Abstract

The availability of pollen in urban-rural landscapes is an essential factor that influences the population dynamics of insect pollinators. The amount and diversity of pollen play a pivotal role in the foraging ecology of pollinators for their growth and health, but investigations on the spatio-temporal patterns of foraged plants remain rare, especially in cities as neo polylectic -ecosystems. Here, we explored the temporal foraging habits of a highly pollinator (*Apis mellifera* L.) in Tokyo, including different landscape classes from rural to urban areas. Mixed-pollen samples in each month and each location (N = 17) were analysed using DNA meta-barcoding to identify plants visited by honeybees. The results showed that the landscape class (rural, suburban and urban areas) explains spatial variations in pollen source-plant composition foraged by honeybees, but not in taxa richness. Furthermore, pollen diversity and pollen source-plant composition showed a strong seasonal dependence. A higher plant richness and foraged woody taxa was found to occur in spring, which was mainly dominated by the genera *Prunus* and *Acer*. In summer and autumn, the genera *Trifolium* and *Plantago* of the herbaceous stratum were the most visited plants. The Fabaceae, Rosaceae, Brassicaceae, Plantaginaceae, and Onagraceae plant families were the most frequently observed in all combined samples. The present study contributes to a deeper understanding of the foraging ecology of *A. mellifera* colonies across urban-rural gradient surrounding mega-cities such as Tokyo.

Keywords: Pollen, metabarcoding, community structure, foraging ecology, *Apis mellifera*, urban ecology

Introduction

Ongoing urbanisation is one of the main drivers of landscape degradation and pollinator biodiversity loss (Ahrné et al., 2009; Concepción et al., 2015; Fortel et al., 2014; Potts et al., 2010; Sánchez-Bayo and Wyckhuys, 2019). Indeed, floral resources are becoming scarcer under the pressure of urban fragmentation, and the increase in impervious surfaces is rendering nesting sites inaccessible to pollinators (Harrison and Winfree, 2015; Wenzel et al., 2020). However, recent studies have revealed that cities can also act as a refuge for pollinators (Hall et al., 2016), particularly for bees (Theodorou et al., 2020): (i) cities are less exposed to pesticides (Fortel et al., 2014; Muratet and Fontaine, 2015), (ii) urban management sustainably permits the maintenance of their floral resources (Baldock, 2020; Pardee and Philpott, 2014), and (iii) urban areas are configured with a heterogeneity of green spaces, which would be favourable to the foraging preferences of bees (Baldock et al., 2015). Moreover, flowerbeds in the urban matrix are highly attractive and are a source of pollen and nectar for insect pollinators (Garbuzov et al., 2015a; Garbuzov and Ratnieks, 2014). With an increasing popularity in beekeeping activities in cities, honeybees (*Apis mellifera* L.) contribute to urban plant pollination, generate profits of by-products, and provide environmental education (Cho and Lee, 2018; Tanaka et al., 2020). However, the massive introduction of urban honeybees has led to growing concerns about detrimental effects on wild pollinators through an increase in floral resource competition and the spillover of shared pathogen agents (Geslin et al., 2017; Ropars et al., 2019).

As a eusocial species, honeybees organise their floral resource collections (i.e. nectar and pollen) through a complex communication system within their colonies. According to plant phenology, honeybee scouts rapidly recruit their siblings to forage on rich new patches of flowers using a characteristic waggle dance (von Frisch, 1965). The foraging decision-making system of the colony can vary from day to day or within the same day following real-time nectar and pollen availability in the surroundings. Throughout its active seasons, the colony constantly maintains a balance between its biomass and energy management according to the availability and the diversity of surrounding floral resources. This strategy ensures sufficient food stores and energy reserves for winter (Alaux et al., 2017; Seeley, 1995). Pollen diversity provides substantial resources in terms of protein, lipid, vitamin, and mineral supplies (Haydak, 1970). Large amounts of pollen (15–30 kg) are collected annually, mainly for brood production during summer (Avni et al., 2014, 2009; Seeley, 1995). The quality and diversity of pollen are also essential for better life expectancy and immunity, as well as the parasite or pathogen tolerance of the bees and the colony (Alaux et al., 2010; Di Pasquale et al., 2013; Wang et al., 2014; Wilde et al., 2003).

The preservation of ecosystem functioning relies on the mutualistic networks of pollinators and plants. Several methods are used to assess these interactions: the observation of floral visits, digital tracking systems to capture floral visits, chemical signatures of pollen, pollen genetic sequencing, and pollen light microscopy (Cornman et al., 2015). Identifying a pollen species or genus by light microscopy from mixed pollen samples, also known as melissopalynology (Ohe et al., 2004), is a time-consuming process, even for well-trained experts (De França Alves and De Assis Ribeiro DosSantos, 2014) that results in low taxonomic resolution, usually at the family or genus rank at best (Bell et al., 2016; Kaškoniene and Venskutonis, 2010; Rahl, 2008; Richardson et al., 2015a). With the advent of high-throughput sequencing (HTS) techniques, DNA meta-barcoding approaches have become reliable methods to obtain faster taxonomic profiles with higher resolution of mixed pollen collected from bees or flowers (Bell et al., 2017; Pornon et al., 2016; Sickel et al., 2015). To elucidate the plant taxonomic composition of mixed-pollen samples, the meta-barcoding process can be based on different genetic markers, such as the *rbcL*a, *matK*, *trnH-*

psbA, *trnL*, and *ITS* regions (mainly *ITS2*), which require high inter-specific and low intraspecific variability (Galimberti et al., 2014; Hawkins et al., 2015; Kraaijeveld et al., 2014; Richardson et al., 2015a; Sickel et al., 2015). These selected loci, the primer set and the differences in available plant reference sequences for each marker used for amplification drive the range of taxonomic inferences (Bell et al., 2016).

The floral plant composition of agricultural or urban landscapes heavily impacts the foraging ecology of honeybee workers (Danner et al., 2017; Lucek et al., 2019; Richardson et al., 2021). Indeed, the fragmentation of urban matrix usually leads to the creation of small, remote, and intensely maintained green spaces (Bastin and Thomas, 1999), which could influence the foraging distance of honeybee workers (Garbuzov et al., 2015b). As the most polylectic bee forager (Butz Huryn, 1997), honeybees can adapt to shortages in floral resources by enlarging their foraging area (Danner et al., 2016; Steffan-Dewenter and Kuhn, 2003). Moreover, seasonal shifts greatly impact the pollen availability for honeybee colonies, according to the phenology of the floral resources (Danner et al., 2017; Sponsler et al., 2020). In temperate climates, the foraged plant characteristics also vary according to the course of the seasons: spring is dominated by trees and shrubs, summer has more herbaceous species, and autumn is characterized by woody vines (Sponsler et al., 2020). Nonetheless, the space-time effects combined with plant characteristics in the foraged plant community have yet to be studied extensively (Richardson et al., 2021). Understanding the floral range dynamics of honeybee colonies according to urbanization gradient would help to better determine the foraging ecology of the honeybee colonies in cities.

To achieve this aim, the taxonomy of pollen foraged by honeybees was identified over the seasons along an urban-rural gradient from different locations in the Kanto region, Tokyo, and its surroundings in Japan. The research questions addressed were as follows: (i) How does the composition of the foraged flower community (and the foraged plant characteristics) vary along an urban-rural gradient? (ii) How does the composition of foraged flower communities and characteristics of pollen forage plants vary according to the course of the seasons?

Material and methods

Study area and experimental set-up

We selected 17 apiary locations, with homogeneous climatic and altitudinal conditions (Table S1), distributed along an urbanisation gradient in the Kanto region of Japan (Fig. 1). One hive per apiary was used for pollen sampling. From March to September 2019, pollen samples were collected using pollen traps at the entrance of the same hive (Fig. S1). This experimental setup was used to collect pollen balls from the hind legs of honeybee foragers with a standardised honeybee-size mesh and tray (Mahmood et al., 2017). Then, the contents of the pollen traps were discharged into labelled 50-ml conical tubes and stored at –20°C. However, due to meteorological conditions and the personal schedules of the beekeepers, the sampling date, collection frequency, and operational time of the pollen trap varied from site to site for a total of 143 collected pollen samples (Table S2). Each corbiculate pollen sample was thoroughly mixed by lightly kneading all collected pollen with a mortar and pestle. Then, all samples were sent to the private company, Bioengineering Lab. Co., Ltd. (https://www.gikenbio.com/, consulted on 20/07/2020) for the processing of the meta-barcoding of the mixed pollen samples.

Landscape analysis

Using remote sensing techniques, the landscape structure was investigated within a 6-km radius around each hive location (Table 1); this distance enclosed 95% of the forage area per colony (Seeley, 1995). With the help of Planet Labs Inc. (Planet Core Team, 2020), we used multi-spectral images (RGB, NIR) with 3-m pixel resolution. To fully exploit the potential of the data, the cloud cover condition was set to a maximum of 5%. Planet data are relevant for computing and mapping high-resolution terrestrial above-ground vegetation at the landscape scale (Miller et al., 2019). For each planet image, the normalised difference vegetation index (NDVI) was computed using the red and near-infrared bands based on band rationing, which allowed for the delineation of the vegetation cover from other types of land cover (Xue and Su, 2017). Classes were created with the function *reclassified* from the *raster* package in R (Hijmans et al., 2020) by defining the NDVI threshold values to distinguish the water (NDVI: from -1 to -0.2), the impervious surface (NDVI: from -0.199 to 0.199) and the vegetation (NDVI: from 0.2 to 1) (Hashim et al., 2019; Taufik et al., 2016). A majority filter, with a 6 × 6 filter kernel size, from the *whitebox* package in R (Lindsay, 2016), was applied to smooth the result and aggregate regions of high uncertainty. Landscape classifications at the site level were performed using demographic data (i.e. number of inhabitants per admin units) and landscape metrics from the *lconnect* (Mestre and Silva, 2019) and *landscape metrics* (Hesselbarth et al., 2019) packages in R. To classify our sites along an urban-rural gradient (Bastin and Thomas, 1999; Hadley and Betts, 2012), we retained selected data: number of inhabitants per km^2 (dpop), the integral index of connectivity (IIC) (Saura and Pascual-Hortal, 2007), the effective mesh size (MESH) (Spanowicz and Jaeger, 2019), Shannon's evenness index (SHEI) (Shannon, 1948), the vegetation cover proportion (veg cover), the vegetation patch density (Threlfall et al., 2015), and the median vegetation class NDVI (NDVI median) (Table S3).

We conducted principal component analysis (PCA) of the landscape dataset to visualise the differences among our study sites. The unsupervised k-means clustering method was applied to delineate the landscape category along the urban-rural gradient into k groups. Before initiating the analysis, the data were standardised using the *scale* function in R to make variables comparable. As a result, the clustering algorithm was independent of any variable unit. The number of k groups required to be defined as the first step was determined using the elbow method (Kodinariya and Makwana, 2013). The k-means partitioning analysis was performed using the *k-means* function with 25 random sets (Strickland, 2014) and the *factoextra* package in R for PCA graphical representations (Kassambara and Fabian, 2020).

Molecular techniques

DNA extraction

First, pollen samples (0.5 g) were lyophilized using a lyophilizer freeze dryer VD-250R (TAITEC, Koshigaya, Saitama, Japan). After being ground at 1500 rpm for 2 min using a ShakeMaster NEO homogeniser (bms, Shinjyuku, Tokyo, Japan), DNA was extracted using the protocol of MPure Bacterial DNA Extraction Kit (MP Biomedicals, Irvine, CA, USA). DNA purification of the samples was performed using the MPure-12 Automated Nucleic Acid Purification System (MP Biomedicals, Irvine, CA, USA). Quality control of DNA extracts was conducted using Synergy H1 (BioTek, Winooski, VT, USA) and QuantiFluor dsDNA System (Promega, Madison, WI, USA).

Library preparation and DNA sequencing

One hundred and forty-three libraries were produced using a 2-step tailed polymerase chain reaction (PCR) method. The first PCR amplification was conducted using internal transcribed spacer (ITS1) primers designed by Masamura *et al.*, (2014) (Table S4), coupled with MiSeq-specific adapters. This primer pair was selected for its effectiveness (Maeda and Takahashi, 2017) to identify more Japanese plant species compared to ITS 1 and ITS 2 region designed by Cheng et al. (2016). The second PCR amplification was conducted using index primers.

PCR reactions were carried out in a reaction volume of 10 µL containing 1.0 µL of 10× Ex Buffer, 0.8 µL of nucleoside triphosphate dNTPs (each at 2.5 mM), 0.5 µL for both forward and reverse primer at a concentration of 10 µM, 2.0 µL of DNA template normalized at 0.5 ng/µL, 0.1 µL of DNA polymerase ExTaq at 5 U/µL (TaKaRa, Otsu, Shiga, Japan) and 5.1 µL of double-distilled water. The PCR profile was as follows: 2 min of denaturation at 94°C, followed by 30 cycles with 30 s of denaturation at 94°C, 30 s of annealing at 57°C, 30 s of elongation at 72°C, and a final elongation at 72°C for 5 min. The PCR products were purified using MPure XP with ratio of beads of 0.7x (Beckman Coulter, Brea, CA, USA). The second PCR profile was follows: 2 min of denaturation at 94°C, followed by 10 cycles with 30 s of denaturation at 94°C, 30 s of annealing at 60°C, 30 s of elongation at 72°C, and a final elongation at 72°C for 5 min. Library concentrations were determined using a Synergy H1 microplate reader (BioTek, Winooski, VT, USA) and a QuantiFluor dsDNA System (Promega). Library quality was evaluated using a fragment analyser (Advanced Analytical Technologies, Ankeny, IA, USA) with a dsDNA 915 Reagent Kit (Agilent, Santa Clara, CA, USA). The generated library was sequenced using MiSeq Illumina technology (Illumina, San Diego, CA, USA) through a 2× 300 paired-end run.

Data processing

For subsequent analysis, the sampling dates were discretely pooled by month and analysed in R version 4.0.2 (R Core Team, 2020). We used Spearman's rank-order correlation between the number of taxa per sample and the sampling length in hours to test whether the data could be treated independently of the sampling length. Furthermore, we used "FASTX Barcode Splitter" from Fastx toolkit, a short-reads pre-processing tool, to extract only the target and index sequences from MiSeq reads (Hannon, 2010). Next, the reads were denoised and filtered using Sickle software (Joshi and Fass, 2011) with an overlap quality value of 20.

Trimmed reads with fewer than 150 bases were discarded. The remaining reads were merged using FLASH (version 1.2.11) paired-end merge script (Magoč and Salzberg, 2011) using the following conditions: fragment length after merging of 420 bases, read fragment length of 280 bases, and minimum overlap length of 10 bases. The open-source bioinformatic pipeline Qiime 2.0 (Bolyen et al., 2019) workflow script was used for taxa creation and taxonomic assignment for the filtered reads. The filtered read pairs were clustered with USEARCH 9.0.2132 (Edgar et al., 2011) to remove low quality data with more than 97% sequence identity. Taxon sequences were searched on the 1st February 2020 against the NCBI nucleotide database (Benson et al., 2013) using BLASTN ver. 2.9.0. and the 10 best matches were retained. Only the top 1 taxon was considered if it belongs to Tracheophyta group (i.e. vascular plants), other taxa belonging to other kingdoms were removed. Following the taxonomy classification step, taxa-abundance data and operational taxonomic unit (OTU) data were applied to the R environment (R Core Team, 2020). First, the assignment of all OTUs below the identity threshold of 97% was discarded (Danner et al., 2017; Smart et al., 2017). Next, the number of reads was sorted by genus and sample (i.e. site and collection date), and was then expressed as the ratio between the read count and the sum of read number per sample for each genus. Genera accounting for less than 0.05% of the total number of reads for a single sample were excluded to prevent false positives and two samples were removed because it accounted for less than 1000 reads to limit inferences from insufficient sequencing depth (Sponsler et al., 2020).

Taxonomic analysis

Read-matches to plant taxa were classified to genus, where possible, because ITS1 reference sequences were not enough to identify some species in the region. They were treated as incidence (i.e. presence/absence binary arrays) data because PCR biases (Bell et al., 2021).

The richness of the pollen samples (i.e. the number of distinct taxa of foraged plants) was analysed as a function of the month (i.e. converted in integer variable) and landscape variables (i.e. explanatory variables). The non-collinearity among the predictors was assessed using Pearson's correlation coefficients with a range value from -0.7 to 0.7 as selection criteria (Dormann et al., 2013). Four landscape variables were selected: dpop, SHEI, the proportion of impervious surface (Cov_urb) and the urban patch density (pd_urb) (Fig. S2). Given that the independence of our measurements could not be controlled because of the pseudoreplication of the temporal data (Table S2) and the unbalanced experimental design, general mixed-effects models (GLMMs) were used (Grueber et al., 2011; Harrison et al., 2018). The pollen collection sites were specified as random effects (1| site). Negative binomial distribution was chosen as link function to explain the distribution error of the foraged plant richness (Lindén and Mäntyniemi, 2011) using *glmmTMB* R package (Brooks et al., 2017). The selected predictors were specified as fixed effects as a function of the months and landscape variables (i.e. explanatory variables). For the model selection, Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) metrics were assessed. The lowest values of theses metrics optimize the trade-off between the fit and the complexity of the constructed models (Richards, 2008). Finally, the residual distributions of all mixed-effects models assumptions (i.e. over- underdispersion, deviance) were inspected and checked with *DHARMa* R package (Hartig, 2021).

For multivariate analysis, the pollen source-plant taxonomic composition of the samples was studied across sites, sampling periods and landscape classes using the Jaccard dissimilarity metric from the *vegan* package in R (Oksanen et al., 2019). This asymmetric distance coefficient addresses the problem of double zero, which is essential when studying data on community composition along a gradient. Differences in pollen source-plant composition between sampling periods and landscape classes were investigated by permutation-based multivariate analysis (N = 999) of variance using the *adonis* function (Anderson, 2001). If the PERMANOVA results were significant, a post-hoc multilevel pairwise analysis with Bonferroni correction was performed using the *pairwiseAdonis* package in R (Martinez Arbizu, 2020). The dissimilarities in the structures of pollinated plant communities were displayed using non-metric multidimensional scaling (NMDS) with 999 permutations. All graphics were generated using the *ggplot2* package in R (Wickham, 2016).

Indicator species and trait-based analysis

Similarity percentage (SIMPER) analysis was applied to identify how the taxonomic composition differed from the environmental conditions (landscape type) and changes (season). This step allowed us to identify the sampled taxa that contributed significantly to the dissimilarities among the months or landscapes. Finally, to analyse the characteristics of pollen forage plants, taxa were further classified by their traits including herbaceous (no woody stems above ground) or woody taxon (tree, shrub, liana), and including native, alien, or cultivar taxa. The plant trait database was built using information from Ylist (Yonekura and Kajita, 2007) and ©Species2000 (Roskov et al., 2019) for the Japanese plant dataset. To determine if the proportion between the different traits varied with the seasons and landscape types, the G-test of independence for contingency table was performed using the *RVAideMemoire* package in R (Hervé, 2020). The G-test is based on the log likelihood ratio and tests whether the relative proportions of one categorical variable (i.e. plant nature or native status) are independent of the second categorical variable (i.e. season or landscape). Next, post-hoc pairwise comparisons were conducted between pairs of proportions using the Bonferroni correction of the p-values (MacDonald and Gardner, 2000).

Results

Landscape classification

The method of differentiating vegetation from impervious surfaces using NDVI provided convincing results after crosschecking, even in the complex environment of an urban matrix. The two first dimensions of the landscape PCA from landscape variables of our sampling sites described a high percentage of the variance (axis 1 = 81.6% and axis 2 = 11.0%; Fig. 2). According to the elbow method (Fig. S3) of the k-means partitioning, we classified our sampling sites into four landscape classes according to a rural-urban gradient: rural (N = 3), suburban (N = 3), urban (N = 5), and urban centre (N = 6). The urbanised locations were driven by a much higher demographic density compared to the other landscapes (Fig. S4). Moreover, following the decrease in the proportion of vegetation along the rural-urban gradient, it can be assumed that the higher patch density in the cities was induced by the presence of many smaller plots, such as private garden patches. In contrast, the rural sites demonstrated a higher connectivity between the patches.

Taxonomic analysis

Illumina sequencing generated a total of 8,179,602 paired-end raw reads for the 143 pollen samples from the 17 sites throughout the 7 months of pollen sampling. After assembling and filtering, 6,799,314 reads (83.2%) were obtained for analysis, with a mean count of 47,548 ± 27,464 (SD) reads per sample. After taxonomic assignment of the meta-barcoding dataset, we identified 307 plant flower taxa from 74 families and 187 genera. Prior to the analysis, the richness was not correlated with the duration of sampling (Table S2), showing a very weak relationship (rs [143] = −0.17, $p < 0.05$), allowing us to consider the statistical independence of all our pollen samples. Plant richness ranged between 3 and 42 pollinated plant taxa per sample, with an average of 12 (*SD* = 6.2). GLMMs were not able to detect a significant interaction between landscape variables and collection time on taxa richness (Table 1). Only, the pollen richness diminished significantly as the seasons progressed (Table 1, Fig. 3A) while the impervious surface proportion has no impact on the pollen richness (Fig. 3B).

NMDS displayed high variability in the composition of foraged pollen across the months and seasons (Fig. 4). The greatest discontinuity separated spring (March, April, and May) from autumn (September). Concerning the floral composition foraged by the honeybees, May and August served as transition months to subsequent seasons. The permutation tests revealed that the month period ($F = 6.87$; $R^2 = 0.23$; $p < 0.001$), site ($F = 1.27$; $R^2 = 0.1$; $p < 0.01$), and landscape class ($F = 2.01$; $R^2 = 0.03$; $p < 0.001$) were significant explanatory variables of pollen source-plant composition in the samples; however, the sampling period was attributed a larger proportion of the variance. From pairwise comparisons (i.e. letters from Fig. 4), the urbanised sites hosted similar plant communities. Moreover, the structure of the plant communities varied significantly over the months until late summer and early autumn (i.e. August and September), when the floral composition harboured similar foraged plant communities.

Indicator species and trait-based analysis

The characteristics of pollen forage plants varied significantly according to their stratum ($G = 99.0$, $p < 0.001$) and native status ($G = 69.1$, $p < 0.001$) over the study months (Fig. 5B), while only the plant strata showed significant differences ($G = 10.7$, $p < 0.05$) according to the landscape classes (Fig. 5A).

Effect of landscape

Significantly more herbaceous plant taxa structured the rural landscape (Fig. 5A), even if pollen source-plant composition traits were independent of landscape type. A total of 35 plant taxa were shared among all the landscapes over the sampling months such as some *Trifolium* spp. or *Plantago asiatica* L., while 151 plant taxa were exclusively related to specific landscapes, corresponding to 27%, 22%, 24%, and 26% of the total plant taxa for rural, suburban, urban, and urban centre landscapes, respectively (Fig. S5). *Sagittaria natans* Pall. and *S. trifolia* L. taxa are tied to rural areas. Plant families, such as Fabaceae, Rosaceae, Brassicaceae, Asteraceae, Plantaginaceae, and Onagraceae, were the most frequent taxonomic families encountered in all samples. However, their proportions varied according to the landscape (Fig. S6). In urban and urban centre areas, leguminous plants prevailed more than in rural and suburban landscapes. However, the suburban landscape showed a higher frequency for the Brassicaceae, Ranunculaceae, and Rosaceae plant families. Surprisingly, anemophilous plants of the Poaceae family were more frequently foraged in the urbanised landscape than in the countryside (Fig. S6). *Trifolium* genus showed constant occurrence throughout the landscape classes while other main genera foraged by honeybees were more frequent in distinct landscapes such as *Allium*, *Sagittaria* and *Helianthus* taxa for rural landscapes, *Veronica* and *Papaver* taxa for suburban areas, *Plantago* and *Oryza* taxa for urban center landscape (Fig. 6A).

Effect of sampling period

The proportion of foraged woody taxa decreased significantly over the seasons ($G = 87.5, p < 0.001$), with a peak of 46% in April and a low of 10% in September. Over the sampling months, honeybees foraged mainly on alien plant taxa (Fig. 5B). Cultivar taxa were more foraged in spring than in the other two seasons ($G = 32.9, p < 0.001$). The most visited plant genera in March and April included *Prunus*, *Helleborus*, *Brassica*, and *Acer* taxa (Fig. 6B). In contrast, four of the 11 most frequent genera emerged in late spring in May. Between April and June, a noticeable phenological turnover in the pollen composition (Fig. 6B) was observed, with May serving as a transition bridge. This has already been highlighted by the discontinuities in the NMDS ordination (Fig 4). Following this shift, the genus *Trifolium* spp. was highly dominant in the June and July samples. In addition, the herbaceous genera

Plantago and *Oenothera* spp. were also found in large proportions in combination with the woody genera *Mallotus* and *Hydrangea* spp. In August, the taxa from the genus *Oenothera* were the most represented with *Trifolium,* despite a reduction in its occurrence. A shift in pollen composition trends, with a reduction in highly proportional occurrence genera, was observed in August and September. In other words, plants detected in August and September were more distributed between the genera. Only the genus *Allium* showed an increase from August to September. Finally, *Trifolium* spp., *Rosa* spp., and *Allium* spp. were the only genera that were observed throughout the study period.

Discussion

This study revealed interesting patterns of honeybee foraging habits along the urban-rural gradient throughout their active season. In the present study, we used an unconventional approach, namely k-means clustering from landscape metrics, to categorise our sampling sites into four landscape classes. These landscape variables allowed the estimation of the effects of some ecological processes at the landscape level (i.e. foraging and plant dispersal) in assessing the diversity, connectivity, and aggregation of the patches (Baguette et al., 2012; Doherty and Driscoll, 2018). Despite the convincing results of the grouping method, several reservations are worth mentioning. First, the selected foraging radius of 6 km accentuated the spatial autocorrelation issues on the landscape variables by increasing the foraging area overlaps among the sites (Plant, 2012). This was not tested in the present study because we considered that there was no spatial influence on foraged plant communities between each sampling colony due to the high variations in their foraging behaviour (Oldroyd et al., 1992; Visscher and Seeley, 1982), even at the local scale for colonies of the same apiary (De Vere et al., 2017). Indeed, the foraging behaviour of honeybee colonies is mainly driven by: (i) the temporal colony needs; (ii) the high density of pollen and nectar resources available near the colony; (iii) the rapid decision making by colony for the most profitable flower patches; and (iv) the ability to tightly modulate its pollen reserves to protect the colony from seasonal pollen breaks (e.g. long rainy periods, rarefaction of floral resources at the end of summer) (Seeley, 1995). Second, the 3-image resolution from satellites may result in some limitations, especially in complex landscapes, such as urban matrices. Therefore, this spatial resolution issue could be counterbalanced with the use of specific cameras, such as red-green-blue (RGB) or multispectral cameras, mounted on unmanned aerial vehicles to characterise floral identification and surfaces (Chen et al., 2019; Librán-Embid et al., 2020). Nonetheless, this research domain is still in its infancy, particularly in the data processing of imagery classification by deep learning (Pritt and Chern, 2017). However, despite these spatial limitations, the approach led to satisfactory classification, which paves the way for further investigations. The use of DNA meta-barcoding with one pair of ITS1 primers (Masamura et al., 2014) allowed for the identification of a great diversity of plant resources and highlights foraging patterns, regardless of the landscape variables or classes. This method yields to the identification of 307 taxa, which is higher than previous studies (Danner et al., 2017; Richardson et al., 2021). This could be explained by the important sampling effort of pollen samples (N = 143) over the seasons combined with contrasting landscapes (i.e. urban to rural landscapes). Moreover, the lack of a Tokyo regional plant list and associated ITS1 references to compare our sequences with could have increased the number of detected taxa by introducing false-positive BLAST alignments (Richardson et al., 2015a).

We observed a strong seasonal effect on plant richness (Fig 3), the foraged plant community, and plant characteristics (Fig 4 and 5B). We observed higher plant richness and foraged woody taxa in spring than in the other two seasons (Fig 3 and 5B), as reported previously (Brodschneider et al., 2019; Lau et al., 2019; Sponsler et al., 2020). The genera *Prunus* spp. and *Acer* spp. (Fig 5D) dominate the foraged woody stratum during this season, as these taxa constitute an important source of pollen for the early season (Brodschneider et al., 2019; Requier et al., 2015; Richardson et al., 2015b; Sponsler et al., 2020). Indeed, bee breads with high proportions of both genera were positively correlated with a higher protein content (Donkersley et al., 2017). Particularly, the complexity and high range of foraged plants is known to be beneficial to the “nutritional value” of these bee bread stocks, and thus to honeybee immunity (Alaux et al., 2010). After the spring period, the proportion of foraged herbaceous strata gradually substituted woody taxa to reach approximately 90% of herbaceous foraged taxa in September, in agreement with previous studies (Brodschneider et al., 2019; Sponsler et al., 2020). This growing herbaceous stratum is mainly dominated by

Trifolium spp. and *Plantago* spp. which, because of their long flowering period, might explain the lower richness of taxa foraged in summer and autumn (Brodschneider et al., 2019; Donaldson-Matasci and Dornhaus, 2012; Liolios et al., 2015). Also late summer and early autumn are known to be characterized by a depletion of floral resources in temperate regions which could explain specific foraging on the remaining floral communities such as *Trifolium* or *Allium* genera (Requier et al., 2015; Sponsler et al., 2020). Moreover, clover species (i.e. *Trifolium* spp.) are highly ubiquitous in grasslands, such as meadows for rural areas or parks and gardens for urban areas (Brodschneider et al., 2019; Critchley et al., 2007) and may contribute to the concentration effect of amino acid content (Donkersley et al., 2017). Concerning the temporal dynamics of the biogeographic traits, the observations point to a decrease in the frequency of cultivars in favour of exotic taxa, which contradicts previous studies (Urbanowicz et al., 2020; Williams et al., 2011). The highly anthropized and fragmented environments of the Tokyo region and its surroundings could explain the predominance of non-native floral species. However, this statement should be mitigated and requires further investigation, such as a complete plant inventory of the study site. Finally, we observed a transitional change from August by a collapse of the dominant flower prevalence, which may correspond to the seasonal dearth of floral resources (Fig 4 and 5B) (Brodschneider et al., 2019; Park and Nieh, 2017). The honeybees mitigate this effect by increasing their foraging range, requiring extra effort for sometimes worthless rewards (Park and Nieh, 2017). Finally, the study of other co-variables, such as brood monitoring or estimating the pollen collection/reserve of each colony, could be used to compare the conditions of each sampled colony and improve our understanding of the foraging patterns of the colonies (Delaplane et al., 2013).

The landscape variables did not influence forage plant richness, as reported in previous studies (Danner et al., 2017; Steffan-Dewenter and Kuhn, 2003). However, considering the spatial variation of taxa composition among the sites of our area of interest (i.e. beta diversity) (Legendre, 2014), our results showed a spatial structure of foraged plant communities in countryside, suburban, and urban environments (i.e. by merging the urban and urban centre areas) (Fig 4). It is likely that honeybee colonies modify their foraging preferences due to the high prevalence of unattractive ornamental flowers in urban landscapes (Garbuzov et al., 2015a). The urban matrix also offers smaller spread patches and less dense floral resources, which contribute to the foraging change of workers (De Vere et al., 2017; Lucek et al., 2019). This shift in the prospected flora is also coupled to fulfil the nutritional demand with a diverse and complementary floral diet (Donkersley et al., 2017; Hendriksma and Shafir, 2016). Therefore, it shows the importance of taking beta diversity and not only the local richness into account to understand the community structure of foraged plants throughout space and time scales (Richardson et al., 2021; Socolar et al., 2016). Despite the landscape structure of the foraged plant community, the trait-based analysis revealed no significant pollinated plants traits for honeybee colonies, except for herbaceous plants in rural landscapes (Fig 5A). However, the forest cover is high in rural areas (Enokisawa and Kuwata=42.2%, Ichihara=35%), mainly composed of artificial forest stands (Enokisawa and Kuwata=53%, Ichihara= 33%) (Chiba, 2022). Artificial forest mainly consists of conifers *Cryptomeria japonica* (L. f.) D.Don and *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. which do not provide floral resources for bees, meaning that the attraction of herbaceous plants would be facilitated. Moreover, the inconclusive result of trait-based analysis can be explained by the number and selection of functional traits selected from the foraged plants. The selected characteristics of pollen forage plants are few and not very informative in the context of plant-pollinator relationships. An evaluation of the functional structure of foraged plants with relevant floral characteristics (which honeybee colonies depends on) such as floral symmetry, floral shape, etc. would have helped to refine the understanding of the attractive floral structure (E-Vojtkó et al., 2020; Fornoff et al., 2017).

In all the samples, 35 plant taxa were shared throughout all the landscapes studied, corresponding to 45% of all read counts. The top three plant families were Fabaceae, Rosaceae, and Brassicaceae which are known to be in the foraging preference of honeybee (Richardson et al., 2021; Sponsler et al., 2020). The presence of grasses (Poaceae) may seem surprising among the most frequent families in the samples, given that these plants are considered unsuitable for *A. mellifera* resource needs (Decourtye et al., 2010). In view of their dominance, it seems unlikely that anemophilic pollen is the result of contamination by pollen blown from flowers onto the body of the bees, such as the rice paddy field in Japan (Kimura et al., 2014). Other recent studies have shown that pollinators (i.e. bees and syrphids), particularly *Apis* bees, interact with wind-pollinated plant species for their nutrient or nesting requirements (Saunders, 2018). Honeybees foraged floral resources ranging from 28 to 45 taxa, specific to each defined landscape. For example, some parts of rural landscapes in our study correspond to wetlands which are suitable to the population development of *S. natans* and *S. trifolia* (Chen, 1989).

Conclusion

This study has shown that the foraged plant richness was mainly explained by the season progression. In addition, it reflects large plant communities dominated by the occurrence of alien species independently of the landscape classes. Woody species offered an important source of pollen to the honeybees in the early growth season while herbaceous plants dominated foraged plant structure in summer and autumn. The cultivar species occurrence tends to decrease constantly over the seasons, while native species are sparsely foraged. These findings are consistent with the literature addressing this topic with similar datasets (Requier et al., 2015; Sponsler et al., 2020). Our work revealed that the foraged floral composition is also driven by the landscape context suggesting that bee diet is locally constrained by ecological drivers although 35 foraged plant taxa were exploited by honeybees in all defined landscapes. During periods of floral dearth, honeybees increase their foraging radius and their number of floral sites which require extra effort from the foragers sometimes for worthless rewards (Park and Nieh, 2017). Indeed, the nutritional need for the colony is a pivotal factor for the colony health which is mainly affected by the variations in pollen depletion and quality rather than its pollen richness (Di Pasquale et al., 2016). Therefore, in an urban greening context, it would be relevant to put in place measures to alleviate this seasonal dearth by ensuring enough high-quality floral resources close to the apiaries which may also benefit in their tolerance against pathogens or pesticides (Barascou et al., 2021; Di Pasquale et al., 2013).

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