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Vertical distribution of airborne microorganisms over forest environments: A potential source of ice-nucleating bioaerosols

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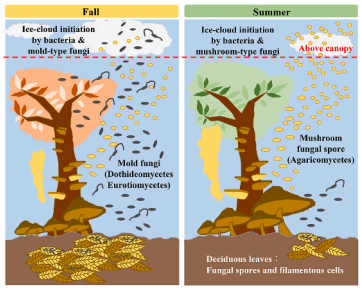
HIGHLIGHTS

- Bioaerosols were collected from ground to high altitude at the forest environments.
- Terrestrial and phyllospheric bacteria were dominated vertically over forest.
- Mushroom- and mold-type fungi in airborne fungal communities changed seasonally.
- Ice-nucleating microorganisms were obtained from the aerosols at heights of 500 m.

GRAPHICAL ABSTRACT



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ABSTRACT

Airborne microorganisms transported from forested areas can influence cloud formation by forming ice nuclei. However, the vertical transportation of airborne microorganisms over forested areas is not well understood. We collected aerosols at three heights, [ground (2 m), canopy top (20 m), and above canopy (500 m)] during the summer, fall and winter, to analyze the airborne microbial communities that were distributed vertically over the forest. During summer and fall, the microbial particles maintained similar concentrations in the forest zone (canopy top and ground) and decreased to 1/10th of the microbial concentration in the above canopy area. The particle concentrations in winter indicated efficient vertical mixing below 500 m. High-throughput DNA sequencing revealed that the airborne microbial communities were composed of terrestrial and phyllospheric species associated with the degradation of decaying plant litters. Regardless of the three seasons, the above canopy was dominated by atmospheric stress-resistant bacteria from the phyla Actinobacteria and Firmicutes. Unlike bacteria, the mushroom-type fungal members of Agaricomycetes grew in relative abundance above the canopy, primarily throughout the summer and winter, while mold-type fungal Dothideomycetes species were often found at all three heights during the fall. The *Fusarium, Pseudomonas*, and *Bacillus* isolates, which were obtained from air samples at three heights, indicated high activities of ice nucleation in the water-drop freezing

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assay. Therefore, ice-nucleating microbial taxa likely originated from fungal and bacterial communities in the soil litter and plant surfaces of the phyllospheric environments.

1. Introduction

Bioaerosols containing bacteria, fungi, plants, and animals account for 20–70% of atmospheric aerosols (Prospero et al., 2005). Microbial particulates (Möhler et al., 2007; Creamean et al., 2013; Joly et al., 2013; Huang et al., 2021) and plant products (Lim et al., 2005) are the most active ice nuclei, possibly contributing to the initiation of precipitation by heterogeneous ice nucleation in ice clouds. Ice-cloud formation plays an important role in atmospheric radiative transfer and the geochemical cycles of atmospheric constituents (Hoose and Möhler, 2012; Murray et al., 2012). The rapid ice-cloud formation at low temperatures between −8 and −3 °C (Hallet and Mossop, 1974) is thought to depend on the ice-nucleation of airborne microorganisms (Möhler et al., 2007; Delort et al., 2010; Creamean et al., 2013). Indeed, the ice-nucleating cell components of some microbial isolates in the *Pseudomonas* and *Fusarium* species associated with plants exhibit high nucleation activities, initiating ice formation at relatively warmer temperatures (from −5 to −2 °C) (Pouleur et al., 1992; Morris et al., 2008) than inorganic ice nuclei such as potassium feldspar (approximately −18 °C) (Atkinson et al., 2013). Hara et al. (2016) previously demonstrated that airborne bacteria carried by westerly winds over East Asia could initiate high levels of ice nucleation. Bioaerosols with high nucleation activities are found to increase during rainfall, and some exhibit fungal spore forms under a microscope (Iwata et al., 2019). Therefore, some ice-nucleating bioaerosols are believed to contribute to ice formation in tropospheric clouds more efficiently than inorganic substances at warmer atmospheric temperatures (Murray et al., 2012; Christner et al., 2008). An aircraft survey demonstrated the effects of bioaerosols on ice particle concentrations at high altitudes in the stratiform region (Patade et al., 2022).

Surveys using assays, such as mass spectrometry and fluorescent detection systems, have revealed that the organic matters in aerosols from forest environments is biological in origin (Elbert et al., 2007; Huffman et al., 2013; Prenni et al., 2013). The bioaerosols collected under the alpine forest canopy were found to contain mainly fungal and bacterial cells related to variations in ice nucleic concentrations (Seifried et al., 2021). The forest ice nuclei characteristics change seasonally in correspondence with bioaerosol abundance (Schneider et al., 2021). Therefore, bioaerosols originating from forests are expected to have a primary influence on ice formation in clouds. However, the community structures of airborne microorganisms, which are distributed vertically over forested areas, remain poorly understood. Additionally, there are no studies on ice-nucleating microorganisms isolated from air particles suspended at higher altitudes in forested areas. Moreover, the sources, vertical mixing conditions, and controlling factors of airborne microorganisms in the air within the canopy zone (from the ground to the canopy top) should be elucidated to establish airborne microorganisms transported from the canopy zone to the open atmosphere. High-throughput DNA sequencing techniques, which allow for higher-resolution analyses of airborne microbial communities, are powerful tools for characterizing airborne microorganisms in forest environments (Igarashi et al., 2019). Indeed, culture-independent analyses of bacterial taxonomic composition revealed that the high diversity of bacterial communities in cloudy air (Bowers et al., 2009) and dust samples (Maki et al., 2018) possibly influenced ice-nucleation in high-elevation areas. The airborne microbial composition at the dryland also changes after rainfall, which affects ice nucleic characteristics (Tang et al., 2022).

In this study, to investigate the vertical distribution of airborne bacterial and fungal communities in a forest environment, air samples were collected above the canopy (500 m), as well as at the canopy top (20 m) and ground level (2 m) from the forest sampling area of Tsukuba Botanical Garden in Japan. The vertical distribution of bioaerosol concentration was determined using fluorescent microscopy and an optical particle counter (OPC). The structures of the bacterial and fungal communities in the air samples were analyzed using high-throughput DNA sequencing targeting bacterial 16S rRNA genes and fungal internal transcribed spacer (ITS) regions. Finally, the ice-nucleation potentials of the microbial isolates were evaluated using dilution culturing and water-drop freezing assays.

2. Materials and methods

2.1. Aerosol sampling and environment condition analyses

Aerosol sampling surveys were performed in the forest of Tsukuba Botanical Garden (36° 6' 11" N, 140° 6' 41" E) during the summer (June 26–28, 2018; June 1–3, 2019), fall (October 29–30, 2018; November 7–8, 2019; November 11–13, 2020), and winter (January 6–7, 2020) (Table S1). Air samples were collected at a height of 500 m above the forest canopy using a helicopter sampling technique (above-canopy sample), as well as at the canopy top (20 m; canopy sample) and botanical ground surface (2 m; ground sample). The Tsukuba Botanical Garden possesses an experimental forested area in which the forest ground and canopy can be accessed simultaneously using a building, and high-altitude sampling by a helicopter can be performed using a nearby heliport.

Since the ground and canopy sampling sites were located in the middle of the experimental forested area, aerosols originating from the forest can be collected mainly to avoid outside influences of the Tsukuba Botanical Garden. Helicopter sampling processes were employed in our previous survey (Maki et al., 2017) (Fig. S1); the washdown-wind turbulence by the helicopter rotor is negligible during the forward movement at speeds greater than 60 km/h (Watanabe et al., 2016). During the helicopter sampling period, the helicopter navigated around a 3 km-diameter circle area at the center of the ground and canopy sampling site. Aerosols were placed into the helicopter inlet with an uptake hole (3 cm in diameter) and collected onto the polycarbonate filters. The OPC measurements demonstrated that the same concentrations of aerosols could be trapped in the inlet at speeds below 90 km/h (data not shown). Bioaerosol samplers with filter holders and an air pump were set on the botanical ground surface and held on the top of the canopy via the building rooftop. For the sampling procedures, aerosols were collected through three or eight sterilized polycarbonate filters with a diameter of 13 mm (0.22-μm pore size; Whatman, Tokyo, Japan) (three filters: canopy sample and ground sample, eight filters: above-canopy sample). The sampling periods at the three altitudes accompanied by helicopter sampling were 45–200 min, and the other sampling periods at the canopy top and botanical ground surface were 270–1000 min. The sampling rates were 0.6 L/min for each filter. Finally, 31, 26, and 7 samples from the ground, canopy, and above-canopy, respectively, were collected.

The temperature, potential temperature, wind speed, and wind direction around the sampling sites were analyzed using meteorological data from the Japan Meteorological Agency, and the mixture conditions of air masses above the canopy were evaluated using back-trajectory analyses (Kajino et al., 2021). Air particle concentrations at each height were measured using OPC (Rion, Tokyo, Japan). The OPC device was connected to the window of a helicopter and placed on a box on the forest ground (ground sampling) and the top floor of the building (canopy sampling). The ground and canopy sampling matching the helicopter sampling period was performed for the short periods ranging

from 45 to 200 min. Since large changes in aerosol numbers and meteorological conditions in this forested area were observed frequently during the initial phases of daytime or nighttime, the influences of sampling period variations during daytime or nighttime are thought to be avoided in this survey. For example, samples 19TkU-5 and 19TkU-H collected for 435 and 60 min, respectively, indicated similar microbial community structures.

Fluorescent particles stained with 4, 6-diamidino-2-phenylindole (DAPI) were counted using epifluorescence microscopy. After 0.2 mL of 1% paraformaldehyde solution was added directly to a filter in one filter holder to fix the aerosols (with a 1 h incubation time), the aerosol was stained by directly adding 0.5 μg/mL DAPI solution and incubated for 15 min (Russell et al., 1975). Filter slides were prepared using low-fluorescence immersion oil, and particles on the filter were observed using a fluorescence microscope (Olympus, Tokyo, Japan) with a UV excitation system. A filter transect was scanned, and the three categorized particles – including small particles with blue fluorescence (bacterial particles) and fungal-spore forming particles with blue or yellow fluorescence (fungal particles) on the filter transect – were counted using a previously reported observational technique (Igarashi et al., 2019). The detection limit of aerosols was 1.32×10^2 particles/filter (from 1.50×10^2 particles/m^3 to 7.00×10^3 particles/m^3), which can detect at least one particle under every view at the area of 1.0 mm × 1.0 mm.

2.2. Analyses of bacterial and fungal community structures

After the aerosol particles collected on another filter holder (270–1000 min of sampling) of the ground and canopy samples, and six samples (54–200 min of sampling) of the above-canopy sample were suspended in 3 mL of sterile 0.9% NaCl solution, the particles were centrifuged at 20,000×*g* for 10 min to pellet them. Sodium dodecyl sulfate, Proteinase K, and lysozyme were used to extract DNA from pelleted aerosols, which were then purified using phenol-chloroform extraction (Maki et al., 2017). Fragments of bacterial 16S rRNA genes (variable region V4; approximately 300 bp) and fungal ITS region (variable ITS1 regions; approximately 400 bp) were amplified from the extracted genomic DNA (gDNA) by 2 steps of PCR amplification. For the first PCR, the universal bacterial primers 515F (5′- Seq A - TGT GCC AGC MGC CGC GGT AA -3′) and 806R (5′- Seq B - GGA CTA CHV GGG TWT CTA AT -3′) (Caporaso et al., 2011) and the universal fungal primers ITS4 (5′- Seq A - TCC TCC GCT TAT TGA TAT GC -3′) and gITS 7 (5′- Seq A - GTG AAT CAT CGA RTC TTT G -3′) (Toju et al., 2012) were used. Seq A and Seq B represent nucleotide sequences targeted by the PCR primers used for the subsequent PCR steps. The first thermal cycle 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 52 °C for 2 min, and extension at 72 °C for 2 min for 23 cycles. In the second PCR, the targeted sequences in PCR products were amplified again using the second PCR forward primers (5′- Adaptor C - < Tag sequences > − Seq A -3′) and reverse primer (5′- Adaptor D - Seq B -3′), where Adaptors C and D were used for the sequence determination. Eight nucleotide sequence markers for sample identification barcoding were included in <Tag sequences>. The second thermal cycle was performed under the following conditions: denaturation at 94 °C for 1 min, annealing at 59 °C for 2 min, and extension at 72 °C for 2 min for 15 cycles. The PCR amplicons were purified using a MonoFas DNA purification kit (GL Sciences, Tokyo, Japan). PCR amplicons were used for high-throughput DNA sequencing on a MiSeq Genome Sequencer (Illumina, CA, USA). Paired-end sequences were grouped based on the tag sequences for each sample. In the PCR analysis steps, negative controls (template from unused filters that just set filter holders at air-sampling circumstances) contained no fragments of 16S rRNA genes and ITS amplicons. This indicates the absence of artificial contamination, such as aerosol contamination during sampling and experimental procedures.

Sequencing data for PCR amplicons were processed using the R package DADA2 v1.14 (Callahan et al., 2016). Primer sequences were removed using Cutadapt v2.8 (Martin, 2011). The reads were uniformly trimmed to 185 bp (forward) and 130 bp (reverse), and then filtered by removing reads exceeding the maximum expected errors (>2 for forward reads and >5 for reverse reads) or reads containing ambiguous N symbols. The reads were used to train the error model and then dereplicated to acquire unique sequences, which were then used to infer amplicon sequence variants (ASVs) using the trained error model. The forward and reverse reads were then merged, and the potential chimeric sequences were removed. Representative ASV sequences were identified using the RDP classifier (Wang et al., 2007) implemented in DADA2. SILVA nr v132 and UNITE 0.4.02.2020 databases were used to determine taxonomic compositions (Yilmaz et al., 2014), before the Chao 1 index and PCoA plots were obtained.

2.3. Isolation and identification of microorganism strains

The cell densities of culturable microorganisms in the air samples were determined using the Most Probable Number (MPN) procedure. Trypticase Soypeptone (TS) and potato dextrose (PD) liquid media (9 mL) were dispensed into the tubes used for the MPN procedure and autoclaved. The TS liquid medium for bacterial growth contained 17 g trypticase peptone, 5 g phytone peptone, 2.5 g K_2PO_4, 2.5 g glucose and 3.0 g NaCl in 1 L pure water. On the hand, PD liquid medium for fungal growth contained 4 g potato infusion (from 200 g potato) and 20 g glucose in 1 L pure water. The particle solution (1.0 mL) was injected into 9.0 mL of each liquid medium and 10-fold serially diluted to 10^{-3} in liquid media, after which the particles on the filter were suspended in 3.0 ml of liquid media. Diluted sample water (200 μl) was added to 24 wells of a 96-well microtiter plate and incubated at 20 °C at each dilution step from 10^{-1} to 10^{-3} dilution. After 14 days, the microplates were incubated at 20 °C in the dark, and the microbial growth in each well was evaluated by turbidity based on visual inspection. The concentrations of viable (culturable) microorganisms in the air samples were calculated using the MPN table (de Man, 1975). Additionally, microbial isolates were obtained from the growing wells of the microplates using the culture plate isolation technique.

After the isolates were incubated in 10 ml of culture medium for 3 days, microbial cells were collected by centrifugation at 20,000×*g* for 5 min gDNA was extracted from microbial cell pellets using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Fragments of the 16S rRNA gene (approximately 1450 bp) and ITS region (approximately 2000 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 (Caporaso et al., 2011) and SR1R, 5′-TAC CTG GTT GAT QCT GCC AGT-3′; ITS4, 5′-TCC TCC GCT TAT TGA TAT GC-3′ (Toju et al., 2012). Thermal cycling was performed using a Program Temp Control System PC-700 under the following conditions: denaturation at 94 °C for 1 min, annealing at 56 °C for 2 min and extension at 72 °C for 2 min for 30 cycles. The PCR amplicons were sequenced using a Dye Deoxy™ Terminator Cycle Sequencing Kit (ABI, CA, USA) and an ABI Prism 373A DNA Sequencer, according to the manufacturer's recommended protocols. The bacterial (27F and 1492R) and fungal primers (SR1R and ITS4) were used as sequencing primers. Amplicon sequences were investigated against the DNA Data Bank of Japan (DDBJ) using BLAST.

2.4. Water-drop freezing assay

The microbial cells of the isolates on the culture plates were suspended in 1.0 mL of sterile pure water. Suspended solutions were diluted using the pure water diluted 0.1 to 20-fold, which were then adjusted to the total cell densities of approximately 5.0×10^5 particles/mL liquid (from 1.0 to 2.0 μg/mL) and confirmed using fluorescence microscopic counts. In a sterile 96-well microplate, a 50 μL of liquid aliquot was

poured into each of the 24 wells. The 96-well microplate was placed onto an aluminum plate, and the measured temperature was decreased from 0 to −25 °C at a rate of 1.0 °C/min (Vali, 1971). For each assay, wells containing Arizona test dust (ATD: 2.0 μg/mL) and pure water were prepared as positive and negative controls, respectively. The percentage of isolates frozen at each temperature was calculated by dividing the number of isolates freezing more than one well by the total number of isolates.

2.5. Data processing

The Chao 1 values and DAPI-count particle concentrations were statistically compared among the three air sampling heights. After the total differences among the samples were assessed using a one-way analysis of variance (ANOVA), the survey series indicating significant differences using one-way ANOVA were used for Dunnett analysis to statistically estimate the differences among the three heights.

2.6. Accession numbers

All MiSeq sequencing data have been deposited in the DDBJ database, and the accession numbers of submissions are DRA014081 (ITS region) and DRA014080 (16S rRNA genes). The DNA sequencing data of microbial isolates have been also submitted as the accession numbers from LC757699 to LC75701, from LC759078 to LC759098 and from LC759603 to LC759637.

3. Results

3.1. Atmospheric conditions and vertical aerosol distribution

During the summer and fall, the coarse particles (>5.0 μm) measured by OPC maintained higher concentrations of more than 3.0×10^4 particles/m^3 at the canopy zone (the canopy top and ground level in the Tsukuba Botanical Garden) than above the canopy (500 m) (no more than 5.0×10^3 particles/m^3) (Fig. 1a). In contrast, coarse particles in the winter fluctuated at low concentrations of 10^3 particles/m^3 at all heights (Fig. 1a). Similar to the coarse particles, the fine-particle (0.3–5.0 μm) concentrations were higher in the summer and fall in the canopy zone than those in the above canopy, while those in winter were similar among all heights. Aerosols with a microbial size (>1.0 μm) were distributed vertically over the forested area, and the aerosol decrease rates from the canopy zone to the above canopy were much larger in the summer and the fall than in winter. The back trajectories during the sampling periods were from the Kanto plain area (including the sampling site) or the mountainous areas around the Japanese Honshu main island (Fig. S2). Changes in potential temperature, temperature, and wind direction were not observed at heights greater than 500 m above the ground during the helicopter sampling periods (Fig. S3). These results indicated that the atmospheric boundary layer vertically mixed the air mass above the sampling site.

Under fluorescent microscopy observation, bacterial cells, fungal spores and fungal filamentous cells with diameters ranging from 1.0 to 3.0 μm were observed in the air samples collected simultaneously (Fig. S4). The bacterial concentrations in the air samples collected at the canopy top (canopy sample) and ground (ground sample) showed similar concentrations between 3.4×10^5 and 5.0×10^6 particles/m^3 from the summer to the fall and decreased to 1.2×10^4 particles/m^3 in the winter. The fungal concentrations in the canopy and ground samples ranged from 14 to 82% of the bacterial concentrations and dropped from 2.7×10^5 to 4.3×10^3 particles/m^3 from the summer to the winter. The bacteria and fungi in the air samples collected at a height of 500 m (above-canopy sample) in the summer and fall decreased to concentrations ranging from 3.7×10^4 to 2.3×10^5 particles/m^3 (Fig. 1b); in the winter, the concentrations were similar regardless of the height status (Fig. 1b). Fungal concentrations in the above-canopy samples were significantly different from those in the canopy-top and ground samples (Dunnett analysis; $P < 0.01$).

Viable microbial cells were abundantly detected by the diluted-liquid culture method, such as the MPN procedure. The microorganisms in cloud drops or wet conditions are expected to grow more efficiently in the liquid culture, because the liquid culture method can avoid the stresses that microorganisms experience during inoculation onto solid culture. The living cell concentrations of bacteria and fungi in the summer and fall were similar, ranging from 1001 to 8255 mpn-particles/m^3 in the canopy and ground samples. This concentration

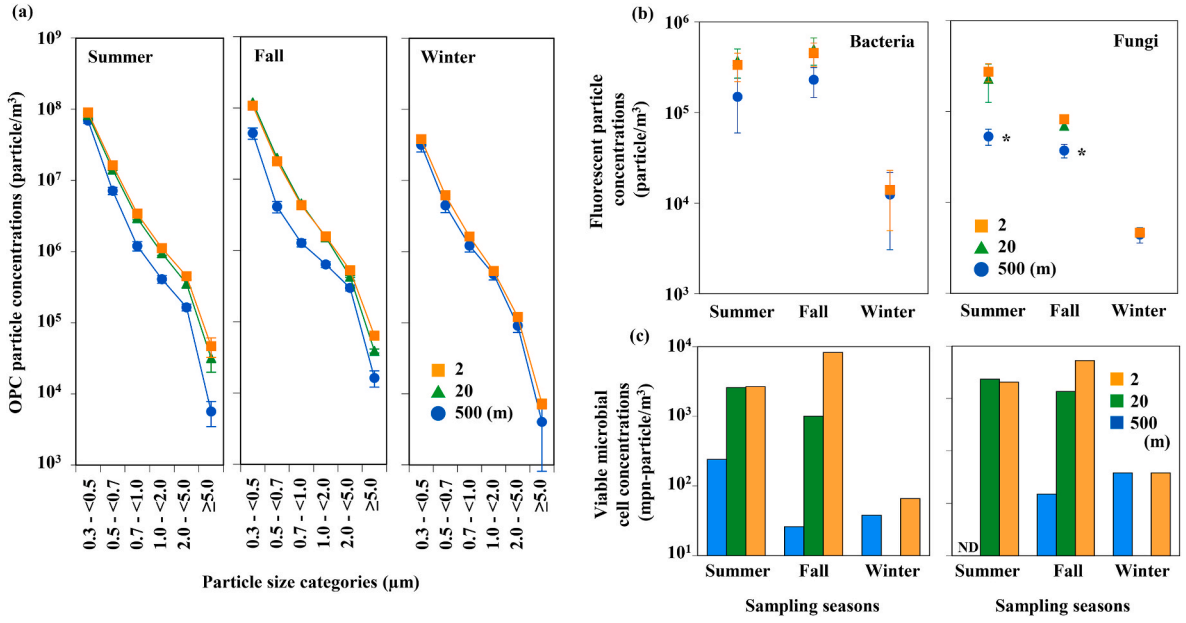

Fig. 1. Vertical variations in concentrations of optical particle counter measure aerosol particles with the six-categorized diameters (a), fluorescent bacterial and fungal cells (b), and most probable number (MPN) counted viable microorganisms (c) at the heights of 2 m (orange), 20 m (green), and 500 m (blue) over the Tsukuba Botanical Garden during summer, fall and winter. Asterisks show significant differences among altitudes (Dunnett analysis; $P < 0.01$). ND indicates below-detectable concentrations.

decreased to less than 241 mpn-particles/m^3 in the above-canopy sample (Fig. 1c).

3.2. Bacterial community structures among three heights

The numbers of reads from bacterial 16S rRNA gene amplicons, which were recovered from a total of 41 samples (Table S1), ranged from 1985 to 305,927 sequences and were represented by 6–676 bacterial ASVs with higher than 99% identity. The rarefaction curves, which indicated an increase in ASVs numbers corresponding to the sequence numbers, were saturated. This indicated that the determined sequence numbers were sufficient for comparing the bacterial compositions among samples. The bacterial community richness estimated by the Chao 1 index decreased from 88 to 19 ASVs from the ground to the above-canopy throughout the three seasons (Fig. 2a, Table S2). The above-canopy sample showed significant differences in bacterial community richness between the canopy-top and ground samples (Dunnett analysis; $P < 0.01$). Airborne bacterial composition in the canopy and ground samples formed an overlapping cluster in the PCoA ordination plot, which differed from the communities in the above-canopy samples (Fig. 2b). In contrast, seasonal changes in the bacterial compositions at these sampling sites were not clearly observed (Fig. 2c).

The relative abundance of the phylum Actinobacteria accounted for no more than 28.2% in the canopy and ground sample communities and increased to 54.9% in the above-canopy samples (Fig. 3a, Fig. S5). In addition, the Firmicutes (particularly, Bacilli) sequences maintained high relative abundances of up to 42.0% in the above-canopy samples, and the families Staphylococcaceae (*Staphylococcus* spp.), Streptococcaceae (*Streptococcus* spp.), and Bacillaceae (*Bacillus* spp.) in Firmicutes were common among all the three heights. Additionally, the above-canopy samples sometimes contained high relative abundances of the Alphaproteobacteria and Gammaproteobacteria sequences (14.5% and 29.3%, respectively) (Fig. 3a).

3.3. Fungal community structures among three heights

The number of reads from the fungal ITS region amplicons ranged from 1769 to 209,845 sequences per sample. The saturated rarefaction curves demonstrated that the obtained sequence numbers were sufficient for comparing fungal compositions among samples. These counts were represented by fewer ASVs (3–332) than those in the bacterial dataset. The Chao 1 index estimated fungal richness ranging from 8 to 38 ASVs, with a significant difference between the canopy-top and above canopy zones (Fig. 2d, Table S2). The fungal richness of the above-canopy samples differed significantly from that of the canopy-top and ground samples (Dunnett analysis; $P < 0.01$).

Unlike the bacterial communities, the fungal communities in the winter samples formed a different cluster in the PCoA ordination from the fall and summer samples (Fig. 2e). The mushroom-type fungi of the class Agaricomycetes increased in relative abundance during the summer and winter, whereas the mold-type fungi belonging to the classes Dothideomycetes and Eurotiomycetes showed an increasing trend during the fall (Fig. 3b, Fig. S6). In the class Agaricomycetes sequences, the dominant sequences of the orders Agaricales, Polyporales, Cantharellales and Trechisporales were detected randomly from the canopy and ground samples during each sampling period and contained a few common species at the three heights. In contrast, the Dothideomycetes and Eurotiomycetes sequencing databases of the three heights were dominated by fungal species of the genera *Cladosporidium, Penicillium*, and *Aspergillus*.

3.4. Ice-nucleation activities of isolates

A total of 95 isolates (71 fungal isolates and 24 bacterial isolates) that

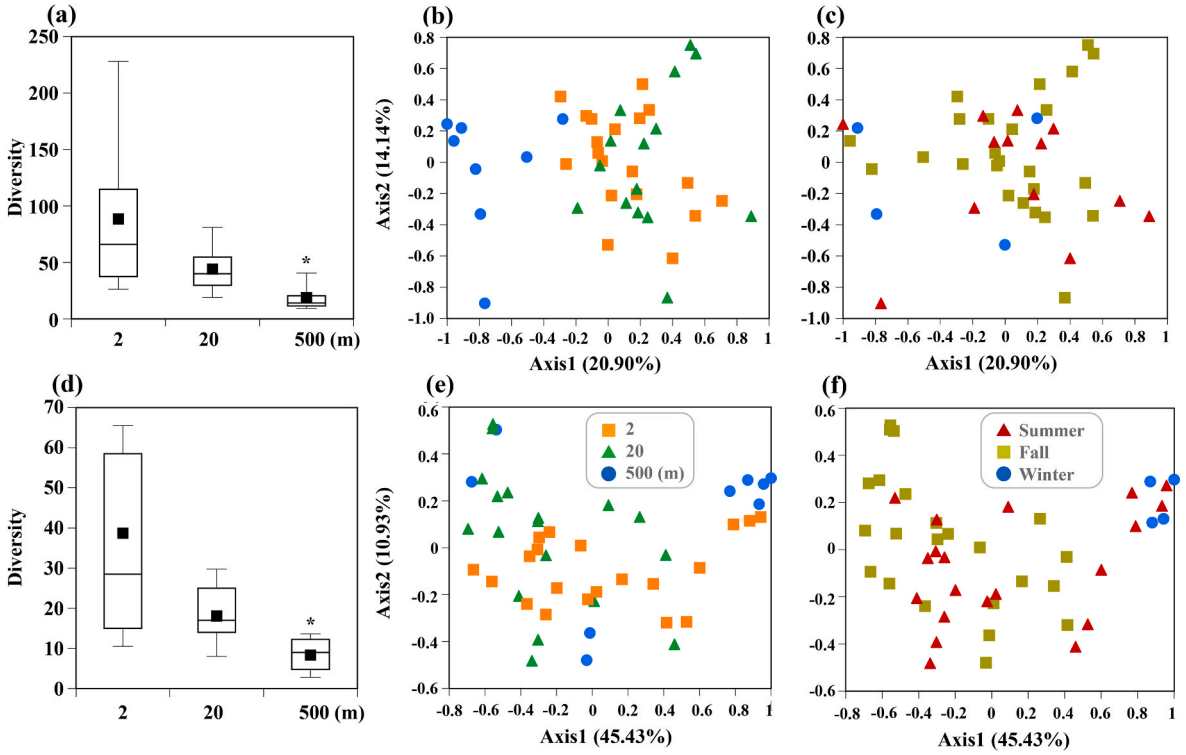

Fig. 2. Diversity of bacterial (a) and fungal (d) communities observed in the air samples collected from the Tsukuba Botanical Garden during the six sampling periods (Table S1). Species were binned at 97% sequence similarity level. Bray-Curtis analysis of bacteria (b, c) and fungi (e, f) displaying phylogenetic clustering by the air samples collected from the Tsukuba Botanical Garden. The graphs (b) and (e) depict the samples collected at above canopy (blue circle), forest-canopy top (green triangle), and forest ground (orange square). The graphs (c) and (f) indicate the samples collected during summer (red), fall (yellow), and winter (blue). Asterisks show the significant differences among altitudes (Dunnett analysis; $P < 0.01$).

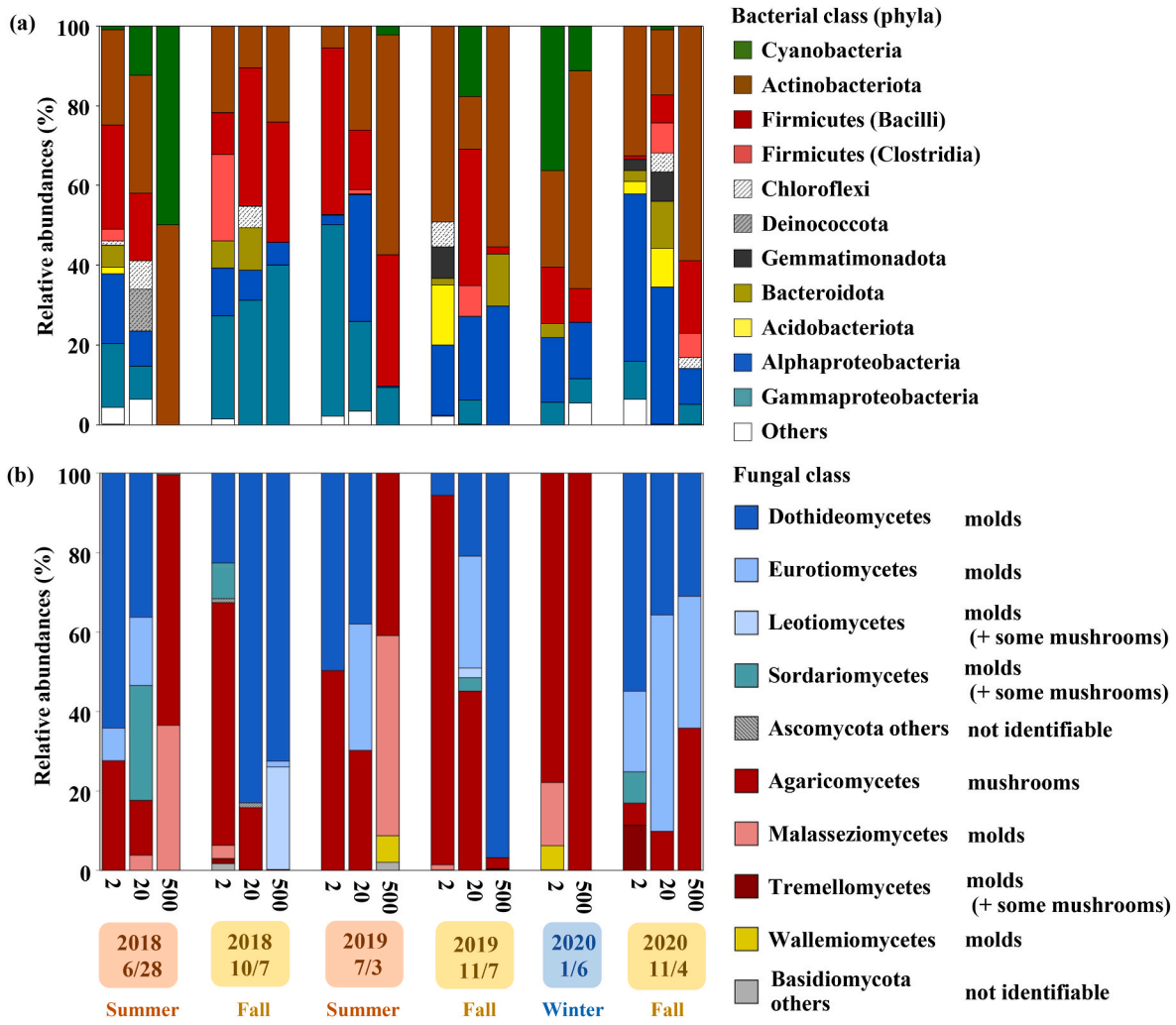

Fig. 3. Bacterial (a) and fungal (b) compositions at class level of the partial sequences in the MiSeq sequencing database (ca. 450 bp) targeting the 16S rRNA gene and ITS region, respectively, obtained from air samples collected simultaneously at the heights of 2 m, 20 m, and 500 m over the Tsukuba Botanical Garden. Others were composed of the sequences that were classified into unknown classes or belonged to the classes occupying no more than 5% of relative abundances. The letters after fungal-class names indicate the types of molds or mushrooms, which the class members include mainly.

were isolated using MPN culture methods (Table S3) were used to initiate ice formation in water drops at temperatures ranging from 0 to −25 °C. Microscopic observations confirmed that the tested microbial cultures were composed of vegetative and spore (endospore) cells. Five isolates of the genera *Fusarium*, *Bacillus* and *Pseudomonas* (>99.7% similarity) exhibited particularly high ice-nucleation activities at the temperatures between −5 and −10 °C, where ice-cloud formation is initiated in the actual atmosphere (Fig. 4a–c). Additionally, microbial isolates with high ice-nucleation activities (freezing temperatures between −7 and −10 °C) showed higher rates among total isolates obtained in the summer and winter (9.5−38.5%) than those in the fall (0%) (Fig. 4d). Microorganisms isolated from the summer and fall samples with high ice-nucleus activity were closely related to *Bacillus* species known to be associated with dead leaves and terrestrial ecosystems (Table S3).

4. Discussion

4.1. Vertical aerosol mixtures over the forest environment

Biological aerosols are frequently transported from forest environments, where fungal and bacterial communities grow in association with the phyllosphere and leaf soils (Elbert et al., 2007; Huffman et al., 2013; Igarashi et al., 2019). During the survey periods, some aerosol particles were distributed vertically from the forest ground to up to 500 m over forest environments and mixed with the air mass originating from other Japanese regions. The aerosol concentrations showed a decreasing trend from the ground to above the canopy, suggesting that the aerosols were transported mainly upward around this survey site during the sampling period. Microscopic observations revealed that the biological aerosols in the forest environments contained bacterial cells, fungal spores, and fungal filamentous cells (Fig. S4). The bacterial concentrations in the air samples collected from the canopy top (canopy sample) and ground (ground sample) ranged from 10^4 to 10^5 particles/m^3. Additionally, some bioaerosols above the canopy are thought to originate from the agricultural zones located around the Kanto Plain. Agricultural environments are also the main sources of bioaerosols and forest environments (Tignat-Perrier et al., 2019; Li et al., 2020). Airborne microorganisms in the canopy zone of the sampling site exhibited similar concentrations of biological aerosols as in other forest environments in Japan (Igarashi et al., 2019) and North America (Huffman et al., 2013). The bacteria and fungi in the air samples at a height of 500 m (above-canopy sample) decreased in concentration from the summer and fall to winter (Fig. 1b). The main island of Honshu is dominated by mountainous areas with broad-leaved deciduous trees (Hirata et al., 2008). During the summer and fall, broad-leaved deciduous trees that

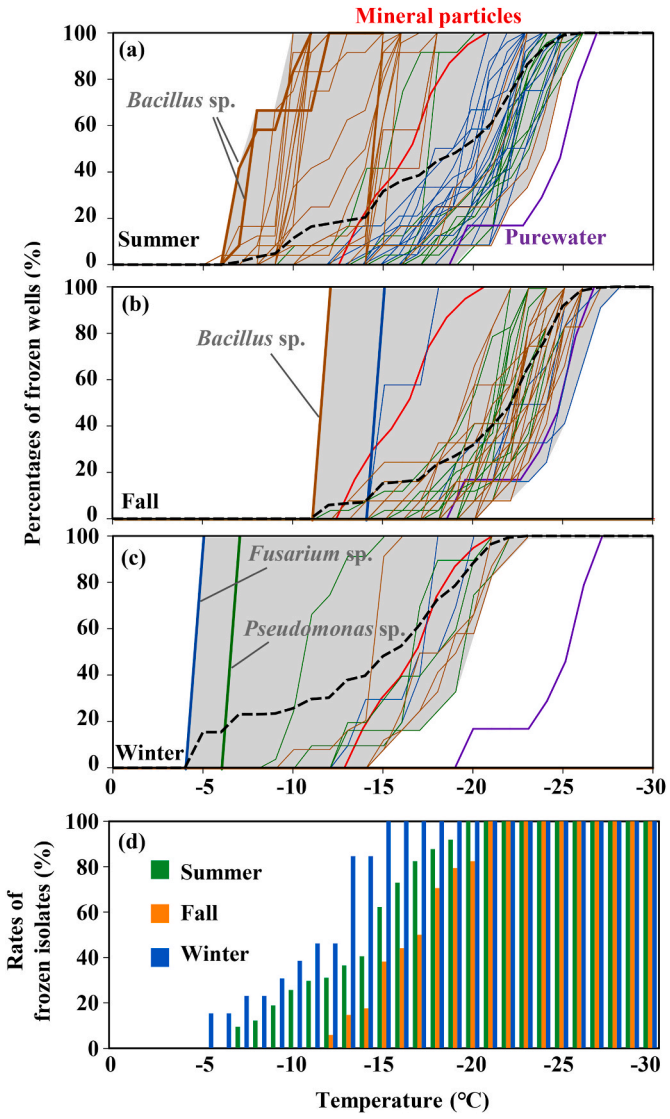

Fig. 4. Frozen-well percentages for the water-drop assay using the isolates obtained from the air samples collected at the heights of 2 m (orange lines), 20 m (green lines), and 500 m (blue lines) over the Tsukuba Botanical Garden during summer (a), fall (b), and winter (c). Gray areas cover the all lines of frozen-well percentages, and the dotted lines indicated the average frozen-well percentage of each temperature. The high ice-nucleus active isolates are indicated using bold lines and the genera names. Red and purple lines indicate the use of Arizona test dust (standard dust mineral particle) and pure water, respectively, in the experiment. The ratio of isolates that freeze the water drops in all wells for every temperature change (d). The isolates were obtained during summer, fall, and winter.

cover the canopy zone release bioaerosols from the Kanto Plain. The loss of leaves from fall to winter was assumed to accelerate the vertical circulation of particles from plant bodies or forest terrestrials upward out of the canopy.

The MPN procedure revealed that living microbial cells were suspended at similar concentrations in the canopy and ground samples and decreased in the above-canopy sample (Fig. 1c). The number of culturable microorganisms detected by the MPN procedure was 1/100th of the total microbial concentration. This result supports previous knowledge that the culturable microorganisms ranged from 1 to 10% of total environmental microorganisms (Olsen and Bakken, 1987). In the winter, the culturable microbial concentrations in the ground samples decreased to 1/10th of the concentration, while the above-canopy samples maintained similar or higher concentrations than those in the summer and fall. In contrast, the fluorescence microscopic count indicated that the microbial concentrations in the summer and fall were higher than those in the winter (Fig. 1b), suggesting that the abundance of culturable microorganisms increased in the winter. The microbial taxonomic compositions are known to change seasonally depending on local climate characteristics such as vegetation, temperature, and humidity (Fröhlich-Nowoisky et al., 2009; Samaké et al., 2021).

4.2. *Vertical distributions and seasonal changes of airborne bacterial communities*

Airborne bacterial communities in the above-canopy samples indicated lower diversities and compositions compared to those of the canopy and ground samples (Fig. 2ab, Table S2). The airborne bacteria above the canopy are expected to originate from several environments, such as agricultural and urban areas, as well as forested area, and are selected by atmospheric stressors. In contrast, there were no seasonal changes in the bacterial composition (Fig. 2c), suggesting that the main origins of airborne bacteria vary according to environmental conditions, which could change daily or on a scale that is much shorter than that of seasonal changes. The bacterial composition in urban areas has been reported to change seasonally owing to snow fall, and plant growth (Bowers et al., 2011). Airborne bacteria in the forest zone are thought to originate from the phyllosphere (plant bodies, leaves, and rhizosphere), and terrestrial ecosystems (fallen and decaying leaves, forest soils).

The airborne bacterial communities in the canopy and ground samples were dominated by the phylum Actinobacteria sequences at higher relative abundances than those in the above-canopy samples (Fig. 3a, Fig. S5). The two major actinobacterial families Corynebacteriaceae and Micrococcaceae are often detected in aerosols at high altitudes (Maki et al., 2015, 2017) and terrestrial areas (Puspitasari et al., 2015). The Firmicutes (particularly, Bacilli) sequences, which were composed mainly of *Staphylococcus* spp., *Streptococcus* spp., and *Bacillus* spp., increased in relative abundance from the ground to the above canopy. Actinobacteria and Firmicutes members on plants and/or terrestrial surfaces can form aggregates (biofilms), which protect bacterial cells against atmospheric stressors such as UV irradiation (Matulova et al., 2014). Additionally, the above-canopy samples sometimes included predominantly Alphaproteobacteria and Gammaproteobacteria sequences (Fig. 3a), which are often dominant in phyllospheric bacterial communities (Vorholt, 2012). Consequently, airborne bacteria originating from the phyllospheric and terrestrial environments are thought to mix below the canopy. In particular, some bacterial populations of Actinobacteria and Firmicutes form aggregates (biofilm) that are transported from the forest canopy and outside the forest, such as agricultural areas and traffic roads, into the open atmosphere.

Bacterial and fungal communities in bioaerosols at high altitudes and local airborne microbial communities are frequently mixed with long-range transported microbial populations (Maki et al., 2019; Tignat-Perrier et al., 2019).

4.3. *Vertical distributions and seasonal changes of airborne fungal communities*

The fungal diversities of the above-canopy samples were differed from those of the canopy-top and ground samples (Fig. 2d, Table S2). Unlike the bacterial communities, the fungal communities in the winter samples varied from those in the fall and summer samples (Fig. 2e), reflecting the previously reported seasonal shift in fungal community composition in response to variations in vegetation, temperature, precipitation, and wind direction (Fröhlich-Nowoisky et al., 2009). The mushroom-type fungi of the class Agaricomycetes were dominant during summer and winter, whereas the mold-type fungi of the classes Dothideomycetes and Eurotiomycetes increased during fall (Fig. 3b, Fig. S6). These fungal taxa are composed of several species that decompose organic plant material in decaying leaves and wood detritus (Bani et al.,

2018). The fungal spores and cells in the forest atmosphere are released from mushrooms and molds that are attached to plant bodies or decaying litter in the forest zone. The class Agaricomycetes sequences contained several species of mushroom-type fungi randomly at the three heights, while the Dothideomycetes and Eurotiomycetes sequences at the three heights were dominated by mold-type fungi (*Cladosporidium, Penicillium*, and *Aspergillus* species). Similar populations of airborne mold-type fungi are thought to be constantly suspended in the forest zone, and some are carried from the canopy zone to the higher canopy.

The major fungal species in the above canopy samples shifted from mushroom-to mold-type fungi above the forest canopy from the summer to the fall. Mushroom-type fungi (Cantharellales) dominated at the ground and above the canopy in the winter (Fig. 3b, Fig. S7). Our Dothideomycetes sequences matched typical cosmopolitan molds, such as *Cladosporium* species, which are associated with plant bodies, leaves, and decaying leaves (Bensch et al., 2010). This coincides with the fall, when mold-type fungi proliferate and disperse due to leaf senescence and low metabolic activities in forest plants around the Kanto plane. Indeed, *Cladosporium* isolates were found to be regulated on young leaves and exhibited better growth on the older leaves (Fig. S8). Additionally, there are a few plants in agricultural areas, and the mold type of fungi is constantly dominant in the atmosphere of urban environments. This suggests that the *Cladosporium* species came from agriculture and urban environments as well as the forested environment. In the summer, Agaricomycetes are dominated by members of the order Polyporales, which form brackets or shelfs frequently on the bodies of forest plants (Binder et al., 2013; Hibbett et al., 2014). Indeed, microscopic observations have revealed that forest aerosols contain high abundances of fungal spores (Iwata et al., 2019; Seifried et al., 2021). The fungi that adhere to the plant body are likely to be transported to the above canopy from forest plants or agricultural crops.

4.4. Variations in ice-nucleation activities of airborne microorganism

Among the total 95 isolates, five isolates of the *Fusarium*, *Bacillus* and *Pseudomonas* species possessed high ice-nucleation activities with freezing temperatures between −5 and −10 °C (Fig. 4a–c). Some *Bacillus* spp. have been frequently identified in cloud water (Matulova et al., 2014) and snow (Maki et al., 2018; Tanaka et al., 2019). In addition to *Bacillus*, members of the genera *Fusarium* and *Pseudomonas* (particularly *Pseudomonas syringae*) are globally ubiquitous in the phyllosphere and terrestrial habitats. Additionally, they are the principal isolates of high-ice-nucleus active microorganisms, such as *Fusarium* species (Pouleur et al., 1992; Kunert et al., 2019) and *Pseudomonas* species (Morris et al., 2008; Maki et al., 1974; Šantl-Temkiv et al., 2015). In this study, *Fusarium* and *Bacillus* isolates were obtained from above-canopy samples. The high-throughput DNA sequencing databases of the above-canopy samples also contained the dominant *Bacillus* sequences. These results strongly demonstrate that these known ice-nucleating microorganisms, such as *Fusarium* and *Bacillus* species, disperse at heights of 500 m, where ice clouds form frequently.

Microbial isolates with high ice-nucleation activity were obtained more frequently in the summer and winter samples than in the fall samples (Fig. 4d). Different microbiota, moisture, air temperature, and terrestrial conditions – all of which vary seasonally – can influence both the microbial and physicochemical degradation of decaying leaves, as well as the production of ice nuclei particles released from the leaf matters (Conen et al., 2017; Hassett et al., 2015). Indeed, several fungal species inhabiting forest environments have been shown to form nuclei-like water drops on their spore surfaces in the atmosphere (Hassett et al., 2015). Microorganisms isolated during summer and fall with high ice-nucleus activities were closely related to *Bacillus* species known to be associated with dead leaves and terrestrial ecosystems (Table S3). During the early defoliation period of the fall sampling, *Cladosporium* species were frequently isolated, and their ice-nucleation activities were found to be weaker at freezing temperatures of no more than −15 °C. The green leaves could regulate the growth of the *Cladosporium* species, whereas the leaves that have changed color in the fall may have lost their regulatory activities against fungal growth (Fig. S8). Consequently, the rates of microbial isolates with high ice-nucleation activity, such as *Bacillus* species, decreased during the fall sampling. After the defoliation period during winter sampling, *Fusarium* and *Pseudomonas* isolates with high ice-nuclei activity were obtained. The seasonal median concentrations of airborne ice nuclei particles in forest environments increased after the vegetation index declines (Conen et al., 2017). Biological ice nuclei in forest environments vary quantitatively and qualitatively between the summer and the winter (Schneider et al., 2021). Decaying leaves in terrestrial ecosystems are thought to be a major contributor to ice-nucleating microorganisms in the atmosphere.

However, the relative abundances of *Fusarium* and *Pseudomonas* sequences from the amplicon data were low (<2.0%) among the entire air sample communities. As described, generally, a substantial portion (by biomass, 90–99%) of environmental microorganisms was estimated to be viable but non-culturable (Olsen and Bakken, 1987). This suggests that the currently identified ice-nucleating microorganisms are a minor component of airborne microbial communities (but are responsible for the majority of ice nucleation activity) or that other currently unknown and uncultured microorganisms are also involved in the ice nucleation process.

5. Conclusion

This is the first study to investigate seasonal variations in the vertical distribution of microbial communities from the forest ground to the open atmosphere above the forest canopy. Throughout all seasons, the bacterial communities from these samples were dominated by terrestrial and phyllospheric bacteria, some of which are thought to be transported to above the canopy and resist to the atmospheric stressors. In contrast, fungal communities in this stratum predominantly contained mushroom-type fungi (members of Agaricomycetes) during the summer and the winter, and mold-type fungi (members of Dothideomycetes) during the fall. Additionally, we isolated *Bacillus*, *Fusarium* and *Pseudomonas* strains with high ice-nucleation activities to identify and characterize the transportation of ice-nucleating microorganisms at a substantial height (500 m above ground) over the forest sampling site. However, there are still many topics to explore based on this study, that can be investigated in future studies. First, the isolate species had low multiplicity in the amplicon metagenomic sequence dataset. Thus, we need physiological isolate characterization (including those that were not included in taxa previously known to promote ice nucleation) and the metagenomic analyses (targeting ice-nucleation relating genes). Additionally, the vertical distribution processes of ice-nucleating microorganisms remain unclear because environmental and anthropogenic data are limited. Future studies should employ extra surveys monitoring detailed meteorological and chemical factors to increase the robustness of the results obtained on vertical distribution processes of ice-nucleating microorganisms over forested areas. Lastly, microbial origins at high-altitude samples could not be clearly determined because there are only a few studies that report on the specialization of airborne microbial communities released from natural ecosystems (e.g., forests) and agroecosystems (e.g., agricultural crops). This apparent contradiction warrants further investigation to determine the index of airborne microbial populations released from vegetative types or species.

CRediT authorship contribution statement

Teruya Maki: Supervision, supervised all surveys and performed all sampling, molecular biological experiments and writing paper. **Kentaro Hosaka:** managed the sampling site and wrote paper. **Kevin C. Lee:** Formal analysis, supported the analysis of sequencing data and wrote paper. **Yasuhiro Kawabata:** Formal analysis, analyzed the metrological

data. **Mizuo Kajino:** Formal analysis, analyzed the air-mass trajectory data and wrote paper. **Maoto Uto:** Formal analysis, analyzed phylogenetically the DNA sequences of microbial isolates. **Kazuyuki Kita:** supported some sampling. **Yasuhito Igarashi:** supported some sampling and produced the meeting for making this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atmosenv.2023.119726.

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