10 m to approximately 4,000 m, information regarding weather conditions (temperature, relative humidity, and pressure) was obtained for the comparative analyses of air masses.

To track the transport pathways of air masses, 72-h back trajectories were calculated using the NOAA HYbrid Single Particle Lagrangian Integrated Trajectory (HYSPLIT) model (http://www.arl.noaa.gov/HYSPLIT.php). Suzu was used as the location of the back trajectory starting point at altitudes of 3,000 m, 1,000 m, and 10 m above ground level for estimating the accurate trajectories of air masses at the three altitudes.

2.3. Determination of particle abundance

The air particles at each altitude were counted using an optical particle counter (OPC). To measure air particles at the altitude of 3,000 m, outside air was transferred by injection into the OPC located in an aircraft. A balloon was similarly used to measure air particles at the altitude of 1,000 m. The air particles at the altitude of 10 m were measured directly using the OPC.

Aerosols on one of the two filters were washed off by shaking the filter with 10 mL of sterilized water containing 0.6% (w/v) NaCl. After washing, aliquots were fixed with paraformaldehyde for 1 h and stained with 4',6-diamidino-2-phenylindole (DAPI) for 15

min. These sample solutions were filtered through a polycarbonate filter (0.22-μm pore-size; Whatman) and stained with Sudan Black (Russell et al., 1974), and particles on the filter were observed using a fluorescence microscope (Olympus, Tokyo, Japan) with UV excitation system. Mineral particles, yellow particles and bacterial cells were counted using a previously reported observation technique (Maki et al., 2014). The detection limit of aerosols was 1.1×10^4 particles/m^3 of air.

2.4. Analysis of bacterial community structures using pyrosequencing analysis targeting 16S rDNA sequences

After the aerosol particles on the other of the two filters were suspended into 10 mL of sterile 0.6% NaCl solution, the particles were pelleted by centrifugation at 20,000 × *g* for 5 min. genomic DNA (gDNA) was extracted from the particle pellets using sodium dodecyl sulfate, proteinase K, and lysozyme, and purified by phenol-chloroform extraction as previously described (Maki et al., 2008). Bacterial community composition was determined using pyrosequencing, which facilitates multiplexed partial sequencing of 16S rDNA. Fragments of 16S rDNA (approximately 500 bp) were amplified from the extracted gDNA by PCR using the following oligonucleotide primers: 27F, 5′- Adaptor I - Index - CAG AGA GTT TGA TCM TGG CTC AG-3′; 519R, 5′- Adaptor II - GWA TTA CCG CGG CKG CTG -3′ (Maidak et al., 1997). Adaptor I is CCA TCT CAT CCC TGC GTG TCT CCG ACT and Adaptor II is CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG. The air samples collected at 3,000 m, 1,000 m, and 10 m were analyzed using Index 1; CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG, Index 2; CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG, and

Index 3; CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG, respectively. Thermal cycling was performed using a thermocycler (Program Temp Control System PC-700; ASTEC, Fukuoka, Japan) under the following conditions: denaturation at 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 2 min for 30 cycles. PCR amplicons were purified using the MonoFas DNA purification kit (GL Sciences, Tokyo, Japan). PCR amplicons from each sample were pooled at approximately equal amounts into a single tube for pyrosequencing on Genome Sequencer FLX system (454 Life Sciences, Roche, Branford, CT, USA) machine. With this protocol, we obtained ca. 400 bp of read length, which covered the variable regions V1, V2, and V3 of the 16S rRNA gene region. These read sequence regions provide an accurate taxonomic assignment to at least the family level of bacterial composition (Liu et al., 2008). Negative controls (no template and template from unused filters) were prepared in all steps of the process from DNA extraction to check for contamination. We performed the experiments of DNA analyses under strict sterile conditions and confirmed no artificial contaminations at each experimental step.

Before the analysis of bacterial community structures, we removed sequences that were <200 bp in length, those with a phred-equivalent quality score of <25, those containing ambiguous characters, those with an uncorrectable barcode, or those without the primer sequence. The remaining sequences were clustered into phylotypes using QIIME (ver. 1.8.0) with a minimum coverage of 99% and a minimum identity of 97%. The bacterial compositions of phylotypes were analyzed by comparing against the DNA Data Bank of Japan (DDBJ) using BLAST. Moreover, the bacterial diversity in each sample was estimated using Chao 1 (Colwell and Coddington, 1994).

2.5. Bacterial 16S rDNA clone libraries

Full-length sequences of 16S rDNA (approximately 1,450 bp) were amplified from the extracted gDNA by PCR using the following oligonucleotide primers: 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; 1492R, 5′-GGY TAC CTT GTT ACG ACT T-3′ (Maidak et al., 1997). PCR and amplicon purification were performed as described for the pyrosequencing analysis section above, PCR amplicons corresponding to 16S rDNA fragments were cloned into *E. coli* using a commercially available vector plasmid with a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. More than 100 clones were obtained for each sample. The amplicons were sequenced using an ABI Dye Deoxy Terminator Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) and an ABI Prism 373A DNA Sequencer (Life Technologies) according to the manufacturer's recommended protocols. The M13 forward primer was used as the sequencing primer.

The amplicon sequences without chimeras were assigned as phylotypes based on bacterial species with more than 97% sequence similarities. Sequences were assigned to phylotypes using BLAST to identify their closest matching sequences. The coverage of the clone library was calculated using the formula [1 – (n/N)] x 100, where n is the number of phylotypes represented by a single clone and N is the total number of clones (Röling and Head, 2005). More than 90% of coverage indicated that the clone library would represent dominant microbial populations in environmental samples. The nearly full sequences of some clones relating to the dominant phylotypes of pyrosequencing analysis were determined using the M13 reverse primer. Phylogenetic trees including

clone sequences were constructed according to the neighbor-joining algorithm using TreeViewPPC (Saitou and Nei, 1987).

2.6. Accession numbers

The DDBJ accession numbers for the 16S rDNA clone sequences determined in this study are shown in Table 1. All sequences of pyrosequencing data have been deposited in the DDBJ database and the accession number of submission is DRA002535.

3. Results

3.1. Environmental conditions

According to metrological data, the relative humidity over Wajima increased to more than 90% from 766 m to 1,450 m at 9:00 JTC on March 10, 2012, and the layers of high relative humidity moved to 1,423–1,891 m at 21:00 JTC, indicating that cloud layers formed between 700 m and 1,900 m on the sampling date (Fig. 2). In Moderate Resolution Imaging Spectroradiometer pictures, a significant cloud area was observed over the Noto Peninsula on the sampling date (Fig. S1). Because of this cloud layer, light detection and ranging (LiDAR) could not be used to measure the light intensity beyond 500 m over Toyama, Japan (Fig. S2). These results suggest that the boundary layer below 2,000 m separated the air mass at 3,000 m on March 10, 2012. The boundary layer was certainly disturbed by the convective motion of moist air and free tropospheric constituents containing bioaerosols largely differed from those of the boundary layer.

A 3-day back trajectory analysis indicated that during the sampling period, the air mass at 3,000 m over Suzu, Japan, came from the desert region of Central Asia and had immediately passed over the Sea of Japan (Fig. 1b). The back trajectories of 1,000 m and 10 m were maintained over the Sea of Japan for approximately 3 days before their arrival at the Noto Peninsula. The wind direction of the upper 2,000 m over Wajima was constantly west (data not shown). Analysis using an Ozone Monitoring Instrument (http://jwocky.gsfc. nasa.gov/) revealed that a dust event occurred over the Chinese desert region 3 days before the sampling date (March 7, 2012), and low densities of aerosol particles were observed over Japan on March 10, 2012 (Fig. S1). Moreover, the LiDAR measurements at Matsue and Nagasaki displayed a slight increase in depolarization rates, consistent with the occurrence of a weak dust event over the Noto Peninsula during the sampling period (Figs. 1a and S3).

3.2. Vertical distribution of aerosol particles

OPC measurement detected and measured aerosol particles of varying diameters in the atmosphere at 3,000 m, 1,000 m, and 10 m over the Noto Peninsula (Fig. 3a). The distribution and concentration of particles between 0.3 μm and 5.0 μm were similar between 10 m and 1,000 m, indicating that atmospheric constituents were mixed below 1,000 m. The concentration of particles between 0.3 μm and 5.0 μm at 1,000 m and 10 m was one order higher than the concentration at 3,000 m. The number density of relatively large particles larger than 5.0 μm significantly decreased from $(1.45 \pm 0.43) \times 10^4$ particles/m^3 to $(1.22 \pm 0.57) \times 10^3$ particles/m^3 from 10 m to 1,000 m, and slightly decreased to $(3.59 \pm 2.59) \times 10^2$ particles/m^3 at 3,000 m. In contrast, the relative rates of

large particles (>5.0 μm) at 3,000 m and 10 m were higher than that at 1,000 m.

DAPI-stained mineral particles collected at the altitudes of 3,000 m, 1,000 m, and 10 m were observed to emit white-blue autofluorescence (Fig. S4). The total density of white particles at 3,000 m and 1,000 m was approximately 1.81×10^5 particles/m^3 (Fig. 3b). This density was higher than the total density at 10 m, which was $(1.25 \pm 3.01) \times 10^5$ particles/m^3. Yellow fluorescent particles, which potentially indicate organic matter, displayed similar values of $>10^5$ particles/m^3 at 1,000 m and 10 m, which decreased to $(4.53 \pm 5.01) \times 10^4$ particles/m^3 at 3,000 m. In contrast, black carbon was only observed in the air sample at 10 m. DAPI-stained bacteria were observed as coccoid-like particles with a diameter of <1 μm and bright blue fluorescence. The density of bacterial particle at 3,000 m and 10 m was $>4.8 \times 10^5$ particles/m^3; this density was slightly higher than that at 1,000 m, which was $(3.29 \pm 0.58) \times 10^5$ particles/m^3. The air samples collected at 3,000 m on 10 March, 2012 included higher concentrations of bacterial, yellow and white particles than those collected during the non-dust event (Fig. S5a). The particle concentrations at 1,000 m were similar between the periods of dust event and non-dust event.

3.2. Analysis of bacterial communities using pyrosequencing analysis

Pyrosequencing analysis of gDNA extracted from the air samples of 3,000 m, 1,000 m, and 10 m yielded a total of 18,459, 20,058, and 14,350 sequences of 16S rDNA amplicons, respectively. Then, the unanalyzable sequences such as chimeras, short-length (<200 bp) sequences and junk sequences, were eliminated from the total sequences of each sample. Finally, 12,104, 13,848, and 9,684 sequences remaining in

the air samples obtained at 3,000 m, 1,000 m, and 10 m, respectively, which were clustered into 60, 69, and 69 phylotypes, respectively, with a minimum identity of 97%. After identification of the representative phylotype sequences using BLAST, the sequences were separated into 14, 10, and 11 Classes, respectively, and 26, 16, and 13 Families, respectively. We found similar patterns of rarefaction curves among the three air samples (Fig. S6a). However, the numbers of bacterial phylotypes, as estimated using Chao 1 analysis, were 270, 410, and 310, respectively, indicating that the diversity at 3,000 m was relatively lower than the diversities at 1,000 m and 10 m. A comparison of bacterial communities by phylogenetic analysis by UniFrac highlighted that the air sample at 3,000 m contained a bacterial community that differed from those found in air samples at 1,000 m and 10 m (Fig. S6b). At the PCR-analysis steps using negative controls (no template and template from unused filters), 16S rDNA amplicons were not appeared demonstrating no artificial contamination during experimental processes.

Almost all phylotypes belonged to the nine bacterial classes, namely, *Cyanobacteria*, *Deinococci*, *Actinobacteria*, *Bacilli*, *Bacteroidetes*, *Flavobacteria*, and *Alpha-*, *Beta-*, and *Gamma-proteobacteria*, which are typically generated from atmospheric, terrestrial and marine environments (Fig. 4a). The phylotypes belonging to the class *Bacilli* accounted for high relative abundances ranging from 28.6% to 49.9%, and were mainly composed of members of the families *Bacillaceae* and *Staphylococcaceae* (Fig. 4b). *Actinobacteria* phylotypes composed of several bacterial families were predominantly detected at 3,000 m with a high relative abundance of 17.9%, while the relative abundances at 1,000 m and 10 m were low (<0.5%). Phylotypes belonging to the class *Flavobacteria* also specifically appeared at 3,000 m

with a relative abundance of 6.1%. In contrast, the typical airborne bacteria in the class *Deinococci* and marine *Cyanobacteria* were specific to the air samples at 1,000 m and 10 m, while these phylotypes were not detected at 3,000 m. In the phylotypes of the class *Alpha-proteobacteria*, the air samples at 1,000 m and 10 m predominantly included pelagic bacterial members of the SAR clade at relative abundances of 22.4% and 15.7%, respectively. During non-dust event, atmospheric bacterial communities at high altitudes were predominantly composed of the members of *Actinobacteria* and *Proteobacteria*, which include minor populations during the dust event (Fig. S5).

3.3 Phylogenetic analysis of airborne bacteria using 16S rDNA clone library

The full-length fragments of 16S rDNA in the air samples were amplified by PCR using the primers targeting eubacterial 16S rDNA. The PCR amplicons were cloned into *Escherichia coli*, and 331 clones including eubacterial 16S rDNA fragments were obtained from the three air samples. Sequences of the 16S rDNA clones indicated that the bacterial populations were divided into 28 phylotypes (sequences with >97% similarity; Table 1). Reasonable coverage of the three samples was over 95 %, showing that the major bacterial population was represented in the clone libraries. The bacterial compositions of clone libraries mostly agreed with those of the pyrosequencing database, while the relative abundances in class and family compositions differed between the analytical techniques (Fig. 4). The clone library technique failed to detect the minor sequences detected by pyrosequencing analysis. Some representative clones that were closely related to the dominant phylotypes in the pyrosequencing database were selected from the clone libraries recovered from the air samples. Their complete

16S rDNA sequences were determined and phylogenetically analyzed using the known sequences belonging to the classes *Bacilli*, *Actinobacteria*, *Alpha-proteobacteria*, *Deinococci*, and *Cyanobacteria* (Fig. 5).

Among the 331 clones, 127 were closely related to *Bacillus* species (>99.7% similarity). Members of the *Bacillus* species dominated in the clone library of each air sample (Table 1). The complete 16S rDNA sequences of the *Bacillus subtilis* clone exhibited high similarities of >99.7% with the sequences of *B. subtilis* detected hundreds of meters above the Taklamakan Desert, China, and from the dust layers in the snow cover of Mount Tateyama, Japan (2,450 m) (Fig. 5a). Moreover, in the class *Bacilli*, three clones belonged to the family *Staphylococcaceae*, exhibiting striking similarities with *Staphylococcus epidermidis*. Among the 15 clones belonging to the class *Actinobacteria*, four were closely related to *Propionibacterium acnes*, and 11 were related to *Microbacterium oxydans*. The sequences of representative clones in the class *Actinobacteria* showed >99.8% similarity to the sequences of *P. acnes* or *M. oxydans* (Fig. 5a).

The sequences of 10 clones derived from the air samples at 1,000 m and 10 m exhibited >98.9% similarity with the sequences of *Deinococcus caeni*; the representative clones clustered with some sequences of *D. caeni* (Fig. 5b). The air samples at 1,000 m and 10 m also included 71 clones belonging to the genus *Synechococcus* in the class *Cyanobacteria*, and their representative sequences exhibited low similarities of 96.0% to the sequences of *Synechococcus* species (CP0000977).

Sequences belonging to the class *Flavobacteria* were recovered only from the air samples at 3,000 m and 1,000 m and occupied 31 clones in the clone libraries. In the

class *Flavobacteria*, 25 clones were related with *Cloacibacterium* species at >99.0% sequence similarity, and the representative clones were clustered with members of the genera *Cloacibacterium* and *Chryseobacterium* (Fig. 5c). Among the 36 clones belonging to *Alpha-proteobacteria*, 32 were related to the pelagic bacteria of the SAR clade such as bacterium SHI-7 at low sequence similarities of <92.0%, and the representative clones formed a single cluster with the sequences of pelagic bacteria (Fig. 5c).

4. Discussion

4.1 Atmospheric conditions and vertical aerosol distribution

Westerly winds blowing over Asian regions disperse airborne microorganisms to downwind environments (Iwasaka et al., 2009) and potentially influence ecosystem dynamics, human health, agricultural productivity, and climate change (Rodó et al., 2011; Brown and Hovmøller, 2002, Liu et al. 2014). The transport of particulate minerals by westerly winds has been reported to increase the amounts of airborne bacteria at the ground level (Hara and Zhang, 2012) and at high altitudes (Maki et al., 2013) during an Asian dust event. According to the analysis conducted using the Ozone Monitoring Instrument (http://jwocky.gsfc. nasa.gov/), a dust event occurred over the Chinese desert region 3 days before the sampling date (March 7, 2012). Back trajectories indicated that the air mass at 3,000 m had traveled from the Chinese desert region to the Noto Peninsula during the 3 days before March 10, 2012 (Fig. 1b). Consequently, low amounts of aerosol particles (dust particles) were observed over

Japan on March 10, 2012 (Fig. S1). In general, the westerly winds blowing over Asian regions reportedly cause a constant weak level of dust events in the free troposphere every spring (Iwasaka et al., 1988). During the sampling period, a cloud layer was observed between 700 m and 2,000 m, and the air mass at 3,000 m, which included dust particles, barely mixed with the boundary layer below 2,000 m over the Noto Peninsula.

In this investigation, air samples were collected at 3,000 m, 1,000 m, and 10 m, allowing a comparison of the airborne bacterial communities at these altitudes. Fluorescence microscopy using DAPI staining revealed the presence of several kinds of particles in every air sample (Fig. S4). Bacterial particles at 3,000 m and 10 m showed slightly higher concentrations compared to those at 1,000 m, indicating that the weak dust event in the troposphere increased bacterial density at 3,000m. Yellow fluorescent particles have been shown to be organic material originating from proteins and other microbial cell components (Mostajir et al., 1995), and are similar to the yellow cells that have appeared in cultures of microorganisms such as *Bjerkandera adusta* and *Bacillus* spp. (Liu et al., 2014). The concentration of yellow particles decreased with decreasing altitude (Fig. 3b). These data suggest that the yellow particles originated from the microorganisms on the Noto Peninsula or in the surrounding sea. Specific appearance of black carbon at the low altitude of 10 m suggested the local human contaminations in Japan such as biomass burning, industrial activities, and vehicle exhaust. The air mass at 3,000 m mainly including China continental aerosols can be compared with those at lower altitudes (1,000 m and 10 m) mixed with Japanese local aerosols. This comparison is valuable for understanding the mechanism of formation of vertical bacterial distributions over downwind regions by westerly winds, after a dust event and

the formation of cloud layers. DAPI staining technique slightly overestimates the airborne bacterial concentrations in the comparison to real-time PCR analysis (Kobayashi et al., 2015; Fig. S4), because some of small-size mineral particles (0.2-1.0 μm) emit blue fluorescence are possibly counted as bacterial particles (Fig. 3S) and the inhibitors, such as humic acid, included in the air-samples would reduce the amplification process of real-time PCR analysis. The overestimation of DAPI staining technique is negligible for discussing about the abundances of airborne bacteria.

4.2 Bacterial community structures among three altitudes

Pyrosequencing and clone library analyses of the air samples collected at the three altitudes revealed that the bacterial communities in every sample were found to be composed of several phylotypes. Chao 1 analysis of the pyrosequencing data showed that the number of bacterial species in the air samples ranged from 270 to 410. In a previous study, microarray analysis detected approximately 400 species in airborne bacterial communities on mountains (Smith et al., 2013), consistent with the estimated numbers observed in this study. In sequential surveys of a mountain site, airborne microbial communities at the higher altitudes of mountains also included highly diverse bacterial populations (Bowers et al., 2012). The main phylotypes in the three air samples were clustered into the classes *Deinococci*, *Bacilli*, *Cyanobacteria*, *Proteobacteria*, *Flavobacteria* and *Actinobacteria* (Fig. 4a), which have been reported to be airborne bacterial populations on European mountains (Vaïtilingom et al., 2012) as well as over Asian rural regions (Woo et al., 2013). In our previous investigations performed over the Noto Peninsula during two dust events, the 16S rDNA clone

libraries recovered from air samples were composed of only a few members of the two classes *Bacilli* and *Actinobacteria* (Maki et al., 2013). In contrast, in the present investigation, the numbers of bacterial phylotypes were higher in every air sample, because the numbers of clones increased and the pyrosequencing analysis was applied.

Some members of terrestrial bacteria such as *Bacilli* and *Actinobacteria* exhibited more sequences in the air samples obtained at 3,000 m than at 1,000 m and 10 m, while marine bacterial members such as *Alpha-proteobacteria* and *Cyanobacteria* were dominant at 1,000 m and 10 m (Fig. 3a). Members of *Bacilli* and *Actinobacteria* were predominantly detected at high altitudes above the Taklamakan Desert (Maki et al., 2008) and above downwind areas during Asian dust events (Smith et al., 2012; Maki et al., 2013). Bowers et al. (2011) investigated airborne microbial communities over several land-used environments demonstrating that the *Bacilli* and *Actinobacteria* sequences in the air samples were mainly originated from terrestrial environments. The phyla *Proteobacteria*, of which some members were detected from pyrosequencing databases in this study, were often reported to dominate in airborne bacterial communities in the air samples collected over mountains (Bowers et al., 2009; Vaïtilingom et al., 2012; Temkiv et al., 2012), on a tower (Fahlgren et al., 2010) and from troposphere (DeLeon-Rodriguez et al., 2013, Kourtev et al., 2011). During the non-dust event, *Cyanobacteria* and *Proteobacteria* occupied most sequences of pyrosequencing data (Fig. S5b), suggesting that these bacterial populations are constantly transported from local areas. According to the back trajectory analysis, in the 3 days prior to sampling, the air mass at 3,000 m moved from the Chinese desert region to the Noto Peninsula and immediately crossed the Sea of Japan, whereas the air masses

at 1,000 m and 10 m were suspended over the Sea of Japan. It is likely that the air mass at 3,000 m contained relatively high amounts of dust particles and was dominated by bacterial populations originating from the terrestrial or desert environments in China. Inside the boundary layer, the air masses at 1,000 m and 10 m were mixed vertically with accompanying airborne bacterial populations, which were transported from marine areas and blown up from local ground surfaces.

4.3 Bacterial populations associated with dust events

Sequences related to members of the genus *Bacillus* dominated in the pyrosequencing database and clone libraries recovered from all air samples, in particular, occupying more than 40% of the total sequences in the air sample at 3,000 m (Figs. 3 and 4a). Some clones recovered from the 3 air samples were identical to *B. subtilis*, which were detected at high altitudes above the Taklamakan Desert (Maki et al., 2008) and were associated with the Asian dust mineral particles collected in downwind areas (Maki et al., 2010; Jeon et al., 2011; Tanaka et al., 2011; Maki et al., 2014; Fig. 4a). Species related to *B. subtilis* were found to be dominant species in the sand samples of the Chinese continental desert (An et al., 2013) and the Antarctic desert (Bottos et al., 2014), and in the air samples collected from the free troposphere during Asian dust events (Smith et al., 2012; Maki et al., 2014). The isolations of *Bacillus* species were also predominantly obtained from dust samples collected in downwind areas (Hua et al., 2007; Gorbushina et al., 2007). Members of the genus *Bacillus* are reported to form resistant endospores that support their survival in the atmosphere (Nicholson et al., 2000). Accordingly, the dominant members of *Bacillus* species at high altitudes have the

potential ability to be transported over long distances and spread their habitant area in downwind environments.

Non-spore-forming bacterial species such as *Staphylococcus hominis* and *Propionibacterium acnes* were also dominant in the high-altitude samples (Figs. 4 and 5a), increasing the relative abundances of the pyrosequencing database at 3,000 m and 1,000 m. Members of the genera *Propionibacterium* and *Staphylococcus* have often been detected in and isolated from aerosol samples collected in downwind areas during dust events (Griffin et al., 2003; Kellog et al., 2004). Possibly, these bacteria, which are attached to or incorporated into large-size particles, are protected against UV radiation and desiccation in the atmosphere.

4.4. Bacterial populations carried from marine regions

Marine bacterial sequences of pelagic bacteria of *Alpha-proteobacteria* (Brinkmeyer et al., 2003) and *Cyanobacteria* dominated in the phyrosequencing data bases obtained from the samples of 1,000 m and 10 m (Figs. 3 and 5bc). The *Cyanobacteria* clones obtained in this study were closely related to the *Synechococcus* spp. that were found in the Sea of Japan and East China Sea (Choi and Noh, 2009; Fig. 4b). In the class *Alpha-proteobacteria*, most clones of the samples of 1,000 m and 10 m belonged to the pelagic bacterial groups of the SAR clade that have been ubiquitously and predominantly detected in the East Sea (Song et al., 2009). As described, the back trajectory analysis showed that the air masses at 1,000 m and 10 m were suspended over the Sea of Japan in the 3 days prior to sampling. Bacterial populations originating from marine areas were found to predominantly increase in cloud droplets in comparison to

those from terrestrial sources, suggesting that the ocean represents a major source of bacteria in clouds (Amato et al., 2007). In this study, the cloud layer between 700 m and 2,000 m was also expected to carry marine bacteria originating from the Sea of Japan to the atmosphere at 1,000 m and 10 m.

Atmospheric aerosols via marine areas were reported to carry marine bacteria belonging to the classes *Cyanobacteria* and *Alpha-proteobacteria*, which were dominant in clone libraries recovered from air samples collected from European (Polymenakou et al. 2008) and Japanese regions (Maki et al., 2014). Sequences of *Cyanobacteria* were reported to dominate in cloud water samples collected at the approximately 3,000 m above ground level indicating the atmospheric transport of *Cyanobacteria* populations (Kourtev et al., 2011). *Cyanobacteria* including *Synechococcus* spp., can eliminate excess peroxide from photosynthesis to resist UV radiation and oxygenic stress (Latifi et al., 2009). The pelagic bacteria of the SAR clade are marine oligotrophs that form ultramicrocells (<1.0 μm) to survive under certain environmental stressors as well as low substrate concentrations (Morris et al., 2002). However, the sequences determined in this study exhibited low similarities (approximately 96%) to the known sequences of pelagic bacteria of the SAR clade (bacterial strain SHI-7) and *Synechococcus* spp. (CP0000977) (Table 1), suggesting that novel bacterial species were transported from the Sea of Japan.

4.5. Ubiquitous bacteria detected in extreme environments

The sequences of *Deinococcus* species predominately appeared in the air samples at 1,000 m and 10 m (Figs. 4 and 5b). However, *Deinococcus*-related sequences were

not detected in the sample of 3,000 m, suggesting that these species originated from local terrestrial areas or marine environments. Members of the genus *Deinococcus* are known to exhibit high resistance to UV and gamma radiation, owing to their red or yellow pigments and their ability to repair damaged DNA (Asker et al., 2008). Air-dried *Deinococcus* cells have exhibited higher resistance to environmental stressors than normal cells (Bauermeister et al., 2011). Accordingly, *Deinococcus* species have often been detected in extreme environments such as high-altitude atmospheric areas as dominant species.

Sequences belonging to the class *Flavobacteria* and the genus *Microbacterium*, both of which are ubiquitous and can survive in extreme environments, appeared mainly at 3,000 m and 1,000 m (Fig. 4). Most of the *Flavobacteria* sequences in the pyrosequencing database and clone library were closely related to *Cloacibacterium* species (Fig. 5c) that have been isolated from the terrestrial samples of Antarctic soil (Yi et al., 2005) and freshwater lake sediments in Nepal (Cao et al., 2010). Thus, there is a possibility that these species were transported from cold environments of continental areas to the atmosphere over the Noto Peninsula. *Microbacterium* species are reported to survive and sometimes dominate in extreme environments such as alkaline groundwater (Wu et al., 2011). In arid areas, minerals, including sodium, were accumulated on dune surfaces, causing alkaline environments (Dianwu et al., 1988). Accordingly, bacterial populations in alkaline desert environments are expected to be one of the sources of airborne bacteria, such as *Microbacterium* species, that may be transported over a long distance.

5. Conclusion

Bioaerosols were collected at the three altitudes of 3,000 m, 1,000 m, and 10 m over the Noto Peninsula, when a weak dust event occurred at high altitudes and cloud layers formed among the altitudes at 700 m and 2,000 m. Bacterial communities in the air samples displayed different compositions among the three altitudes. The air sample at 3,000 m was dominated by terrestrial bacteria, such as *Bacilli* and *Actinobacteria*, which are thought to originate from continental areas, while the air samples at 1,000 m and 10 m contained bacterial populations of marine origin, including members of *Cyanobacteria* and *Alpha-proteobacteria*. Some species of *Bacilli* and *Proteobacteria* are well known as pathogens of plants, animals, and humans (Morris et al., 2008), whereas heterotrophic bacteria in these classes have been found to contribute to geochemical cycling in the oceans and terrestrial areas (Ulrich et al., 2008). Thus, the transport of bioaerosols plays an important role in dispersing bacterial populations that are potentially associated with microbial, plant and animal ecosystems, human health, organic matter cycles, and geochemical processes. Further investigations combining culture techniques with culture-independent analyses are required to accurately evaluate the influence of transported bioaerosols on ecosystem dynamics and human societies in and around downwind environments.

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Figure Legends

Fig. 1. (a) Sampling location and aircraft flight routes during the sampling period from 11:40 JTC to 12:40 JTC on March 10, 2012 (dotted lines). (b) Three-day back trajectories of aerosols that arrived at 3,000 m, 1,000 m, and 10 m at Suzu, Japan from 2:00 UTC and 8:00 UTC on March 10, 2012.

Fig. 2 Vertical variations of temperature (closed squares), relative humidity (closed circles) and pressure (open circles) from 10 m to 4,100 m above ground level over the Noto Peninsula at (a) 9:00 JTC and (b) 21:00 JTC on March 10, 2012.

Fig. 3 (a) Concentrations of aerosol particles with diameter sizes of 0.3–0.5 μm (closed squares), □0.5–0.7 μm (closed triangles),□0.7–1.0 μm (closed circles), 1.0–2.0 μm (closed diamonds), 2.0–5.0 μm (crosses), and >5.0 μm (open circles) in the atmosphere at 3,000 m, 1,000 m, and 10 m above ground level over the Noto Peninsula on March 10, 2012. (b) Concentrations of all bacterial particles (open circles), white particles (closed circles), yellow fluorescent particles (closed triangles), and black carbon (closed squares) in air samples collected at the three altitudes.

Fig. 4. Changes in bacterial compositions at (a) class level and (b) family level of the partial sequences in the pyrosequencing database (ca. 400 bp) and 16S rDNA clone libraries (ca. 600 bp) obtained from bioaerosol samples collected at altitudes of 3,000 m, 1,000 m, and 10 m over the Noto Peninsula on March 10, 2012.

Fig. 5. Phylogenetic tree including the partial sequences of 16S rDNA amplicons obtained from the clone libraries (Szp series) from the bioaerosols collected at 3,000 m, 1,000 m, and 10 m over the Noto Peninsula and the known members of (a) *Bacilli* and *Actinobacteria*, (b) *Cyanobacteria* and *Deinococci*, and (c) *Flavobacteria* and *Alpha-proteobacteri*. The phylogenetic tree was calculated from a dissimilarity matrix of (a) an approximately 1,400-bp alignment (*E. coli* numbering 89 to 1,435), (b) an approximately 1,400-bp alignment (*E. coli* numbering 88 to 1,435), and (c) an approximately 1,100-bp alignment (*E. coli* numbering 95 to 1,098) using a neighbor-joining algorithm. The names of representative clones are indicated in the phylogenetic tree, and the numbers of clones, full-length sequences of which were determined and analyzed, are given in parentheses. Open circles at branch points indicate that bootstrap values obtained by neighbor-joining analysis exceeded 50% (after 1,000 resamplings).

Table 1. Phylogenetic affiliation of 16S rDNA gene sequences obtained from clone libraries.

Category	Clone No.[*1]	Numbers of clones of each samples[*2] 3000	1000	10	Length (bp)[*3]	GenBank accession no.	Closest relative	Similarity (%)[*4]
Cyanobacteria	12Sztp1000-11	0	27	44	1460	LC002531	*Synechococcus* sp. CC9902 (CP000097)	96
	12Sztp1000-66	7	0	7	975	LC002532	*Synechococcus* sp. WH 8109 (CP006882)	98.8
	12Sztp10-22	0	0	2	523	LC002533	bacterium WHC3-9 (JQ269283)	92.1
	12Sztp10-55	0	0	1	682	LC002534	*Halospirulina* sp. EF17(2012) (JX912466)	97.4
Deinococci	12Sztp1000-16	0	2	5	1463	LC002535	*Deinococcus caeni* strain Ho-08 (DQ017709)	98.9
	12Sztp1000-63	0	1	0	839	LC002536	*Deinococcus* sp. MBIC3950 (AB022911)	99.8
	12Sztp10-66	0	0	2	1399	LC002537	*Deinococcus* sp. MBIC3950 (AB022911)	98.8
Actinobacteria	12Sztp3000-33	4	5	2	1485	LC002538	*Microbacterium oxydans* (KF150504)	99.9
	12Sztp1000-64	2	2	0	1425	LC002539	*Propionibacterium acnes* ATCC 11828 (CP003084)	99.9
Bacilli	12Sz1000-15	32	11	3	1509	LC002540	*Bacillus subtilis* (EU257448)	99.5
	12Sztp3000-69	25	9	5	1451	LC002541	*Bacillus subtilis* (JQ403532)	99.4
	12Sztp1000-22	7	16	19	1512	LC002542	*Bacillus megaterium* (HM771662)	99.7
	12Sztp10-68	2	3	5	1440	LC002543	*Staphylococcus epidermidis* (JN644522)	99.8
	12Sztp1000-4	0	1	1	592	LC002544	*Anaerococcus* sp. 8405254 (HM587319)	96.6
	12Sztp10-39	0	0	2	543	LC002545	*Abiotrophia defectiva* (AY879307)	99.1
	12Sztp10-48	0	0	1	529	LC002546	*Bacillus amyloliquefaciens* (KJ572221)	100.0
Flavobacteriia	12Sztp3000-44	8	5	0	1122	LC002547	*Cloacibacterium rupense* (AB682228)	99.0
	12Sztp3000-39	9	3	0	538	LC002548	*Cloacibacterium normanense* (AJ575430)	99.8
	12Sztp1000-14	0	3	0	589	LC002549	*Porphyromonas catoniae* (AB547656)	98.0
	12Sztp1000-13	0	2	0	590	LC002550	*Fibrella aestuarina* BUZ 2 (HE796683)	100.0
	12Sztp1000-29	0	1	0	593	LC002551	Flavobacteriaceae bacterium YMS-2 (EF017801)	99.7
Alpha-proteobacteria	12Sztp3000-7	6	5	12	1374	LC002552	bacterium SH1-7 (JQ269250)	91.4
	12Sztp1000-65	0	4	5	1368	LC002553	bacterium SH1-7 (JQ269250)	91.1
	12Sztp3000-55	1	3	0	543	LC002554	*Sphingomonas* sp. D31C2 (AY162145)	99.6
Beta-proteobacteira	12Sztp3000-46	4	0	0	834	LC002555	*Massilia* sp. 9B11b1 (FR865962)	99.3
	12Sztp10-54	0	1	1	1141	LC002556	*Snodgrassella alvi* wkB2(JQ746651)	93.1
Gamma-proteobacteira	12Sztp3000-50	1	1	0	551	LC002557	*Acinetobacter junii* (KJ147092)	100
	12Sztp3000-49	1	0	0	538	LC002558	arsenite-oxidizing bacterium NT-5 (AY027498)	97

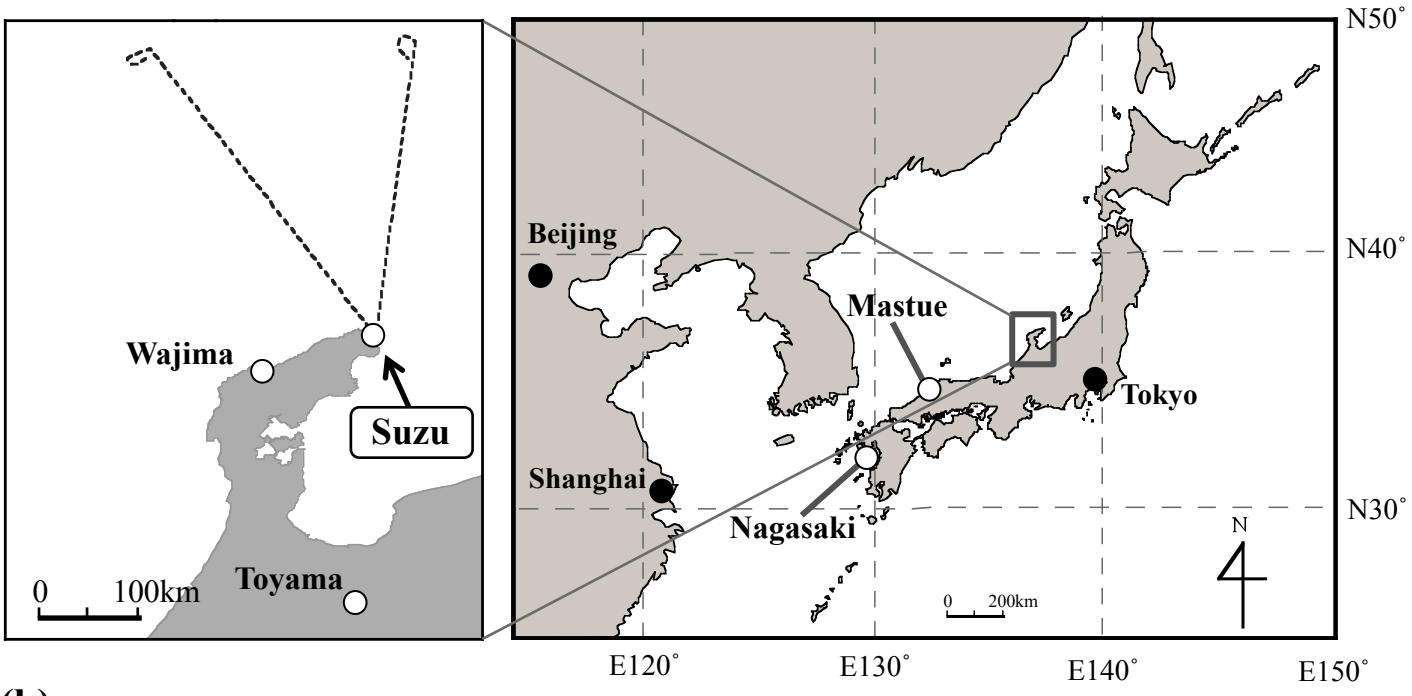
***1 Representative clones in 16S rDNA library indicate phylotepes that were classified with more than 97% similarity, and were named as the 12Sztp series.**

***2 Numbers of the clones in 16S rDNA clone libraries recovered from the air samples collected at altitudes of 3,000 m, 1,000 m and 10 m.**

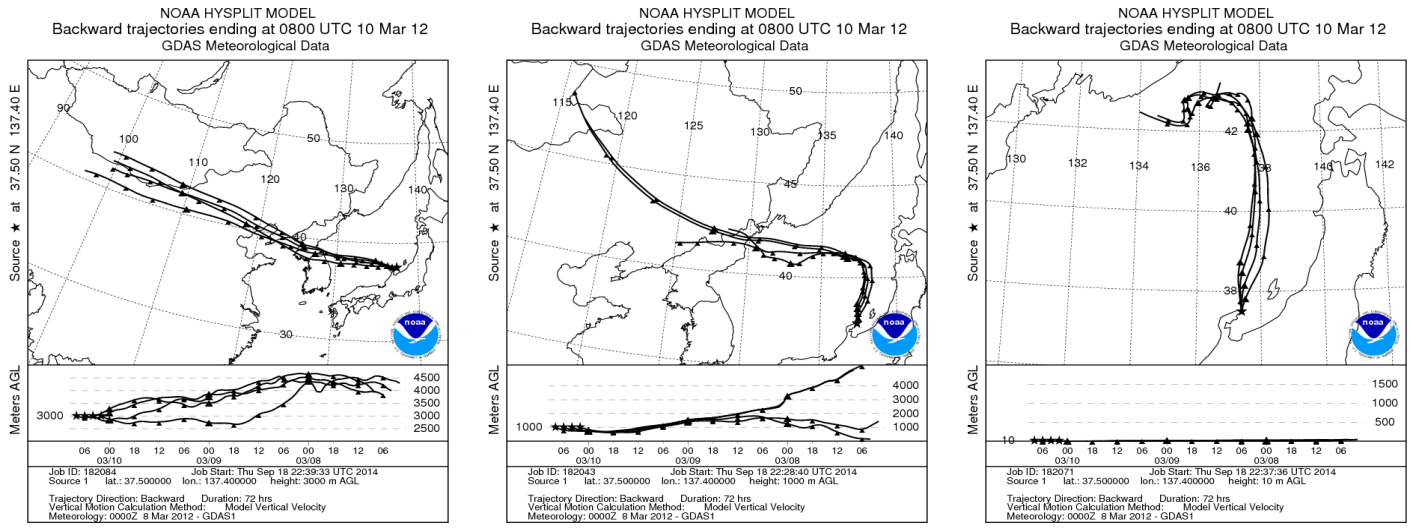
***3 The sequence length of each representative clone.**

***4 Similarity value between each representative clone and the closest relative in databases.**

(a)



(b)



3,000 m **1,000 m** **10 m**

Fig. 1 T.Maki et al.

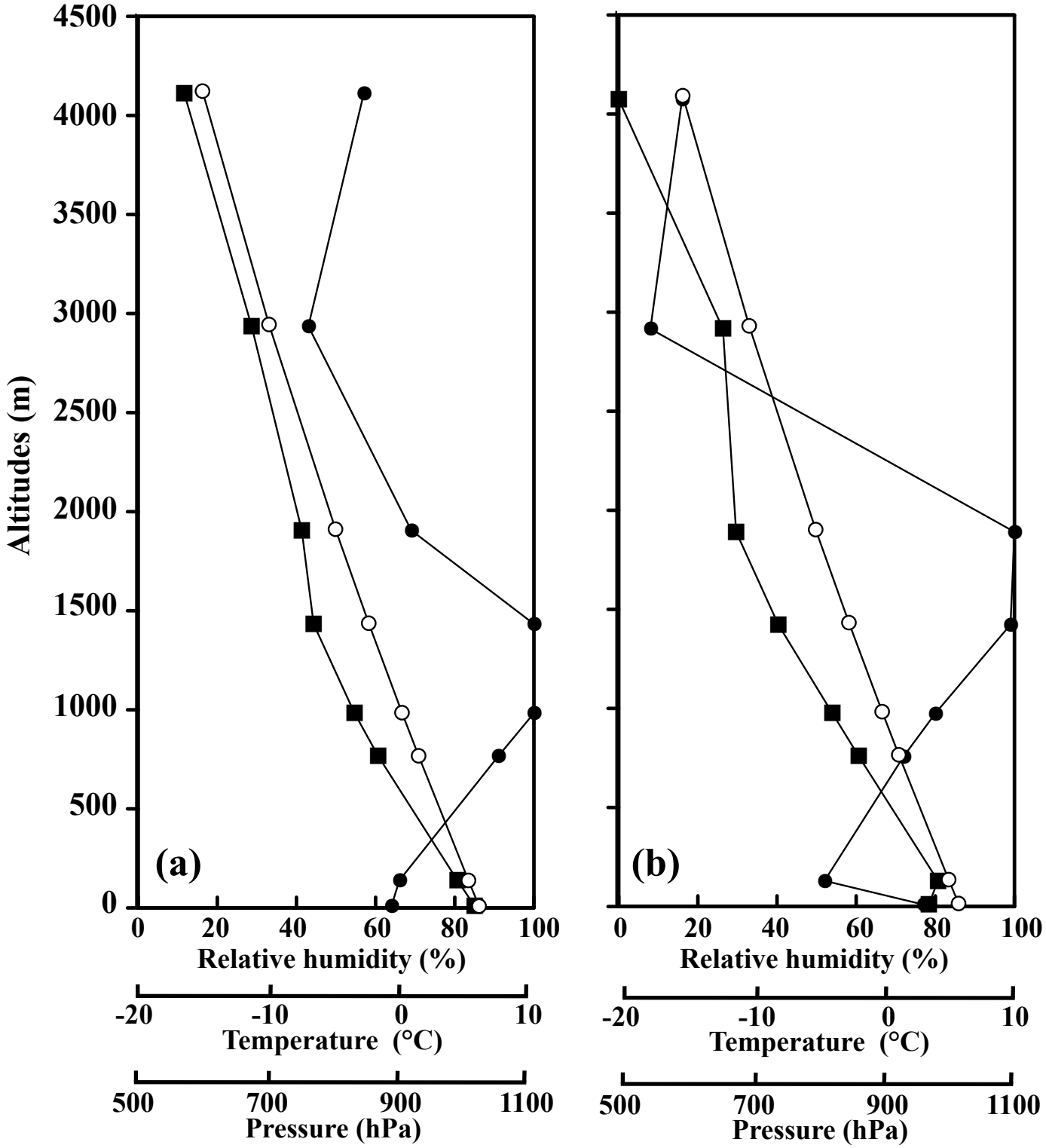


Fig. 2 T.Maki et al.

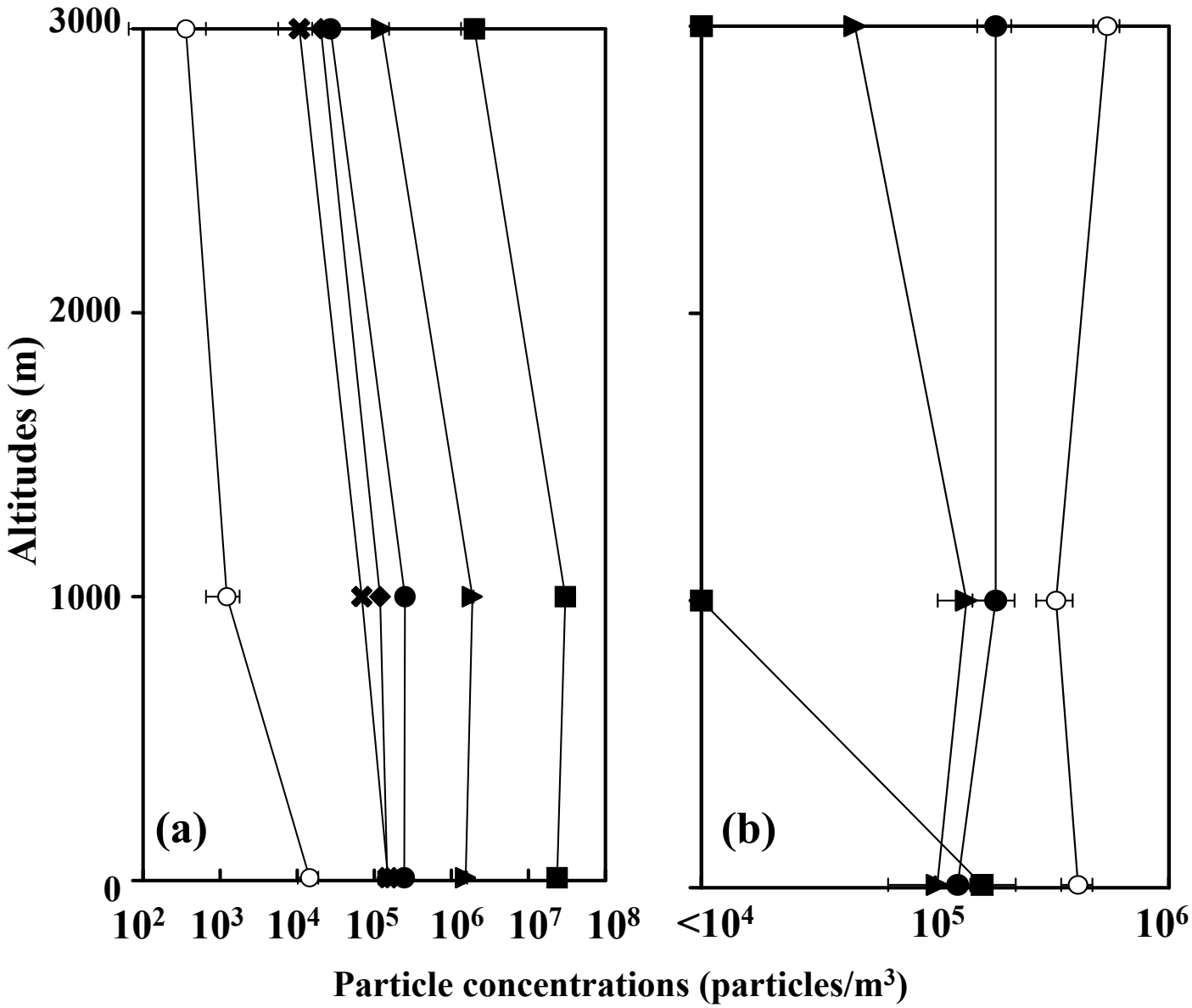


Fig. 3 T.Maki et al.

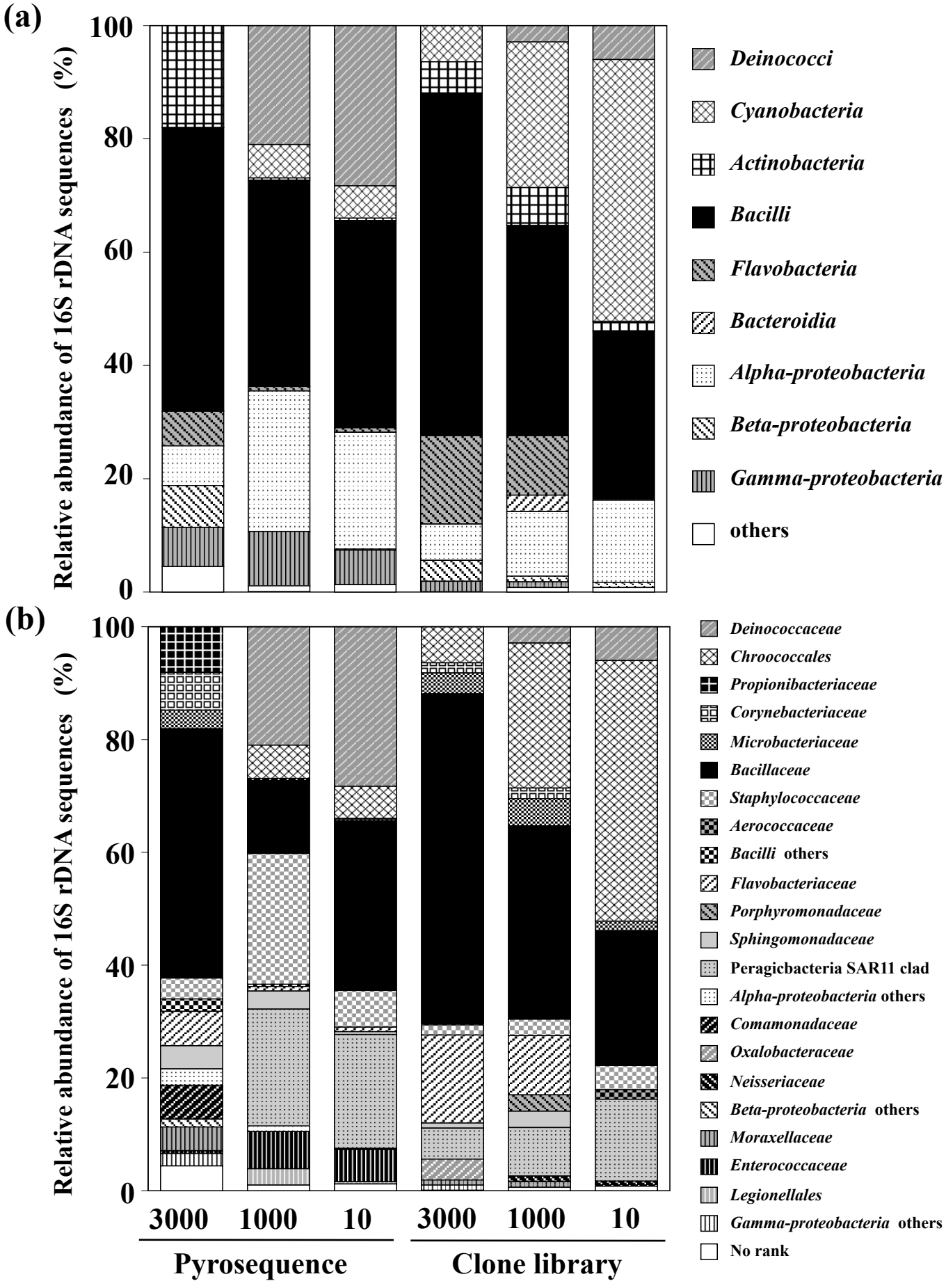


Fig. 4 T.Maki et al.

(a)

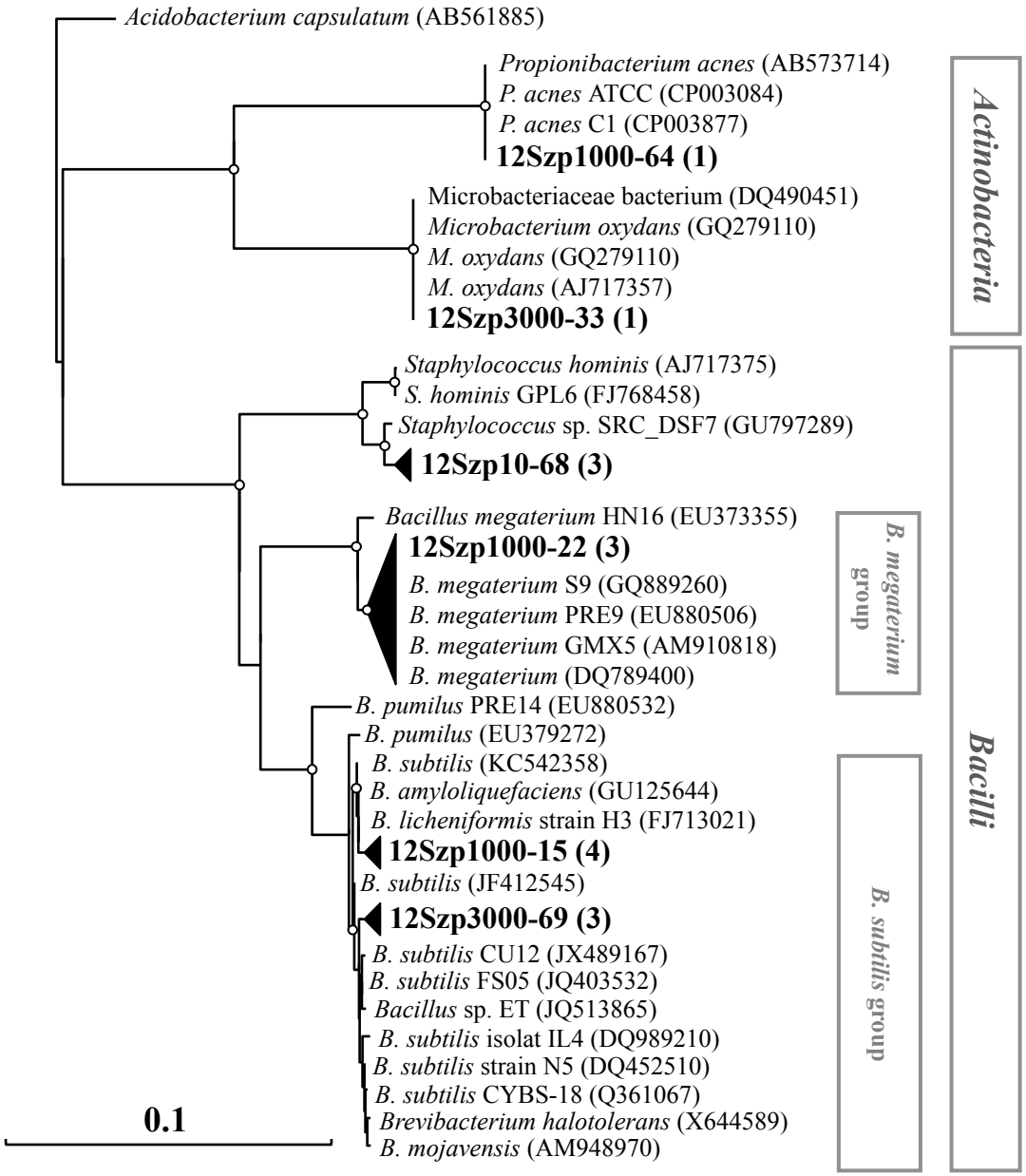


Fig. 5 T.Maki et al.

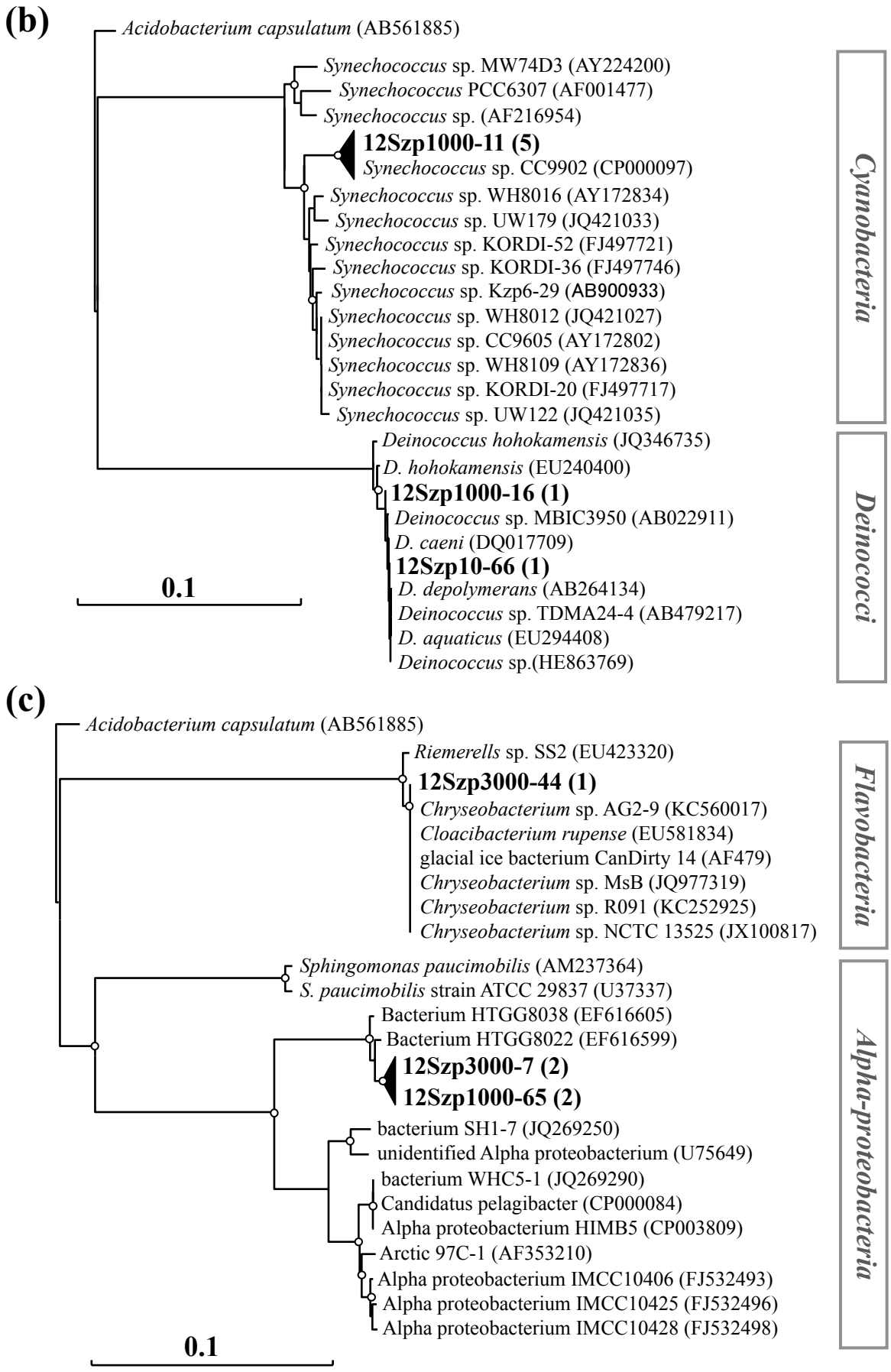


Fig. 5 T.Maki et al.