Visualization of the seasonal shift of a variety of airborne pollens in western Tokyo

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Abstract

Airborne pollens cause pollinosis and have the potential to affect microphysics in clouds; however, the number of monitored species has been very limited due to technical difficulties for the morphotype identification. In this study, we applied an eDNA approach to the airborne pollen communities in the suburbs of the Tokyo metropolitan area in Japan, within a mixed urban, rural, and mountain landscape, revealing pollen seasonality of various taxa (a total of 78 families across the period) in the spring season (February to May). Those taxa distinctly shifted in the season, especially in the beginning of February and the middle of April. Air temperature shift was an obvious key factor to affect the airborne pollen community, while the influence of other meteorological factors, such as wind speed, humidity, and precipitation, was not clear. Taxonomic classification of major Amplicon Sequence Variants (ASVs) indicates multiple pollen sources, including natural forest, planted forest, roadside, park lands, and horticultural activities. Most major ASV belongs to Japanese cedar (*Cryptomeria japonica*), which is the most notable allergen that causes pollinosis in Japan, peaking in mid-February to March. Backward trajectory analysis of air masses suggests that the Japanese cedar and other Cupressaceae plantation forests in the western mountains were a significant source of airborne pollen communities detected at our sampling site. Other major plant pollen sources, including Japanese zelkova (*Zelkova serrata*) and ginkgo (*Ginkgo biloba*), emanated from the nearby parks or roadside regions. This study's approach enables us to visualize the phenology of multiple pollen, including timing and duration. Long-term monitoring of this type would provide additional insight into understanding the role of climate change on pollen transmission and links to flowering events.

Introduction

Aerosolization of pollen is a natural part of the pollination process for the majority of gymnosperms and a smaller part of angiosperms. Anemophilous (wind pollinationing) plants generally have more flowers with more pollen grains than animal pollination plants (Friedman & Barrett 2009), and pollen release and dispersion are controlled primarily by winds, though also through humidity and temperature (Whitehead 1983; Picornell *et al.* 2019). The dispersal range of the pollen increase as the terminal settling velocity decreases (Niklas 1985). Terminal settling velocity is determined by the physical parameters of pollens such as size, shape, and bulk density (Hirose & Osada 2016) and those can change the range of transportation if once into turbulence. For example, Japanese cedar, which is the most common pollen causing pollinosis in Japan, can travel and induce symptoms over more than a 100 km range (Okamoto *et al.* 2009). Furthermore, smaller size pollen such as birch pollen can stay in the air for a few days and be transported much further: 1,000 km (Sofiev *et al.* 2006). The long-range transportation of these pollens affects human health issues such as pollinosis.

Seasonal allergic rhinitis in Japan is caused by pollen of multiple species, including Japanese cedar, Japanese cypress, birch, alder, beech, oak, elm, grass such as mugwort (Kishikawa & Yokoyama 2016). The most notable airborne pollen is Japanese cedar because the pollinosis caused by Japanese cedar pollen dominates in Japan, with 26.5 % of the Japanese population in 2008 having an allergic reaction to Japanese cedar pollen, and prevalence increasing recently (Okubo *et al.* 2017). One reason for the high prevalence of Japanese cedar pollinosis is the large surface area of the Japanese cedar forest (18% of Japan's entire land) (Yamada *et al.* 2014). More than half of the Japanese cedar forest was planted from the 1950s to 1970s due to the high demand for timber after World War II. The prevalence of Japanese cedar

pollinosis is markedly increasing after the 1980s because male flowers become mature to produce more pollen grains after 30 years old. Japanese cedar pollinosis also has become a health issue in Japan and other countries where Japanese cedar was transplanted (Lee *et al.* 2015).

The flowering phenology and pollen initiation (hereafter "pollen seasonality") are changing due to climate change, especially the consequences of air temperature warming. In North America, the length of ragweed (*Ambrosia* spp.) pollen season has been increasing, and this is associated with a delay in the first frost of the fall and lengthening of the frost free period (Ziska *et al.* 2011). In Japan, the length of the *Cryptomeria japonica* pollen season has been increasing too, and the first day of observation has become earlier (Teranishi *et al.* 2000). This pollen season change has been associated with mean air temperatures in the previous July because production of pollen after male flowers bloom during this season is much more in the higher July air temperature.

Therefore, understanding pollen seasonality changes due to warming temperatures is important for determining impacts on human health. However, most of the existing pollen seasonal data is for limited species (e.g., major species that cause pollinosis in Japan, such as Japanese cedar and Japanese cypress. Data is publicly accessible from the Association of Pollen Information: http://pollen-net.com/welcome.html). Furthermore, the pollen seasonality of other species was not measured routinely because the traditional microscopic counting method is time-consuming in order to cover multiple species and requires investigators' expertise to identify their morphotype (Núñez *et al.* 2017).

Recently, comprehensive detection of pollen using an environmental DNA (eDNA) approach via high throughput sequencing (HTS) has enabled acquisition of fine taxonomical resolution of airborne pollens (Kraaijeveld *et al.* 2015; Núñez *et al.* 2017; Banchi *et al.* 2019; Brennan

et al. 2019). For example, Brennan et al. (2019) showed spatio-temporal shifts of airborne grass pollen at a high taxonomic resolution (genera or species-level) in the UK. Heretofore, this has been difficult to identify based on morphology alone, and so was generally categorized only at the family level (Poaceae).

In this study, we focus on the airborne pollen in the suburbs of Tokyo because there is closer to possible source region of Japanese cedar and including or surrounding various vegetation types (e.g., forest, roadside, park lands, and horticulture) that cause seasonal allergic rhinitis. In order to identify the pollen seasonality (i.e., the start date and duration of pollen dispersal) of multiple species, we applied the eDNA approach (next generation sequencing and bioinformatics) to airborne pollen sampled on a fine time scale (every 24 hours during from February to mid-May) and showed a seasonal shift of multiple taxa at the genera or species-level, including identifying the start date and duration of each pollen type.

Method

Bioaerosol sampling

Airborne particles were sequentially collected (every 24h interval from 0:00 Japan Standard Time) on the rooftop of the building of the National Institute of Polar Research (26.7 m AGL. N 35°42'44.6", E 139°24'32") in the western suburbs area of Tokyo (Fig. 1) from February to May 2016 using a sequential aerosol sampler (GS-10N, Tokyo Dylec Corp.). Samples were filtered through 47 mm diameter quartz filters (Tissuquartz™ Filters, 2500 QAT-UP, Pall) mounted in sterilized open NILU (Norwegian Institute for Air Research) filter holders at a flow rate of average 30.2 L /min. In order to remove potential contamination, quartz filters were combusted (500 °C for two hours) before using, and filter holders were hand washed with detergent followed by wiping by DNAaway tissue (Thermo Fisher Scientific Inc.) on a clean bench.

DNA sequencing

In order to avoid contamination, all processes before Polymerase Chain Reaction (PCR) amplification were done in a laminar flow clean bench (PCV-1305BNG3-AG, Hitachi). The clean bench was sanitized with a UV lamp overnight, and pipettes were sterilized in a DNA cross-linker (CL-1000, UVP) box inside the clean bench. Genomic DNA in bioaerosols captured on quartz filters was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). The quartz filter was initially pulverized during the bead beating step, but in order to maximize the yield of DNA, all fragments of the filter were carried over until the final elution step. The ribulose-bisphosphate carboxylase gene (*rbc*L) was amplified using the forward primer: 5'-CTTACCAGYCTTGATCGTTACAAAGG-3' and the reverse primer: 5'-GTAAAATCAAGTCCACCRCG-3' (Erickson *et al.* 2017) with Illumina overhang adaptor sequences attached to each 5′ end, by Ex-Taq HS (Takara, Shiga, Japan). PCR reaction conditions comprised 35 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min and an additional final elongation at 72 °C for 5 min using a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). Subsequent clean-up and indexing of PCR amplicons were performed by following Illumina standard protocol for 16S metagenomic sequencing library preparation. All samples were sequenced at NIPR using a MiSeq (Illumina, San Diego, CA). Raw sequence data are available from the Sequence Read Archive of the National Center for Biotechnology Information (NCBI): PRJNA676177.

Sequence analysis

All sequence libraries from samples were clustered into amplicon sequence variants (ASVs) using the R package "DADA2" (Callahan *et al.* 2016). Taxonomy was assigned by

the Naive Bayes Classifier method in The Ribosomal Database Project (RDP) Classifier (Wang *et al.* 2007) implemented in DADA2 using the *rbc*L database (Bell *et al.* 2017) with sequences of closest relatives of our major ASVs using Basic Local Alignment Search Tool (BLAST) implemented in Geneious R10 (https://www.geneious.com) (https://figshare.com/account/home#/projects/90272). The alpha diversity (the number of ASVs), taxonomy visualization, and Non-metric multidimensional scaling (NMDS) were performed by R package phyloseq (McMurdie & Holmes 2013). Kruskal-Wallis test and Tukey's test were performed by kruskal.test and TukeyHSD functions in R package stat, respectively. The distance-based redundancy discriminate analysis (dbRDA) with environmental data (see "Environmental data" in this section) was analyzed using R package Vegan (Oksanen 2015). The closest relatives of the major 20 ASVs were searched by BLAST and assigned the finest taxonomy for each sequence. The first day of dispersal season of each pollen was defined as the first day of 3 consecutive days, in which relative abundance was more than 1% of maximum relative abundance through the season. And the last day was defined as the day before 3 consecutive days, in which relative abundance was less than 1% of maximum relative abundance through the season. Each pollen period was determined from the days between the first and the last day of the season.

Environmental data

Environmental data was taken by the Automated Meteorological Data Acquisition System (AMeDAS), the Japan Meteorological Agency (http://www.data.jma.go.jp/obd/stats/etrn/index.php). Air temperature, moisture, wind speed, and PM10 were measured at the "Tachikawa" station, the adjacent building of the study site (Tachikawa City Hall). Precipitation was measured at the "Ome" station, 15 km northwest of the study site (Fig. 1b).

Number of pollen grains

The number of pollen grains (grains/m3 of air) was monitored by an automated pollen monitoring system of the Ministry of Environment (see the detail in "Pollen seasonality" in Introduction) at Kodaira City Health Center, 6.5 km northeast from the study site. Hourly total concentration during main pollen season (February - May) since 2006 is publically available at http://kafun.taiki.go.jp/DownLoad1.aspx (in Japanese) and daily sum concentration is shown in Figure 2c. Raw data (Number of pollen.txt) were deposited in https://figshare.com/account/home#/projects/90272.

Vegetation map

Vegetation data (National surveys on the Natural Environments) managed by the Biodiversity Center for Japan, the Ministry of the Environment, are available as KML and Shape data format are from http://www.biodic.go.jp/trialSystem/EN/kmlddl.html. The 6th vegetation survey data in the 2nd mesh point (red square in SI Fig. 1) was used for analysis. Shape data was edited and redrawn using "MANDARA 10"(http://ktgis.net/mandara/index.php) in Fig.1a.

Backtrajectory

The three-dimensional trajectories of air masses arriving at the study site (calculation height = 500 m AGL) were calculated by the Hybrid Single Particle Lagrangian Integrated Trajectory Model (HYSPLIT4) (Draxler & Hess 1998; Stein *et al.* 2015) implemented in R package SplitR v0.4 using 1 degree gridded meteorological data from the Global Data Assimilation System (GDAS). Calculation length of backward air trajectories was 12h.

Result

The number of total and unique sequences

We attempted to undertake PCR analysis on samples from all days between February 1 to May 13 2016, and most of the samples were successfully amplified and sequenced. However, since some samples (February 3-5, 10 and 13-15, March 7 and 26, and April 26) were not amplified, we excluded these samples from sequencing and downstream analysis. On average, 60574 and standard deviation (SD) 10758 sequences were retrieved from each sample. These sequences were assigned to 1121 ASVs and an average of 32.0 ASVs (SD: 24.6) per sample ranging from 3 ASV on March 10 to 110 ASVs on April 25. These ASVs were classified into 78 families, with an average of 10.2 families (SD: 8.7) per sample ranging from 2 families and 37 families. The major families were seasonally shifted (Fig. 2, described in more detail in "Seasonal change of ASVs and taxonomy"**),** especially three major families Betulaceae, Cupressaceae, and Fagaceae, which dominated in the early February, later February to March, and April through May, respectively.

Detailed taxonomy assignment of major ASVs

Twenty ASVs, which were significant in samples (mean relative abundance throughout the whole period> 0.02%) and could be identified at the species, genus, or family level of taxonomy using BLAST against NCBI nr/nt database, were manually selected (SI Table 1, Fig. 3). Taxonomy identification of these ASVs was made as follows. For ASV1, ASV9, and ASV17, a single BLAST search candidate species was categorized with 100% BLAST identity (*Cryptomeria japonica*, *Ginkgo biloba*, and *Morella rubra*, respectively). For ASV2, ASV7, ASV8, ASV15, and ASV23, candidate species were multiple with 100% BLAST identity except for ASV15 (97.1%), but only one species was selectable based on their

habitats (*Chamaecyparis obtusa*, *Chamaecyparis pisifera*, *Zelkova serrata*, *Corylus heterophylla*, *Torreya nuciferaa*). For ASV3, ASV4, ASV5, ASV6, ASV10, ASV11, ASV12, ASV14, ASV18, ASV19, ASV33, and ASV34, candidate species or genus were multiple with 100% BLAST identity, and only genus or family was selectable. The total relative abundance of these selected (only) 20 ASVs was 0.89 out of 1 on the average (SD: +- 0.10) (SI Fig. 2), and fraction of these representing other pollen and plant tissue was much smaller.

Seasonal change of ASVs and taxonomy

The numbers of ASVs showed significant variations during our sampling period (Kruskal-Wallis: $p < 0.001$), and those in April and May were significantly higher than in February and March based on Tukey's test (SI Table 2). An NMDS ordination plot of beta diversity by Bray–Curtis dissimilarity (SI Fig. 3) shows that the airborne plant community was seasonally shifted by month. The numbers of Families also showed significant variations during our sampling period (Kruskal-Wallis: $p < 0.001$), with number in April and May were significantly higher than in February and March by Tukey's test (SI Table 3). Seasonality of 20 selected are shown in Figure 3. For example, the family *Cupressaceae*, *Cryptomeria japonica* (ASV1), the dominant ASV among all ASVs, primarily dominated in Febuary and March. Then, *Cupressaceae's* relative abundance is gradually shifted to *Chamaecyparis pisifera* (ASV7) and *Chamaecyparis obtusa* (ASV2) in March. Duration of dispersal period of each pollen was listed in SI Table 4.

Number of airborne pollen grains

The number of pollen grains measured by the automated pollen monitoring system is shown in Fig. 2 c. March is the highest month by average number (813 +- 529 grains/m3) followed

by April (757 +- 788 grains/m^3), May (392 +- 229 grains/m^3), and February (326 +- 324 grains/m^3). Monthly change was significant (Kruskal-Wallis: $p < 0.001$).

Environmental factors

Environmental data, measured in nearby weather monitoring stations, show the seasonal change of meteorological factors, including the gradual increase of air temperature (SI Fig.4). Among 5 factors (air temperature, moisture, precipitation, wind speed, and PM10), distance-based redundancy analysis (dbRDA) shows that the only air temperature was significantly related to community change ($F = 23.3$, $p = 0.001$, SI Table. 5).

Discussion

Plant type of airborne pollen

Various families of plants were found and the majority of these were likely originated from airborne palynomorph transport, because the sampling site of this study is located on the rooftop of a 26.7 m high building. We found that the majority of the sequences belong to trees plant (Fig. 2a), while the presence of grass families (e.g., Poaceae, Plantaginaceae, Araceae) and mosses (e.g., Hylocomiaceae, Funariaceae) are also identified.

Pollen of tree anemophilous species is commonly found in spring in Japan; however, the main flowering season of grass anemophilous species, including *Urticaceae*, *Poaceae*, and *Ambrosia* is autumn (Kawashima *et al.* 2007). Therefore, grass pollen might be less abundant during our sampling period. Another reason is that our sampling site is relatively close to the mountain region in western Tokyo, where is extensively covered by uniform planted forest (Fig. 1, Cupressaceae: *Cryptomeria japonica*, *Chamaecyparis obtuse, and Chamaecyparis pisifera* plantation). As backward trajectory analysis indicates, air (and contained particles) was predominantly passing from over this mountain region to the site,

especially in February (Fig. 4); therefore, ASVs of tree pollens: *Cryptomeria japonica*, *Chamaecyparis obtusa and Chamaecyparis pisifera* (ASV1, ASV2, ASV7) might be very common in air samples, especially February and March. It is also noteworthy that *Chamaecyparis pisifera* is very commonly used as a hedge in homes, and some amount of pollen of *Chamaecyparis pisifera* may be originated from cultivars around the sampling site.

The seasonal shift of airborne pollen

The taxonomy of airborne pollen changed seasonally (Fig.2 a & 3), and their diversity increased along with the air temperature rise over the season. The seasonal change of plant community structure shown by Bray-Curtis dissimilarity also shows a shift along with months (SI Fig. 3). Although pollen release and transport are thought to be controlled primarily by wind and humidity (Whitehead 1983), our results indicate that among 5 environmental factors (air temperature, precipitation, moisture, wind speed, and PM10), only air temperature is related to the community change. Therefore, the air temperature as a proxy of the seasonal shift from winter to spring is the most influential factor in alpha (number of ASVs) and beta (community structure) diversities.

At the beginning of the sampling season (February 2016), the number of observed ASVs is significantly lower than in other months, and 7 samples (February 3-5, 10, and 13-15) during this period had been failed for PCR, likely due to the low concentration of DNA (i.e., pollen, Fig. 2c). After this period, the pollen of *Cryptomeria japonica* (ASV1) became detectable by PCR, and the number of pollen grains significantly increased around one order higher. This timing is similar to the first days of observation of *Cryptomeria japonica* over 15 years of record (Teranishi *et al.* 2000). Other species of the *Cupressaceae (Chamaecyparis obtusa*), which was also from the same planted forest of *Cryptomeria japonica* (Fig.1b), had lagged with *Cryptomeria japonica* by a month, similar to a previous study (Yamada *et al.* 2014).

Then, *Chamaecyparis obtusa* gradually took over *Cryptomeria japonica*, and their number of ASVs reached the highest level in early April. Following this season, the family *Fagaceae*, *Ulmaceae*, *Ginkgoaceae* increased their relative abundance. ASVs (ASV3 & 6) belonging to Fagaceae were only taxonomy, which could not be classified at the genus level among the major 20 ASVs, and potentially include *Castanea*, *Castanopsis*, *Quercus*, and *Lithocarpus* (only in ASV6). However, these species are generally distributed in the same type of forests, categorized in "Substitutional vegetation of secondary forest" in the vegetation map. This type of vegetation, which is referred to as "Satoyama", is remarkably abundant around reservoirs (Yamaguchi Reservoir and Murayama Reservoir) and in the foothills forest in the City of Ome and Akiruno (5 km north and 10 km west of the study site, respectively) (Fig. 1b). Substitutional vegetation of secondary forest is also spotted in western mountains, especially above 350 m a.s.l. (20 km west of the study site). The backward trajectory of air masses show that the direction of likely particle transport is quite variable in April and May, with no preferred direction. Therefore, these vegetation types would be a significant source of Fagaceae. Otherwise, *Zelkova serrata* in *Ulmaceae* and *Ginkgo biloba* in *Ginkgoaceae* are significant species on the roadside in Tachikawa City and in parks, especially in the Showa Commemorative National Government Park (Fig. 1b, SI Fig. 5), which is a neighboring park of sampling site with 180 ha total area. Therefore *Zelkova serrata* and *Ginkgo biloba* are likely to have originated from the urban area nearby.

Getting information on each pollen dispersal seasonality and their potential source from this study is useful for personal allergic rhinitis treatment. In many cases, the cause of rhinitis is hard to detect except for remarkable seasons such as Japanese cedar. Because pollen types and season are very variable in the country has wide longitudinal range, and very limited information on pollen seasonality is available in public. Otherwise, minor pollen also can

cause allergic rhinitis (Kawashima *et al.* 2007), and that information from the eDNA approach can provide what kind of pollen should be cared for each location and person.

Detection of the phenology of various species

In this study, we found a distinct phenological pattern for each ASV, for example, early start (February 19) and prolonged period (58 days) for *Cryptomeria japonica* (ASV1), and in contrast, a late start (April 15) and the short period (16 days) for *Ginkgo biloba* (ASV9) (Fig. 3). These phenological patterns, such as timing (Clot 2003) and the duration of pollen dispersal (Ziska *et al.* 2011), will be changed by future air temperature increases due to global warming. For example, for *Cryptomeria japonica*, the first day of observation became earlier, and the duration got longer in 15 years of record from 1983 to 1998 due to 394-temperature warming (Teranishi *et al.* 2000). Furthermore, this phenological change follows an increase in the number of pollen grains because the temperature in previous July relates to male flower formation during the next season (Ito *et al.* 2008; Yamada *et al.* 2014), and the average temperature in Tokyo has risen 1.15 °C in the past 100 years, which is higher than 0.6 °C as the global average (Yamada *et al.* 2014).

This study's approach, using the DNA marker as pollen indicator, enables us to show the timing and duration of various taxonomy from various sources, information that difficult to obtain by classical morphological measurement. Therefore, long-term pollen monitoring using DNA markers can supply detail of phenological changes of forest and urban plant communities. However, for minor types which has lower relative abundance, pollen periods were much shorter due to discontinuous distribution. Therefore, the definition of the period in this study should be considered, especially for minor types.

This study supports the potential utility of developing a pollen monitoring system using eDNA measurements. In order to construct a new pollen monitoring system, one needs to

consider the standard protocol. For example, in this study, we only used *rbc*L for the DNA marker, and it is difficult to identify some of the taxonomy (e.g., Fagaceae in this study). Furthermore, detection accuracy varies by markers (Brennan *et al.* 2019); therefore, we recommend using multiple markers such as *rbc*L, *trn*L, and ITS for better comparison. Also, high volume air samplers, with sampling volumes about one order higher than used in this study, have been introduced for use in recent bioaerosol studies (e.g., Mbareche *et al.* 2018; Archer *et al.* 2019), and these can reduce contamination in the extraction and enrichment processes, thereby improving the detection accuracy of rare taxa.

Conclusion

In the air of suburbs of the Tokyo metropolitan area, 78 families were detected during the spring season (February to May) using the eDNA approach. Japanese cedar (*Cryptomeria japonica*) was the most major allergen from mid-February to March, which corresponded to the typical pollinosis season in this region. Backward trajectory analysis of air masses suggests that the Japanese cedar and other Cupressaceae plantation forests in the western mountains were a significant source of airborne pollen communities. And other major plant pollen sources, such as Japanese zelkova (*Zelkova serrata*) and gingo (*Ginkgo biloba*) were distributing nearby parks or roadside regions and emanated from there. The phenology of multiple pollen were detected, for example longer period for *Cryptomeria japonica* (58 days), and the short period for *Ginkgo biloba* (16 days). This approach is very useful to understand the regional difference of multiple pollen dispersal between the major cities and prevention and treatment for allergic rhinitis.

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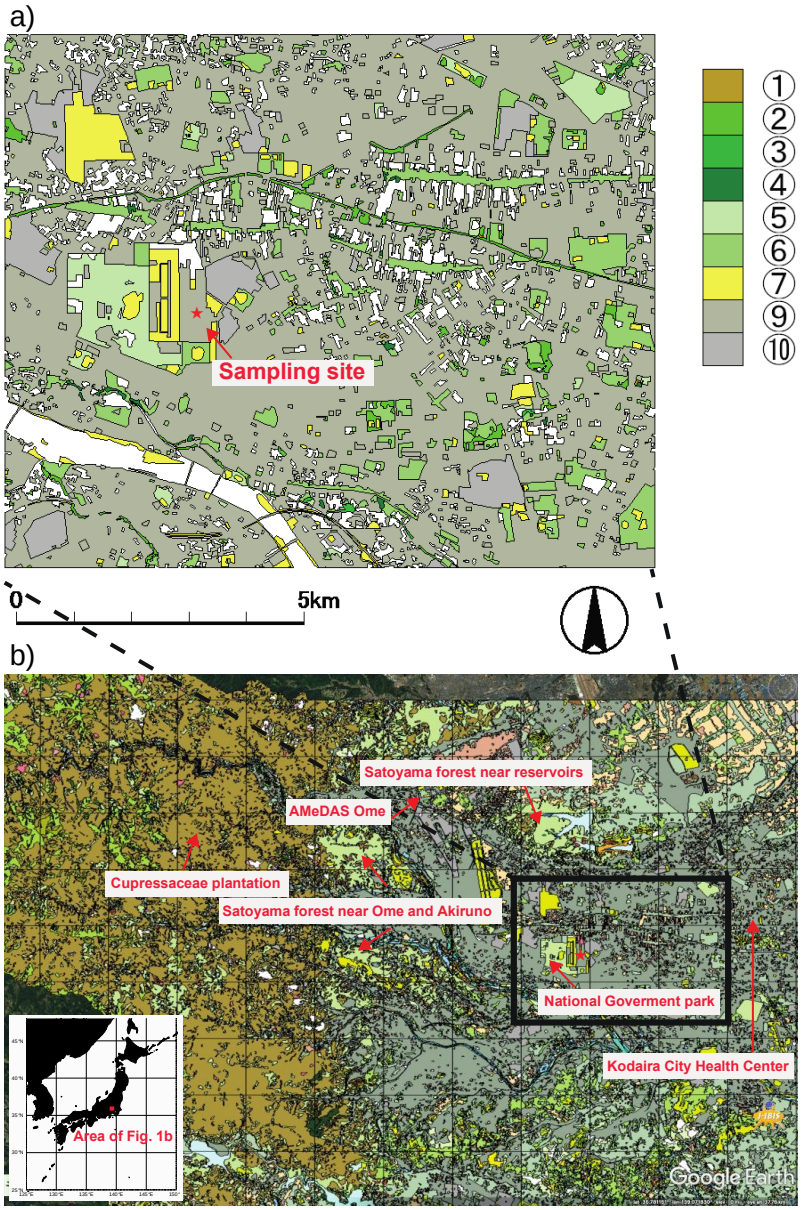

Figure 1: Vegetation map a) near the sampling site, b) in the regional scale. 1: Cupressaceae plantation (*Cryptomeria japonica*, *Chamaecyparis obtusa*, *Chamaecyparis pisifera*), 2: *Quercetum acutissimo-serratae*, 3: *Quercetum myrsinaefoliae*, 4: *Quercus myrsinaefolia premises* forest, 5: Park, Graveyard etc. with residual, planted trees, 6: Urban and residential district with many trees, 7: Golf links and Turf, 8: Plant communities in clear-cut area, 9: Urban district with a few trees, 10: Factory and industrial area. More detail is available from the Ministry of the Environment (detail in Method section).

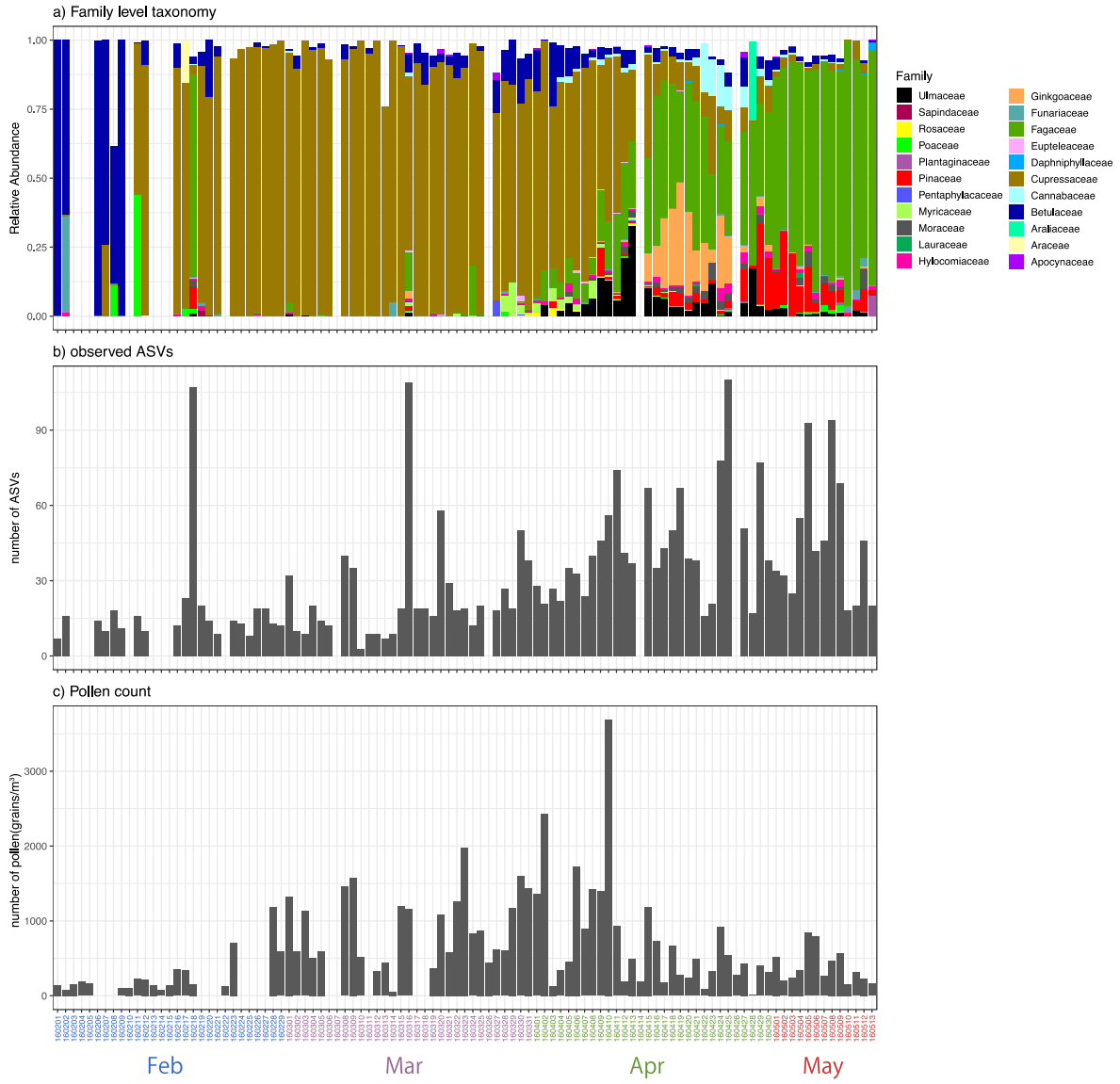

Figure 2: Seasonal change of a) taxonomy at the family level, b) the number of observed ASVs, and c) pollen concentration by an automated pollen monitoring system of the Ministry of Environment.

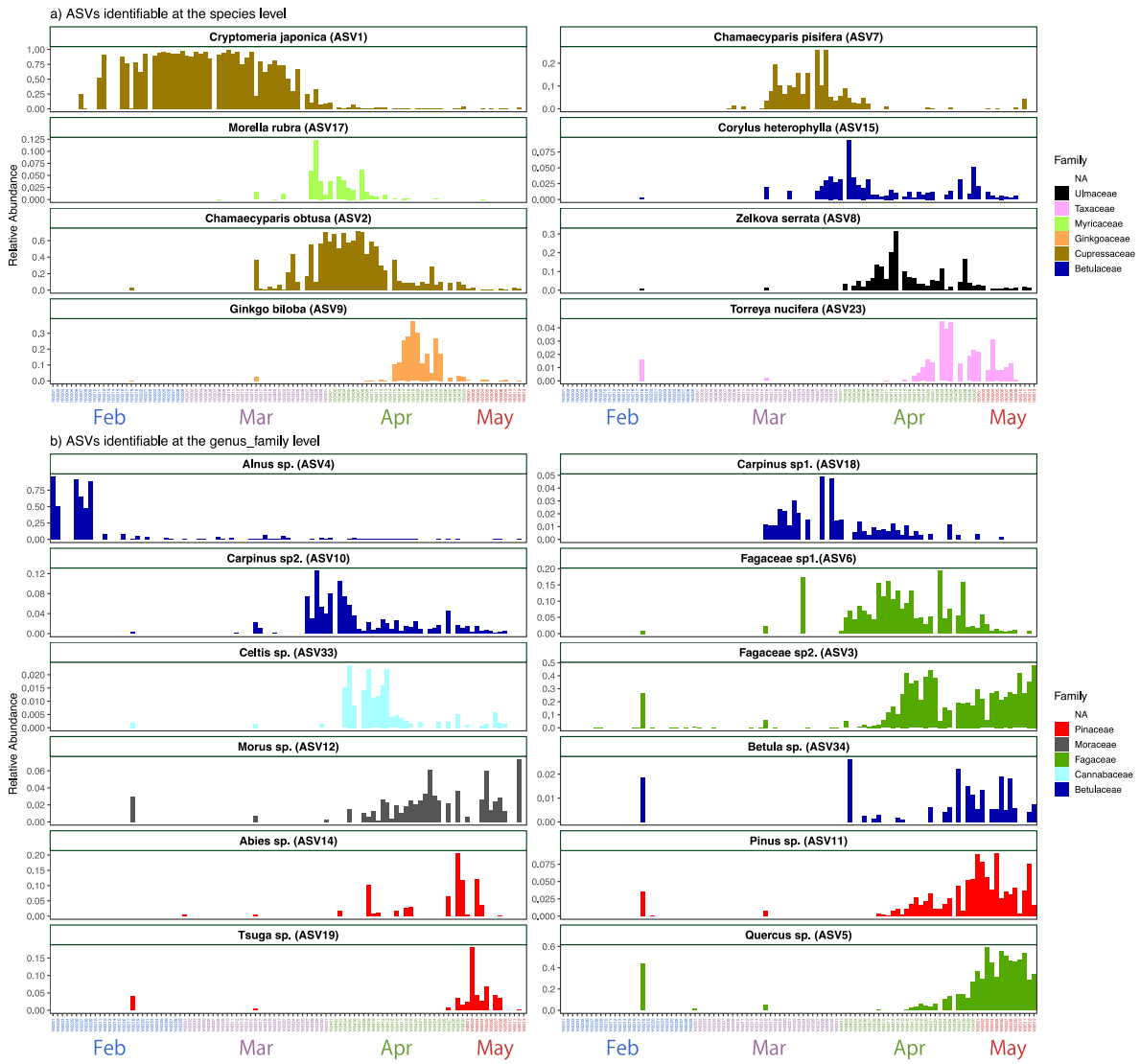

Figure 3: Seasonal change of 20 major ASVs, identifiable at the species level (a), and at genus-family level (b).

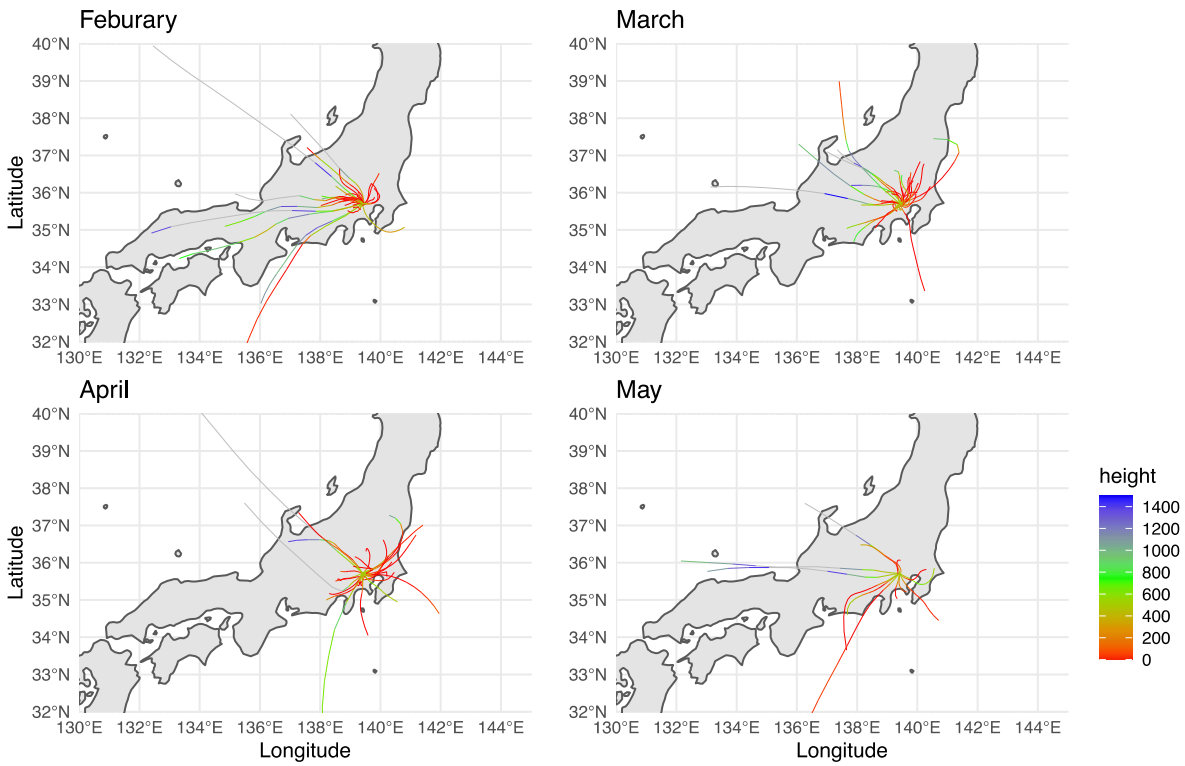

Figure 4: 24 hour back trajectories calculated using HYSPLIT4 with GDAS 1 degree data, initiated above the sampling site (500 m).