

# Using pollen DNA metabarcoding to profile nectar sources of urban beekeeping in Kōtō-ku, Tokyo

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## Research note

**Keywords:** Honeybee, Urban beekeeping, Nectar source, Pollen, DNA metabarcoding, Metagenome, Ribulose 1,5-bisphosphate carboxylase/oxygenase, Chloroplast, Next generation sequencing

**Posted Date:** July 27th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-44772/v1>

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**Version of Record:** A version of this preprint was published on November 10th, 2020. See the published version at <https://doi.org/10.1186/s13104-020-05361-2>.

# Abstract

**Objective:** *Apis mellifera* is a honeybee that has been introduced around the world as an industrial beekeeping species. Recently, urban beekeeping has attracted attention as a means of ecosystem protection and urban greening. This study aimed to investigate nectar sources of an urban beekeeping in Kōtō-ku, Tokyo using pollen DNA metabarcoding.

**Results:** We extracted DNA from pollen collected by the honeybees of a local urban beekeeping. DNA metabarcoding analysis was carried out by sequencing a part of the *rbcL* region of the chloroplast genome. A total of 31 samples collected between mid-March, 2018 and mid-October, 2018 yielded 54 operational taxonomic units (OTUs) comprising 14 families, 32 genera, and 8 species. Whereas 5 OTUs were profiled throughout all seasons, 38 OTUs were season-specific (spring, summer, or autumn). Therefore, we were able to infer seasonal nectar sources for the beekeeping at the family or genus level, and at the species level to a lesser extent. Our pollen-sampling strategy was effective for profiling season-specific nectar sources, with the exception of a few anomalies that can be accounted for by out-of-season flowering by artificial gardening and/or pollen accumulation over multiple seasons.

## Introduction

*Apis mellifera* is a honeybee that has been introduced around the world as industrial beekeeping species. Although *A. cerena* has long been used in Japanese beekeeping as a part of the traditional culture, *A. mellifera* was introduced via the United States in 1877 [1]. Honeybees generally collect nectar and pollen from flowers that they visit to provide the nutrients necessary for colony maintenance and development [2]. Nectar is processed to form honey, the main energy source for the colony. Pollen represents the colony's only supply of protein, and is essential for brood rearing and the development of hypopharyngeal glands in young worker bees [3]. Urban beekeeping has recently attracted worldwide attention as a useful method of bee conservation in urban areas and as a method of promoting urban greening, which is an important countermeasure to heat islands [4–7]. A Japanese urban beekeeping project known as the Ginza Honey Bee Project was initiated in Tokyo in 2006, and is currently being implemented in many cities and communities. In addition to honey harvesting, this project includes four objectives: environmental education, such as providing honey harvesting experience as well as workshops; development of local brands using the harvested honey; promotion of urban greening around the region; and investigation of surrounding nectar and pollen sources [8].

High-throughput sequence technology using next-generation sequencing (NGS) has been variously applied to such uses as novel genomic construction and transcriptomics. Another novel technique that employs NGS technology is DNA metabarcoding, which is based on metagenomic analysis. This technique has enabled investigation into various aspects of biome composition by comprehensively identifying barcode regions common to organisms occupying a particular habitat type (e.g. soil, water, or air). Conventional DNA barcoding based on Sanger sequencing technology has been used to analyse pollen collected by honeybees [9–11]. While this technique can be useful in elucidating information on a

fine scale, it is not practical for large-scale application [12]. To address this limitation, DNA metabarcoding has recently been applied to pollen collected by honeybees [13–16]. This updated approach to pollen analysis can be expected to yield improvements in terms of efficiency, cost, and labour.

Located on the waterfront of Tokyo Bay, Kōtō-ku is a special ward within Tokyo Metropolis, Japan, and has been targeted for development as a green city (Fig. 1). Urban beekeeping has been practised at Musashino University of Kōtō-ku since 2014 as a practical education program that is part of the ‘Living Laboratory for Sustainability’ environmental project. This project explores the potential role of urban beekeeping as an integral aspect of a sustainable landscape design model based on symbiosis among honeybees, plants, and humans. However, knowledge of surrounding nectar-source plants is primary to successful beekeeping. By using DNA metabarcoding to analyse pollen collected by the honeybees, this study investigated the kinds of plants that serve as nectar sources for the Musashino University bee colonies, because honeybee collect both nectar and pollen as described above.

## Main Text

## Methods

### Materials collection

Beekeeping (*A. mellifera*) is carried out on the roof of the second building at Musashino University Ariake Campus in the Ariake district of Kōtō-ku, on the Tokyo Bay coast (Fig. 1). A microspatula-tip full of bee pollen was obtained from an uncovered and a relatively new honeycomb. Pollen was bright orange or yellow in colour, low-viscosity, and low-permeability. Samples were collected three times per month (representing early, middle, and late periods) on rain-free days during daylight hours from mid-March, 2018 to mid-October, 2018, with the exception of the mid-July sample period, which was omitted (Fig. S1).

### DNA metabarcoding analysis

After adding Lysis Solution F (Nippon Gene, Tokyo, Japan), 0.5 mm zirconia beads, and 5.0 mm stainless steel beads to the bee pollen sample, the liquid was shaken at 1,500 rpm for 2 min using a Shake Master Neo (Biomedical Science, Tokyo, Japan), and was then incubated at 65 °C for 10 min. After centrifugation at  $12,000 \times g$  for 10 min, supernatant was collected. Genomic DNA was extracted using an MPure Bacterial DNA Extraction Kit (MP Bio Japan, Tokyo, Japan). The extracted DNA solution was mixed with a final concentration of 10% polyvinylpolypyrrolidone (PVPP) and purified by collecting the supernatant after centrifugation. The DNA concentration was measured with a Synergy H1 (BioTek, Winooski, VT, USA) and a QuantiFluor dsDNA System (Promega, Madison, WI, USA).

An amplicon library targeting a part of the *rbcL* region of the chloroplast genome was constructed by two-step PCR. The 1st PCR primer set was customised within the barcode region as a mini-barcoding system

for amplicon sequencing by short-read NGS [17]. This PCR was carried out in a total volume of 10  $\mu$ l, containing 0.5 ng template DNA, 0.5  $\mu$ M forward primer (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTACCAGYCTTGATCGTTACAAAGG-3' [underline indicates the Illumina adapter sequence]), 0.5  $\mu$ M reverse primer (5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGTAAAATCAAGTCCACCRCG-3' [underline indicates the Illumina adapter sequence]), 0.2 mM dNTP mixture, 1  $\times$  company-supplied buffer, and 0.05 U ExTaq HS DNA polymerase (TaKaRa Bio, Shiga, Japan). The reaction cycles were as follows: initial denaturation at 94  $^{\circ}$ C for 2 min; 30–35 reaction cycles at 94  $^{\circ}$ C for 30 sec, at 50  $^{\circ}$ C for 30 sec, and at 72  $^{\circ}$ C for 30 sec; and final extension at 72  $^{\circ}$ C for 5 min. After clean-up of the 1st PCR product, the 2nd PCR was performed under the same conditions as the first, with the following modifications: a forward primer (5'-AATGATACGGCGACCACCGAGATCTACAC[index 2]ACACTCTTTCCCTACACGACGC-3') and a reverse primer (5'-CAAGCAGAAGACGGCATAACGAGAT[index 1]GTGACTGGAGTTCAGACGTGTG-3') were added, and adjustments were made to some of the PCR programs (12 cycles and annealing temperature at 60  $^{\circ}$ C). After clean-up of the 2nd PCR product, the quality of the constructed library was checked using a Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA, USA) and a dsDNA 915 Reagent Kit (Advanced Analytical Technologies). Multiple libraries were pooled and sequenced by running 2  $\times$  300 bp paired-end reads using a MiSeq platform (Illumina, San Diego, CA, USA).

Raw read data were cleaned by removing primer sequences using the Fastx-Toolkit ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)). In addition, reads with a quality score of less than 20 or length of less than 40 nt were excluded using Sickle (<https://github.com/ucdavis-bioinformatics/sickle>). The clean-paired reads were merged using FLASH [18]. Parameters were merged as follows: (i) minimum overlap length = 10, (ii) average read length = 230, and (iii) average fragment length = 320. Operational taxonomic units (OTUs) were clustered by a 97% similarity threshold using USEARCH (<http://www.drive5.com/usearch/>). The most abundant sequence from each OTU was selected as the representative sequence and was annotated for target species with 97% similarity against the NCBI non-redundant nucleotide 'nt' database using the BLASTN program [19]. For each OTU that was annotated to different species with the same similarity score, the genus or family common to those was assigned. After reads were mapped to representative OTUs, they were corrected by length (count per million [CPM]).

## Results

A total of 31 samples were obtained from each sampling period. Thirteen samples (for example early May) were taken from a single honeycomb on the same day to assess any potential variation in pollen accumulated among different honeycombs. Sequence data for 6,994 – 95,832 paired-end reads were generated as output for each sample (Table S1). Approximately 13%-94% reads per sample were available for OTU profiling. Non-target OTUs, which included sequences that were classified as honeybee, human, *Zygosaccharomyces* sp., Shuttle vector, *Ralstonia pickettii*, and *Trebouxia showmanii*, were excluded from analysis. Those classified as Pinaceae, Cupressaceae, Podocarpaceae, Arecaceae, Poaceae, and Woodsiaceae were also excluded, as these represent wind-pollinated flowers.

A total of 54 OTUs were obtained by DNA metabarcoding analysis (Fig. 2). They were annotated as comprising 14 families, 32 genera, and 8 species. The average number of OTUs per sample was 5.9, with a range of 1–31. Moreover, multiple samples that were obtained from different honeycombs on the same day shared some OTUs in common with each other, although the matches were imperfect. The family Fagaceae and the genera *Salvia*, *Photinia*, *Hydrangea*, and *Trifolium* were detected throughout all three seasons. The family Fabaceae and the genera *Prunus*, *Papaver*, *Spiraea*, *Citrus*, *Celastrus*, and *Phellodendron* were detected only in spring (March, April, and May). The family Vitaceae and the genera *Hypericum*, *Mallotus*, *Passiflora*, and *Erythrina* were detected only in summer (June, July, and August). The families Brassicaceae, Fabaceae, Hydrangeaceae, Verbenaceae, Asteraceae, and Polygonaceae; the genera *Bidens*, *Liriope*, *Abelia*, *Polyspora*, *Phyla*, *Berberis*, *Chloracantha*, *Allium*, and *Eruca*; and the species *Ulmus parvifolia*, *Commelina communis*, *Begonia herbacea*, *Elaeagnus macrophylla*, *Diplotaxis tenuifolia*, and *Berberis thunbergii* were detected only in autumn (September and October).

## Discussion

Although our approach enabled profiling of many families and genera, few identifications were made at the species level. In particular, half of the OTUs annotated at the family level were related to Asteraceae and Fabaceae, which constitute two of the three largest plant families (the third being Orchidaceae) [20]. The restrictive approach to barcoding used here, which was specific to the *rbcl* region of the chloroplast genome, may lead to difficulty in recognising common sequences between species. However, even the more complex approach, which uses portions from both the *rbcl* and *matK* regions of the chloroplast genome along with the ITS2 region of the nuclear genome, has not been completely refined [16]. Therefore, the technique of applying DNA metabarcoding to plants using pollen requires further improvement in terms of the identification and selection of more robust barcoding regions, and the accrual of many more reference genomes.

In this study, five OTUs were detected in all three seasons. These included garden plants such as sage (*Salvia* sp.) and white clover (*Trifolium* sp.), which flower throughout the entire year. In contrast, 38 OTUs were detected as 'seasonal OTUs'. These include park and street plants such as cherry (*Prunus* sp.) and echidna (*Cytisus* sp.) in spring, Mallotus bark (*Mallotus* sp.) and passion flower (*Passiflora* sp.) in summer, and lacebark elm (*Ulmus parvifolia*) and broad-leaved oleaster (*Elaeagnus macrophylla*) in autumn. However, some plants were suggested based on profiled OTUs for seasons other than their typical flowering time. In particular, samples collected during the autumn season tended to yield many such annotations. This could possibly be the result of two factors: out-of-season flowering induced by artificial gardening techniques, and/or pollen accumulation over multiple seasons. As noted above, we found that different honeycombs sampled on the same day tended to share some OTUs in common. However, our sampling method may require improvement, because it allowed arbitrariness in sample selection on the part of the experimenter. One potential improvement could be the use of a pollen trap [21]. We can therefore conclude that the sampling method is a very important consideration in pollen DNA metabarcoding analysis.

The range of honeybee travel has long been believed to be within 2 km [22]. However, recent reports based on new research techniques indicate that bees can forage approximately 10 km further than previously thought [23, 24]. A large variety of plants occupies the 14 parks or grounds that occur within a radius of 2 km from our beekeeping site (Fig. 3). In addition, 26 parks or grounds are located across the sea within a distance of 4 km. Although it is plausible that honeybees could forage from these locations, only a few OTUs indicated that this was the case. We posit that the honeybee foraging range could be limited by beekeeping management practises and inclement weather (Table S2). However, this study did not demonstrate these associations.

In summary, most of the plants used as nectar sources by the urban honeybees studied herein were identified at the family and genus levels; most plants could not be identified at the species level. We conclude that further development of this approach will enable the creation of a full-year pollen calendar. In addition, it is also an effective method for assessing landscape diversity.

## Limitations

The data used in this study were obtained through metabarcoding of plant DNA that was derived from pollen collected by honeybees. However, there is no supporting data regarding the locations of the identified plant species.

## Declarations

### *Ethics approval and consent to participate*

Not applicable.

### *Consent for publication*

Not applicable.

### *Availability of data*

The dataset supporting the conclusions of this article is available in the DDBJ Sequence Read Archive (accession number DRA010035) after publication of this report.

### *Competing interests*

The authors declare no competing interests.

### *Funding*

Musashino University Creating Happiness Incubation supported this work.

### *Authors' contributions*

The manuscript was written by KT and MS-K. The experimental design, including material collection and DNA extraction was conducted by AN, HN, and MS-K. Data analysis for DNA metabarcoding was supported by KT and YS. This study was planned by MS-K as the supervisor. All authors have read and approved the final manuscript.

### ***Acknowledgments***

DNA metabarcoding analysis was performed by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). We thank Dr. Osamu Akashi and the beekeeping group of Dept. Environ. Systems Sciences, Musashino Univ. for granting us permission to sample bee-collected pollen at the Ariake Campus.

## **Abbreviations**

DNA: deoxyribonucleic acid

NGS: next-generation sequencer

PVPP: polyvinylpyrrolidone

*rbcl*: *ribulose-1,5-bisphosphate carboxylase/oxygenase*

PCR: polymerase chain reaction

OTU: operational taxonomic unit

CPM: counts per million

*matK*: *Maturase K*

ITS2: internal transcribed spacer 2

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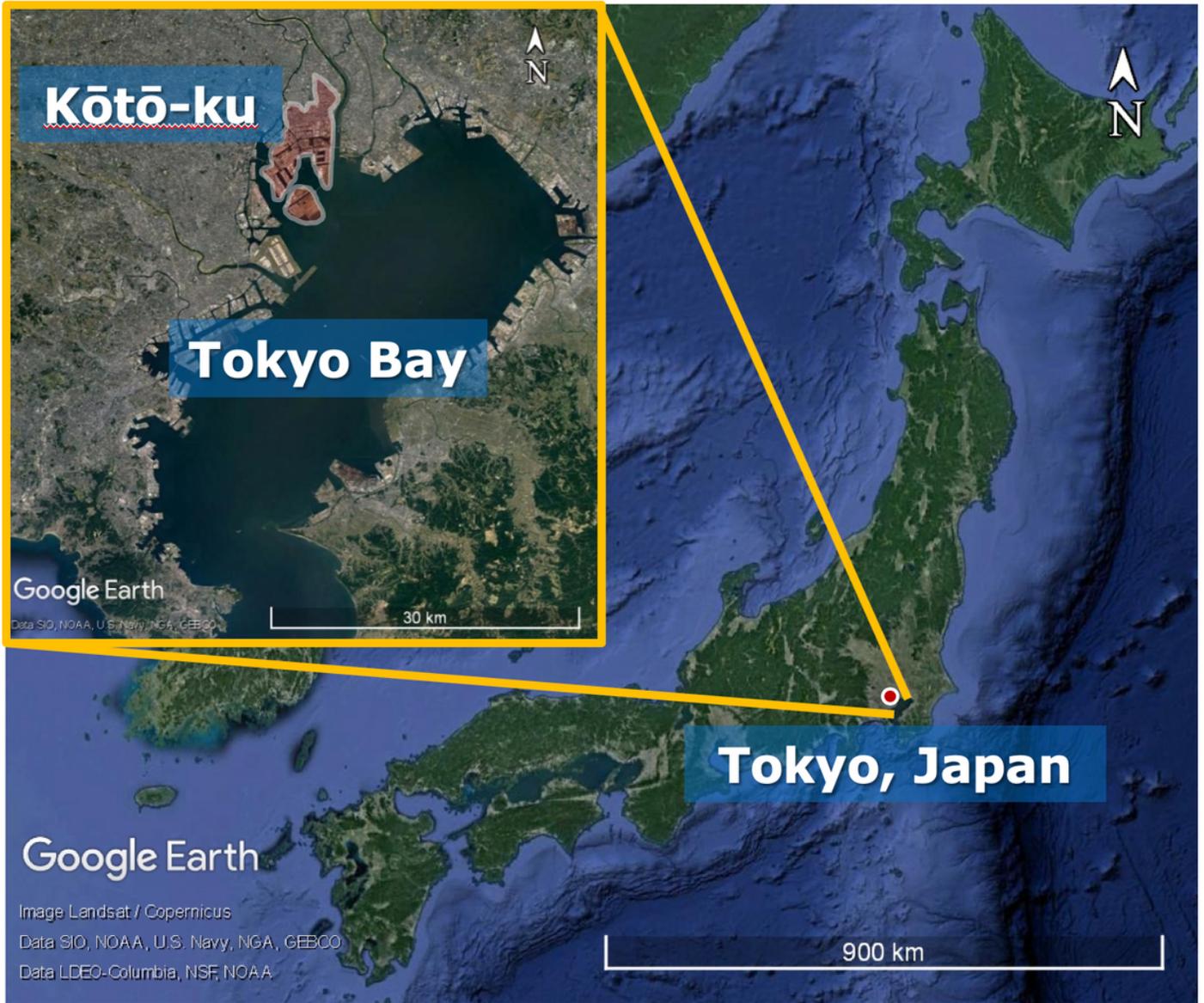
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## Supplementary Tables

Tables S1 and S2 were not provided with this version.

## Figures



**Figure 1**

Topography of Kōtō-ku, located on the waterfront of Tokyo Bay, Japan.

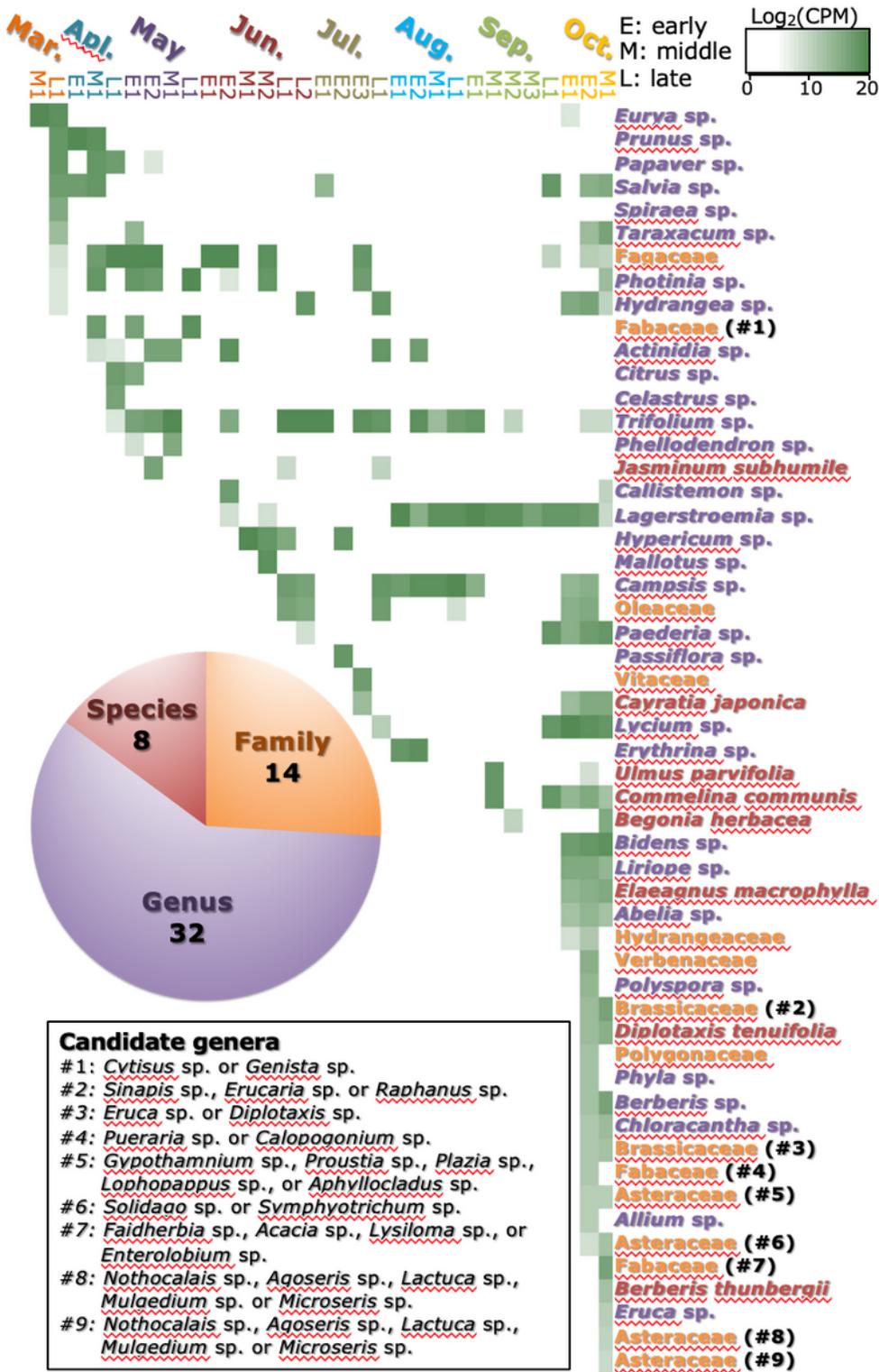


Figure 2

Profiles of annotated OTUs from each sample. The read count for each OTU is indicated by the log<sub>2</sub>CPM. Taxonomic level is indicated by colour; family (orange), genus (purple), and species (red). These categories are shown as pie charts in the lower left portion of the figure. To facilitate visualisation of variations in seasonal patterns, the annotated OTUs are listed in the order of appearance from March to

October. Nine OTUs with the symbol “#” show that they are annotated as different species with same similarity score; candidate genera are described within this figure.

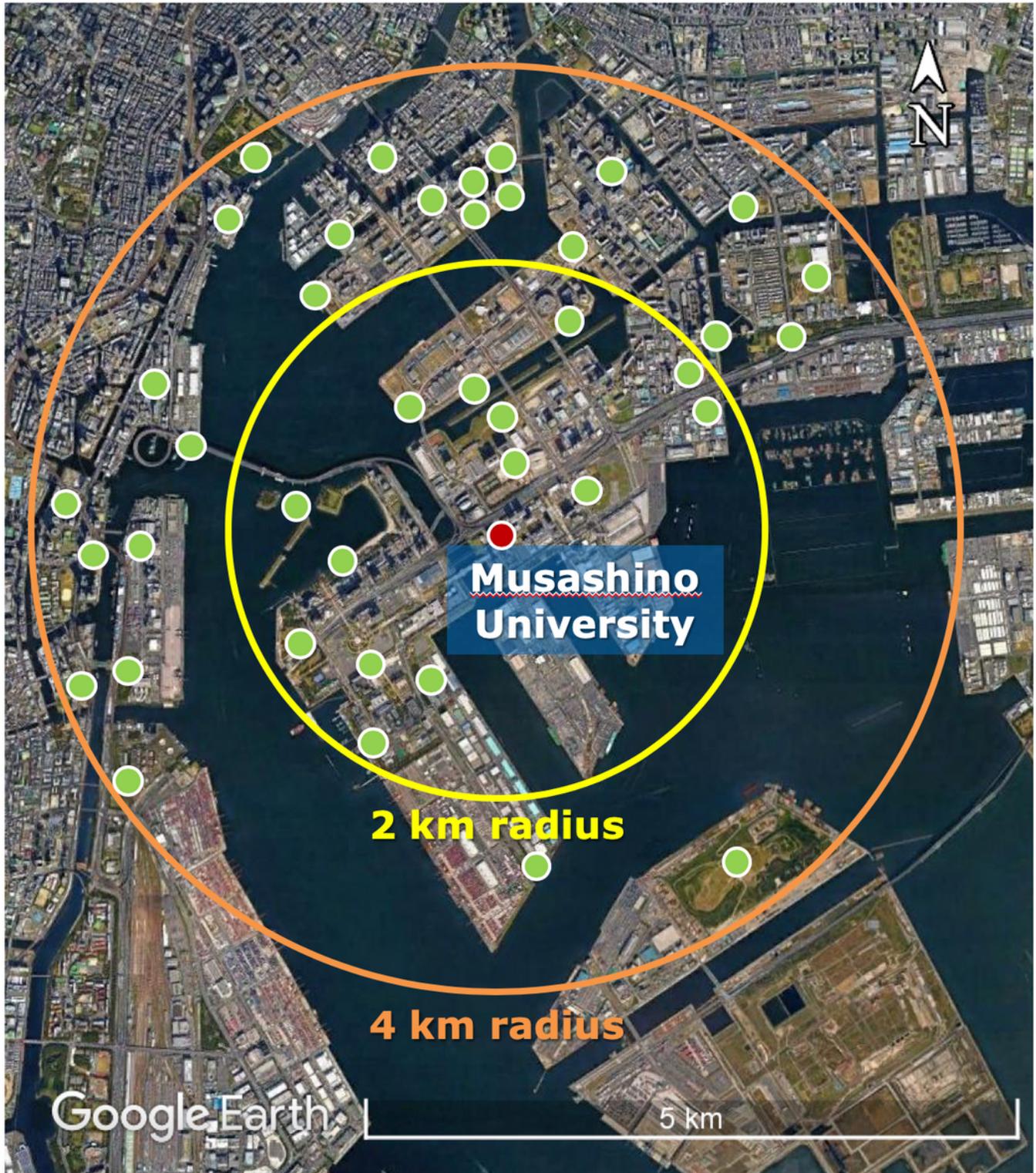


Figure 3

Locations of major planted parks and grounds in Kōtō-ku. The red plot shows the beekeeping site at Musashino University; the green plot shows parks or grounds.

## Supplementary Files

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- [SupplementaryFigure.pdf](#)