Urban honey beekeeping using hive-pollen to identify urban foraging sites and preferred vegetation communities in Japan

Auteur : Pouilloux, Lise
Promoteur(s) : Francis, Frédéric; Garré, Sarah
Faculté : Gembloux Agro-Bio Tech (GxABT)
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URBAN HONEY BEEKEEPING USING HIVE-POLLEN TO IDENTIFY URBAN FORAGING SITES AND PREFERRED VEGETATION COMMUNITIES IN JAPAN

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ABSTRACT

In a context of global biodiversity loss, the primary concern for our society today is to understand what the causes are and to provide pollinators with new refuges. The cities are therefore questioned about their contribution in terms of pollen and nectar resources for pollinating insects and, more particularly, for populations of *Apis mellifera* species in urban beekeeping.

The study realized within the framework of this master thesis was conducted in 2019 in 4 apiaries (Kiba, Kashiwanoha, Yaesu and Nishichiba sites), located in the urban area of Tokyo. This study aims (1) to identify the plant communities used by bees in four sites located in Tokyo's urban area using pollen collected from 4 apiaries during 2017 and 2018 and (2) to characterize the spatial occurrence of the most used species by bees around four urban hives in order to identify the contribution of urban road network to the plant diversity used by *Apis mellifera* populations.

The study includes several analyses, including the description of families and taxonomic species, the proportion of herbaceous and tree species and the proportion of native, exotic and cultivar species used by bees. Two factors will help to make conclusions about the use of certain floral species: the location factor (4 apiaries located in the Tokyo urban area) and the time factor (from March to October). Phyloseq analyses are also performed as part of this master thesis.

In 2017 and 2018, 168 species in 57 taxonomic families were identified as pollen sources for bees in urban areas, with a large number of honey plant species in families such as *Fabaceae* (15 spp.), *Asteraceae* (14 spp.) or *Rosaceae* (8 spp.).

Among herbaceous and tree species used by bees of the four apiaries in 2018, 54% are herbaceous species while 40% are tree species (the remaining 6% include non-floral species and species whose taxonomic level up to the species is not identified). 52% are alien species, 20% are cultivar species and 25% are native to Japan (the remaining 3% include non-floral species and species whose taxonomic level up to the species is not identified). Besides, at the end of the summer, some exotic species are very highly exploited, including *Eucalyptus vicina*, Canadian goldenrod (*Solidago canadensis*) and chickpeas (*Cicer arietinum*).

A study carried out on 60 transects showed that out of 60 species sought, 24 of them were identified along the roads. The urban road network thus contributes to the refuge of a majority of exotic species (63%) but also of a large share of cultivar species (25%) and a minority of indigenous species (13%).

This study underlines the fact that urban beekeeping must remain a sustainable activity and that it is preferable to support biodiversity by creating green spaces, suitable for all pollinators, whether generalists or specialists. Further additional studies are therefore needed to make decisions about which floral species to promote in urban green spaces.

RESUME

Dans un contexte d'appauvrissement de biodiversité à l'échelle mondiale, la préoccupation majeure pour notre société aujourd'hui est de comprendre quels en sont les facteurs et de fournir aux pollinisateurs de nouveaux refuges. Les villes sont donc questionnées quant à leur contribution en termes de ressources de pollen et de nectar pour les insectes pollinisateurs et, plus particulièrement, pour les populations de l'espèce *Apis mellifera* en apiculture urbaine.

L'étude réalisée dans le cadre de ce présent travail a été menée en 2019 dans quatre ruchers (Kiba, Kashiwanoha, Yaesu and Nishichiba sites), située dans la zone urbaine de Tokyo. Cette étude vise (1) à identifier les communautés végétales utilisées par les abeilles dans quatre sites situés dans la zone urbaine de Tokyo en utilisant le pollen collecté auprès de quatre ruchers au cours des années 2017 et 2018 et (2) à caractériser l'occurrence spatiale des espèces les plus utilisées par les abeilles autour de quatre ruches urbaines afin d'identifier la contribution du réseau routier urbain dans la diversité florale utilisée par l'espèce mellifère, *Apis mellifera*.

L'étude regroupe plusieurs analyses dont la description des familles et espèces taxonomiques, la proportion d'espèces herbacées et arborées et la proportion d'espèces natives, exotiques et cultivées utilisées par les abeilles. Deux facteurs permettront aussi de tirer des conclusions quant à l'utilisation de certaines espèces florales : le facteurs lieu (4 ruchers situés dans la zone urbaine de Tokyo) et le facteurs temps (de Mars à Octobre). Des analyses phyloseq ont aussi été réalisées dans le cadre de ce travail.

Au total, 168 espèces dans 57 familles taxonomiques ont été identifiées comme source de pollen pour les abeilles en milieu urbain, avec un grand nombre d'espèces de plantes mellifères dans les familles *Fabaceae* (15 spp.), *Asteraceae* (14 spp.) ou encore *Rosaceae* (8 spp.).
Au niveau des proportions entre espèces herbacées et arborées utilisées par les abeilles des quatre ruchers en 2018, 54% sont des espèces herbacées tandis que 40% sont des espèces arborées (6% restant incluent les espèces non florales et les espèces dont le niveau taxonomique jusqu'à l'espèce n'est pas identifié). 52% sont des espèces exotiques, 20% sont des espèces de culture et 25% sont indigènes au Japon (les 3% restant incluent les espèces non florales et les espèces dont le niveau taxonomique jusqu'à l'espèce n'est pas identifié). De plus, à la fin de l'été, certaines espèces exotiques sont très fortement exploitées et notamment les espèces *Eucalyptus vicina,* le verge d'or du Canada (*Solidago canadensis)* ou encore les pois chiche (*Cicer arietinum)*.

Une étude réalisée sur 60 transects a montré que sur 60 espèces recherchées, 24 d'entre elles ont été identifiées le long des voies de circulation. Le réseau routier urbain contribue donc au refuge d'une majorité d'espèces exotiques (63%) mais aussi d'une grande part d'espèces cultivées (25%) et d'une minorité d'espèces indigènes (13%).

Cette étude insiste bien sur le fait que l'apiculture urbaine doit rester une activité durable et qu'il est préférable de soutenir la biodiversité par la création d'espaces verts propice à l'ensemble des pollinisateurs, qu'ils soient généralistes ou spécialistes. D'autres études complémentaires sont donc nécessaires pour la prise de décision quant aux espèces florales à promouvoir dans les espaces verts en ville.

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INTRODUCTION

During the natural pollination process, pollinators allow fruit growth and seed development in natural and anthropized ecosystems. They, therefore, participate in ecosystem services and have a very significant impact on people's lifestyles (MEA 2005). For instance, in 2005, pollination was estimated to €l53 billion annual economic value (Gallai *et al.* 2009). However, global biodiversity is dramatically declining (between 1 and 10% loss per decade (Kluser & Peduzzi 2007)), and according to the IUCN Red List, already 16.5% of vertebrate pollinators are threatened with extinction worldwide ((1) Potts *et al.* 2016). This decrease is due to several human-induced drivers (Sala *et al.* 2000) such as climate change, pesticides, invasive alien species, or landscape degradation and fragmentation. Thus, rural areas are not considered as welcoming places for pollinators and many studies are questioning cities as new refuges (Hall *et al.* 2016). Indeed, by 2050, 2.5 billion more people will live in cities, which represents 68% of the world's population (United Nations, 2018). Current suburbs will therefore become the densely populated cities of tomorrow, will represent considerable challenges in terms of biodiversity and can be managed to welcome more species (Parson *et al.*2018).

Given the growing international popularity of urban honey beekeeping, many studies are carried out to understand the network of plant-pollinator interactions, essential to evaluate the stability of pollination systems (Bosch *et al.* 2009; Ferreira *et al. 2013*). In many cases, these researches require the identification of large quantities of pollen to reveal natural interactions; for this purpose, new methods are used, such as DNA metabarcoding techniques. In recent years, many researches have focused on plant-pollinator interaction studies (Bosch *et al.* 2009) and are now combined with DNA metabarcoding techniques (Pornon *et al.* 2016). Metabarcoding is also used in several other applications including the determination of floral species of honey products (Hawkins *et al.* 2015) and in the search for resources used by bees (De Vere *et al.* 2017; Richardson *et al.* 2015). In this context, the question of native and exotic resources used by honeybees are questioning in studies (Morandin & Kremen 2013; Salisbury *et al.* 2015). But "the value of native and non-native plants in supporting animal biodiversity is, however, largely unknown" (Salisbury *et al.* 2015). Indeed, it appears that a significant lack of knowledge about the resources used by bees is to be regretted. What proportion of native and non-native species do they use? Where are foraging sites located? In urban areas, are there enough food resources to accommodate wild pollinators and honeybees from popular beekeeping activities? How far do honeybees travel in urban areas: do they have more access to their food resources, or do they have to travel long distances? Moreover, a lack of research on pollen and nectar sources for honeybees is to be regretted in Asia. As Taha *et al.* mention in their study in northern Egypt (2017), some studies have been carried out worldwide, but very few in Asia (in the Philippines by Payawal *et al.* (1991), or in India by Singh (2003)).

The understanding of which flowers are used by honeybees and where the resources available are in cities can help stakeholders to promote green spaces hosting pollinating insect populations and thus contributing to the preservation of biodiversity.

This study is being carried out in Japan and aims to identify the plant communities preferred by bees at four different sites in urban areas through DNA metabarcoding identification, and to determine which species are found along the traffic lanes.

BACKGROUND

1. APIS MELLIFERA

a. Honeybee colony

For hundreds of millions of years, the emergence of bees is correlated to the occurrence of angiosperms, nectar and pollen productions being linked. This co-evolution has played a decisive role in the diversification of angiosperms (Dodd *et al.* 1999; Ollerton 1999; Sauquet *et al.* 2017). For example, orchids are pollinated almost exclusively by bees and there are more than 20,000 species of orchids (largest family of angiosperms) (Le traité rustica de l'apiculture 2009).
Twenty thousand species of bees exist all over the world, classified in seven families (Michener 2007; Danforth *et al.* 2013) and divided into four broad groups: solitary, bumble, stingless, and honeybees (Wilson-Rich 2014). But the most studied and the most popular is *Apis mellifera* (L.), the honeybee. *Apis mellifera* belongs to the Hymenoptera insect order and Apidae family.

Bees from the *Apis* genus are characterized by social behavior (Le traité rustica de l'apiculture 2009; Michener 2007). The haplodiploidy mode of genetic inheritance of bees allows them to regroup individuals from three different castes. In the Spring, a honeybee's colony is composed by female individuals (two sets of chromosomes and inherited genes from both their mother and their father) including a queen bee and some tens of thousands of worker bees as well as thousands of male individuals (one set of chromosomes inherited from their mother), also called drones (Wilson-Rich 2014).

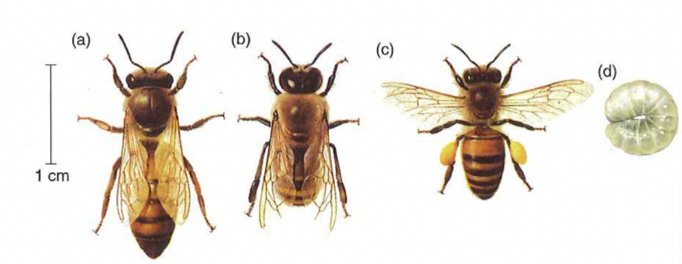

Figure 1: Honeybee (Apis mellifera): (a) queen;(b) drone; (c) worker; (d) larva (from Zanetti 1977)

The morphological, physiological and behavioral adaptations allow individuals of every caste to realize different tasks (Wilson-Rich 2014; le traité rustica de l'apiculture 2009).
Queen bees have a reproductive role. They must lay the eggs and ensure the colony progeny. A queen bee needs 16 days to emerge as an adult individual. Queens and workers own the same genetic patrimony but are differentiated by their diet (extra ration of royal jelly to allow reproductive organs development (Wilson-Rich 2014)). The morphological queen bee is characterized by a huge abdomen, a symbol of its fertility. Also, it has a tiny tongue and inadapted pollen legs, unlike the workers.
Males have a single task in their life: to fertilize queen bees. Males are the result of a parthenogenesis reproduction. They are therefore derived from a haploid egg. A drone has a very short life and is only present if the resources are available and sufficient to host it. Thus, at the end of the season, all the males die. A major characteristic of a male individual is its capacity to fly and to feed from one beehive to another. Male bees – as opposed to female ones - are not linked to a unique beehive, they can be accepted in a different hive from their own.

Worker bees have different roles depending on their age: it is the polyethism of castes. They accomplish all of the other functions necessary and ensure colony survival. A worker bee needs 21 days to reach its adult stage. During its first days, the young bee will clean the cells, seal the cells, take care of the brood and the queen, store the nectar and the pollen and finally build the rays. As it ages, a worker bee will go out of the beehive to guard the beehive or to search pollen and nectar necessary to the survival of the colony.

b. Foraging activities

Bees and plants have always been in a mutualist relationship. When a bee is traveling from flower to flower, some pollen hooked to its body can participate in the fertilization of plants (Wilson-Rich 2014). Pollination is a natural process which consists in a transfer of pollen to allow plant fertilization (Liss *et al.* 2013). The pollen will germinate on the stigma and give birth to a pollen-tube. The fusion between the spermatic nuclei and the ovules lead to the gamete fusion (Lord & Russel 2002).

A worker bee will contribute to pollen collection at about three weeks of its life depending on the beehive need. Foraging activities will last only four or five days. The first step for a foraging bee will be to realize first flies and memorize the close environment of the beehive. To memorize it, a bee can take some marks as topographical data, shape data or the color and the smell of the flowers visited. On average, a bee can realize about ten trips per day, depending on the distance of the foraging site (Le traité rustica de l'apiculture 2009).

Nectar and honeydew are harvested thanks to the long tongue of the bees. Concerning the pollen harvest, worker bees present a body adapted for the collect and the storage of pollen. Bee's last leg shows a tibia transformed into a basket with a rake at its extremity (Figure 2). Moreover, the first article of the tarsi is covered by combs. These adaptations allow the storage of the pollen on the bees. Finally, worker bees go back to the beehive and discharge the precious crop into a pollen cell.

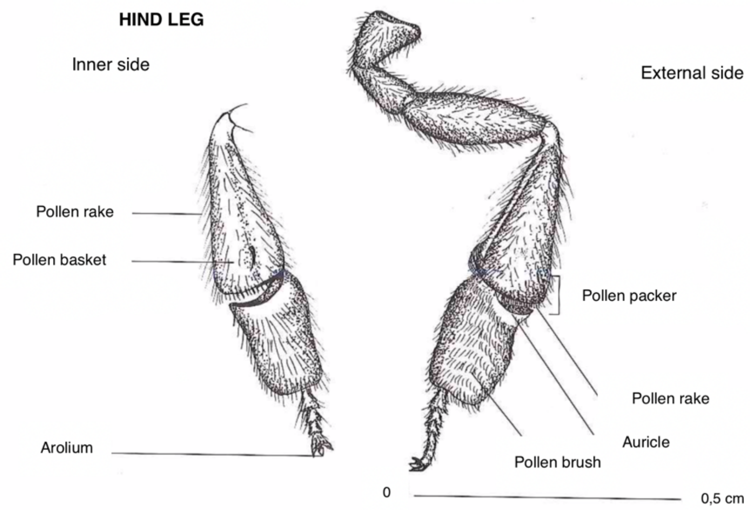

Figure 2: Drawing of the hind leg of honeybees (from Hayoux 2002 and translated in English)

c. Decline of pollinators and its consequences

Pollinator populations are experiencing significant declines (Goulson *et al.* 2015; Potts *et al.* 2010). The multiple factors contributing to this decline are the decay of floral biodiversity availability, the loss of habitats, the contribution of large quantities of pesticides to our landscapes and its consequences on the disease resilience of bee populations (Goulson *et al.* 2015; Vanbergen *et al.* 2013). Moreover, globalization and landscape homogenization disturbing all of the ecosystems seem to be the main cause in the disappearance of pollinators (Brown & Paxton 2009; Potts *et al.* 2010).

Pollination has always been considered as a granted ecosystem services provided by nature for humans. Even if the largest crops are not dependent on insects' pollinators (rice-wheat, corn and sugar cane), "the proportion of crops that requires pollination by animals has increased steadily" (FAO 2018). Currently, more than 85% of the world's floral species are fertilized by animal pollination (Ollerton *et al.* 2011). Ecosystem services provided by nature have a significant impact on people's lifestyles (MEA 2005). According to Gallai *et al.* (2009), in 2005 pollination was estimated to €l53 billion annual economic value, i.e. 9.5% of world agricultural production. Moreover, the benefits that global pollinators provide to humans are more than food services, they also participate in the creation of several medicines, biofuels (an expanding sector), fibers and construction materials (FAO 2018).

This is why, nowadays, it is important to create spaces that can accommodate biodiversity in new urban ecosystems.

2. URBAN BEEKEEPING

a. Urban beekeeping in the world

Despite the dramatic decline of bees, cities welcome diverse communities of wild bees in many parts of the world (Hall *et al.* 2017). Rural areas are no longer considered as good shelters for pollinators due to the landscape homogenization, the loss of habitats and the overuse of pesticides which all contribute to a diminution of floral availability and disturb bee populations (Ollerton *et al*. 2014). Surrounded by rural and suburbs areas, can cities be considered as a refuge for insects? Indeed, the numerous gardens and urban allotments, yards and all-year blossoms provide a great foraging place for pollinators (FAO 2018; Fetridge *et al.* 2008). Moreover, other hypotheses regarding the increase in the survival of bees in cities are stated such as the urban heat-island effect (Collins *et al.* 2000*)*, the lower amount of pesticides (compared to rural landscapes)(McIntyre & Hostetler 2001) or the fact that bees have to travel shorter distances to find their food sources and therefore spend less on energy (Wilson-Rich 2014).

Urban beehive projects are getting more and more famous in the world. According to the FAO (2018), "the number of urban beekeepers is rising by no less than 200% each year". But to consider beekeeping as a sustainable activity, cities need to achieve a balance between environmental protection, economic development and social wellbeing (Wu 2010). The three pillars of sustainable development are related to environment, economy and social conceptions (Purvis *et al.* 2018). Thus, a beekeeping project integrates a legitimate conservation practice by ensuring the pollinator's ability to thrive. Conservation of pollinators will have a huge impact on flora conservation, biodiversity, resilient food system and well-being places in cities (Hall *et al*. 2017). Which brings us to the second component of sustainable development, more responsible and more circular economy. Beekeeping is, therefore, an activity that can be economically profitable, by the sale of derivative products (honey, wax ...) to the local population. Finally, beekeeping can create more social links by teaching professionals to beginners or by interactions between stakeholders in projects (farmers, foresters and gardeners...) (Les maitres des abeilles, Arte 2019). This cooperation contributes to the creation of new interactions and the development of more sustainable activities in line with the environment.

However, several studies demonstrate the importance of being careful when introducing honeybees into natural ecosystems and underline the dangerous impacts on wild pollinators. According to Mallinger *et al.* (2017), "the majority of the studies reach the conclusion that managed bees negatively affect, or have the potential to negatively affect, wild bees through competition, changes in plant communities, or transmission of pathogens".

First of all, cities have the potential to host pollinating biodiversity, but only according to the proportion of impervious surfaces in cities. This proportion is therefore at the root of the fragmentation and reduction of green spaces in cities. Thus, beehive location has to present floral availability resources in enough quantity for the domesticated honeybees and the local wild pollinators. The number of melliferous plants and the choice of species in the beehive local environment must be sufficient and structured to satisfy pollinator's needs. For instance, the city hall of Paris specifies in the rules of urban beekeeping in Paris that it is necessary to "ensure that there are sufficient resources of pollen and nectars: trees, shrubs, street flowers and surrounding parks" for bee honeybee's population (Mairie de Paris 2017).

Secondly, regarding the potential transmission of pathogens caused by honeybee's introduction, managed bees must be controlled for pathogens and parasites and wild bee species of conservation concern must be the priority before the economic value of honeybees (Mallinger *et al.* 2017).

To conclude this part, honeybee's introduction could participate in the local and social economy in the world but must be controlled by rules to avoid negative impacts on natural ecosystems and natural wild pollinators. According to Bruxelles Environnement (2018), the goals is therefore not to increase the number of pollinators but to increase their diversity in ecosystems in order to have more resilient and sustainable systems.

Throughout the world, beekeeping is facing the same issues and must find new, sustainable and resilient solutions.

b. Japan and beekeeping history

Located in the Pacific Ocean, Japan covers an area of 378 000 km². According to the BBC, Japan has the world's third-largest economy and, despite a significant population decline, is still the tenth most populous country in the world with a population density of 335.66 ha/km² (Japan population 2019).

Japan is also a country of a multitude of facets. It's capital, Tokyo, is one of the most impressive places where technologies, modernity and traditions coexist. The capital has a population of 13.5 million people in the city downtown and 38 million people including with the suburbs (Tokyo population 2019). However, globalization does not prevent it from maintaining and promoting its strong traditions, which are rich in history.

Historically, Beekeeping was a very popular activity in Japan and is part of the local food history of the country (Kohsaka *et al.* 2017). The wild native species of Japan is *Apis cerana japonica*. Thus, it is important for Japan to promote a sustainable beekeeping activity to contribute to the preservation of this tradition. The first records of beekeeping in Japan go back to the 7th century. At a very small scale, it's in the Middle Ages that beekeeping is recognized. Afterward, it begins to be more popular during the Edo period (1603 – 1868) when several manuals on beekeeping were published. At the end of the 19th century, one of the biggest changes in the practice of beekeeping in Japan was the import of western honeybees and the modern beekeeping techniques associated with it. This arrival changed the whole way to produce honey in Japan (Kohsaka *et al.* 2017).

Since then, Western bees have been largely popular on the island because of their several advantages. First, at all, Western bees generate a higher amount of honey than Japanese honeybees. European honeybees are larger than the Japanese one and can collect four to five times as much nectar (Shimamura 2009). As mentioned before, European honeybees were introduced along with the Western revolutionary technology which came with them. As opposed to the traditional system of beekeeping, the beehive allows to beekeepers to inspect the bee frames without damaging the hive. Finally, *Apis mellifera* bee is a very resistant bee compared to other species.

Indeed, its natural range now extends from Africa to the Nordic countries. Its only limits are extreme climatic conditions (Le traité rustica de l'apiculture 2009).

After World War II, as the demand for honey was very high, honey production and the use of European bees were very widespread. However, since 1985 a huge decline of beekeepers and bee colonies in Japan led to a high dependency on imported goods, and this, despite the high demand and the promotion policies surrounding beehive activities. According to Kohsaka (2017), in Japan "imports nowadays exceed 10 times the quantity of domestic production". This decline has also contributed to a serious shortage of pollinators in the country's agricultural practices and the decrease of agricultural resilience in Japan. Moreover, a huge quantity of pesticide is used every year in agricultural practices in Japan. These practices are strongly related to a huge decrease of the honeybee population (Taniguchi *et al.* 2012).

To summarize the different issues linked to this decrease of beekeeper and bee colonies, it appears that the Japanese countryside is no longer the best place for beekeeping activities because of non-sustainable agricultural practices (Taniguchi *et al.* 2012), that Japan's economy is strongly dependent on honey imports and presents a non-resilient food system and finally, that the Japanese honey demand is still very high compared to its productivity (Kohsaka 2017).

However, several strategic plans could help the activity to be more sustainable.

- Cities in Japan are places that can host beekeeping activities because of their reduced pesticide context. Pesticides are avoided because of the growing number of people with allergies (Ginza project 2009). Urban areas therefore constitute reduced pesticide zones that are ideal foraging sites for pollinators.
- The introduction of honeybee's activities in cities could be a sustainable alternative for pollination. For instance, according to Ginza project (2009), since the arrival of beekeeping in Tokyo, the entire biodiversity changed with a new production of fruits by cherry blossoms, new arrival of bird and insect populations eating cherries and so on…

- Urban beekeeping can make a significant contribution to the local economy. For instance, in 2009, the amount of honey collected by the Ginza project reached 700 kg and increases every year.
- Japanese bees are once again reconsidered by beekeepers because of their impressive resistances. Indeed, they can easily collect the nectar of a hundred different flowers and are more resistant to infectious diseases such as foulbrood and chalkbrood (Shimamura 2009). One of the best advantages is their resistance to the Japanese giant hornets and other intruders. In contrast to *Apis mellifera*, *Apis cerana* has been able to co-evolve with these species (Fayet 2013). Finally, Japanese honeybees are less sensitive to the extreme temperature of heat and cold than the European ones, thus more suitable for Japan's climate (Ginza project 2009). The main problem remains the access to the Japanese honeybee population because their numbers are limited and restricted to a small zone in Japan. According to Shimamura (2009), this species contributed to less than 10 percent of domestic honey in 2009, and honey in Japan still remains a luxury product. About Western bees, they allow a higher honey production and are less fleeting than the Japanese one. Thus, in the future, working with both of the species could allow to increase the biodiversity and to take advantage of the characteristics of both species.
- Urban beekeeping in Japan is also a very important alternative to sensitize the city dwellers to biodiversity and economic services that bees and wild insects provide. Indeed, one of the Japanese bees' characteristics is also to be less aggressive than the Europeans one which can be a real asset for educational projects with kids…

To conclude, honeybee keeping projects combine educational, economic and environmental purposes to contribute to a more sustainable activity. In 2016, there were more than 100 urban beekeeping projects in Japan (Lessons from the bees, 2016). Also, the suburbs of Tokyo are going to play a major role in the composition and structure of the future city and its capacity to be more sustainable (Schmid *et al*. 2018). As mentioned before, Tokyo and metropolises in Japan could be places to host pollinators if stakeholders continue to work on how to limit the negative impacts of managed bees depending on several factors (selection of native bee species, controlled bee densities, resource availability/landscape diversity/proportion of impervious surface, pathogens/parasites control and conservation concern of wild bee species (Mallinger *et al.* 2017; Geslin *et al.* 2018)).

3. DNA METABARCODING

The melissopalynology is the identification of bee-collected pollen and is used in several applications such as plant-pollinator network (Pornon *et al.* 2016; Bell *et al.* 2017), product authentication (Prosser & Hebert 2017), honeybee nutritional biology (Taha *et al.* 2017) and so on.

Until recently, the usual method was the microscopic examination based on human expertise. Steps required to perform this technique are, firstly, the carrying out of a difficult preparation to identify pollen grains and, secondly, the comparison of these samples with a reference collection of pollen from local taxa. These steps are all described in the work of Erdtman (1943). This process does not allow the identification of large volumes of pollen and, as summarized Richardson *et al.* (2015), tends to be vulnerable to human error, limited in taxonomic precision and highly time-consuming. One alternative is pollen DNA metabarcoding.

At the end of the 1980s, first DNA metabarcoding methods became known. Today, DNA metabarcoding is based on two techniques commonly used in the scientific world: DNA amplification by PCR techniques and the identification of universal primers. Indeed, standard primers are selected and used according to animal or plant applications (Taberlet *et al.* 2012). For DNA plant barcodes, the three regions of the chloroplast genome *rbcL*, *matK* and *trnH-psbA* (Hollingsworth *et al.* 2009; Hawkins *et al.* 2015) and the nuclear ribosomal ITS region (Chen *et al.* 2010) are used separately or in combination.

According to Hawkins *et al*. (2015), metabarcoding has the advantages that it “does not require a high level of taxonomic expertise, a greater sample size can be screened, and it provides greater resolution for some plant families”. Moreover, in the context of plant-pollinator interaction applications, metabarcoding allows revealing natural interactions hard to observe otherwise. According to Pornon *et al.* (2016), metabarcoding can show 2.5 times more plant species involved in plant-pollinator interactions. In the future, DNA metabarcoding applications are set to expand in current issues such as global warming, invasive species, or biodiversity conservation (Bell *et al.* 2016).

However, due to PCR amplification biases and varying copy number of loci, it’s still difficult to affirm that this technique quantifies precisely the relative abundance of pollen collected by honeybees (Bell *et al.* 2017, Bell *et al.* 2018; Richardson *et al.* 2015). Indeed, according to the recent study of Bell *et al.* in 2018, “metabarcoding is largely robust for determining pollen presence/absence, but that sequence reads should not be used to infer relative abundance of pollen grains”. However, in the literature, many studies remain contradictory, even with the same marker used; for example, positive relationships are established between the ITS2 marker and the amount of pollen for Keller *et al.* (2015) study and then contradicted by Richardson *et al.* (2015) study, explained by the fact that specific taxa are overrepresented or underrepresented in metabarcoding method by comparing the same data with microscopic observation method.

4. RESEARCH QUESTIONS

As part of this master's thesis, this project will therefore identify and characterize the vegetation communities used by honeybees in Japanese cities.

According to Geslin *et al.* (2000), honeybees, considered as MIMS (Massively Introduced Managed Species), "seem also more prone to visit invasive or exotic plant species which might favor these plants at the cost of natural species (invasion meltdown)". Indeed, some exotic plants are already integrated into the ecosystem relationship between pollinators and plants and source of nectar and pollen for pollinators (Aizen *et al.* 2008; Stout & Morales 2009). Moreover, the proportion between native and exotic plants in supporting animal biodiversity stays largely unknown (Salisbury *et al.* 2015). In this context, it would be interesting to determine the proportion of native and non-native species used by honeybees in Tokyo urban areas and to identify which species are the most used.

Moreover, given that the majority of ornamental flower communities planted in urban areas are poorly or totally unattractive to insect floral visitors or even behave as invasive species (Geslin *et al.* 2000), which floral species should we promote?

According to Schweiger *et al.* (2010), alien species could represent a food alternative for generalist pollinators in the face of climate change but will not compensate for the multiple negative effects that these two factors (alien species and climate change) can have on natural ecosystems. In this context, an analysis of the use of exotic species used during the year will be carried out to determine whether or not bee populations are dependent on non-native species after summer (end of the flowering of native species).

Urban design features appear to influence the abundance and the diversity of bees (McIntyre & Hostetler 2001). Considering the complexity of urban structures and their complementarity in terms of the floral resources available to pollinators, the research question of this final project will be to characterize the contribution of the Japanese urban road network and its close dependence (private gardens in front of houses) in the diversity of floral resources useful for bees.

To sum up, the research objectives are: (1) to identify vegetation communities used by bees in four sites located in the Tokyo urban area using pollen collected from individuals in 2017 and 2018, (2) to characterize the spatial occurrence of the species most used by bees around four urban hives in order to identify the contribution of the urban road network to the plant diversity used by *Apis mellifera* populations.

MATERIAL & METHODS

1. CHIBA UNIVERSITY PROJECT AND DATA COLLECTED

a. Spatial context

In Chiba prefecture, Chiba University conducted a series of studies on urban beekeeping. During 2017 and 2018, the research team collected a total of 69 samples of pollen at four different sites in Tokyo and Chiba prefecture (Figure 3).

Figure 3: map of the four beehives location in Japan (Yaesu, Kiba, Kashiwanoha and Nishichiba sites)

During 2017, the study begins with the two different sites of Yaesu et Kiba. These two sites are located in the downtown of Tokyo. During 2018, two new sites joined the study, Nishichiba and Kashiwanoha sites, both located in Tokyo's outskirts. The following table summarizes the number of data collected by site during 2017 and 2018 (Table 1).

Table 1: Number of beehives and pollen samples collected per site during 2017 and 2018

Site		Yaesu	Kiba	Nishichiba	Kashiwanoha	Total
Number of hives		6	3	4	20	33
Number of samples	2017	10	11	/	/	21
	2018	11	13	12	12	48

Pollen was collected from each site at an interval of about two weeks. In each site, the pollen was collected from three hives. The dates of each collection are given in Table 2.

Table 2: Samples and dates related of the four sites (Yaesu, Kiba, Nishichiba and Kashiwanoha sites) during 2017 and 2018.

Yaesu		**Kiba**		**Nishichiba**		**Kashiwanoha**	
Date	**Code**	**Date**	**Code**	**Date**	**Code**	**Date**	**Code**
17/05/2017	YP-1	27/06/2017	FP-1	27/03/2018	NP-1	22/04/2018	KP-1
02/06/2017	YP-2	11/07/2017	FP-2	10/04/2018	NP-2	01/05/2018	KP-2
15/06/2017	YP-3	27/07/2017	FP-3	02/05/2018	NP-3	11/05/2018	KP-3
27/06/2017	YP-4	07/08/2017	FP-4	16/05/2018	NP-4	15/05/2018	KP-4
11/07/2017	YP-5	24/08/2017	FP-5	28/05/2018	NP-5	29/05/2018	KP-5
29/07/2017	YP-6	05/09/2017	FP-6	13/06/2018	NP-6	09/06/2018	KP-6
09/08/2017	YP-7	20/09/2017	FP-7	27/06/2018	NP-7	22/06/2018	KP-7
23/08/2017	YP-8	05/10/2017	FP-8	28/06/2018	NP-8	07/07/2018	KP-8
05/09/2017	YP-9	18/10/2017	FP-9	10/07/2018	NP-9	16/07/2018	KP-9
20/09/2017	YP-10	11/04/2018	FP-10	27/07/2018	NP-10	30/07/2018	KP-10
19/04/2018	YP-11	26/04/2018	FP-11	10/08/2018	NP-11	17/08/2018	KP-11
01/05/2018	YP-12	11/05/2018	FP-12	31/08/2018	NP-12	10/04/2018	KP-0
17/05/2018	YP-13	25/05/2018	FP-13				
31/05/2018	YP-14	05/06/2018	FP-14				
14/06/2018	YP-15	19/06/2018	FP-15				
28/06/2018	YP-16	04/07/2018	FP-16				
12/07/2018	YP-17	20/07/2018	FP-17				
26/07/2018	YP-18	01/08/2018	FP-18				
10/08/2018	YP-19	17/08/2018	FP-19				
23/08/2018	YP-20	27/08/2018	FP-20				
13/09/2018	YP-21	07/09/2018	FP-21				
		19/09/2018	FP-22				
		05/10/2018	FP-23				
		16/10/2018	FP-24				

Pollen traps were put at the entrance of each beehive for two hours (Figure 4). Then, pollen samples were gathered in small plastic bags classified by site and by date. All pollen samples were kept in freezers at the temperature of -18°C. There were three samples for each day in each site. The pollen from three beehives was mixed, and the mixture of pollen from three hives was treated as one sample for each day in each site. They were then sent to the laboratory for the identification of the floral species used.

Figure 4: Pollen traps used by Chiba University

b. DNA metabarcoding

In this study, the internal transcribed spacer 1 (ITS1) has been used, located between the 18S ribosomal RNA and the 5.8S ribosomal RNA genes (Masamura *et al*. 2013). Moreover, laboratory methodology used to identify pollen species is the same used in the study by Maki *et al*. (2016), at the specific point 2.6 High-throughput sequencing. PCR technique is used, and a taxonomic level of genus or species is determined. The process of pollen identification results in an OTU table (Operational Taxonomic Unit). An example is given in Table 3. All pollen identification manipulation was performed by scientists in December 2018.

Table 3: OTU table resulting of the pollen identification

#OTUId	YP-1	…	Target_top1	Identity_top1	Alignment_length_top1	Species_top1
OTU_001	1	…	*Trifolium_pratense*	100	342	ムラサキツメクサ
…	…	…	…	…	…	…

A total of 855 OTU is determined. A minimum of 72,945% of certitude rate is given for all of the species identified.

2. STUDY

a. Vegetation community analysis

In order to determine the preferred vegetation communities used by honeybees, the following analyses will be carried out: (1) a spatial one, by comparing communities vegetation from the four different sites during 2018 (2) a second one, by comparing the two years of data collection in 2017 and 2018 at the two sites of Yaesu and Kiba and (3) a last one, by comparing vegetation communities from Mars to October, using both 2017 and 2018 and the four sites data.

Out of the total number of species used, proportions will be calculated and then compared between sites or between months during the year. Besides, ANOVA analysis have been realized on RStudio software (version 1.2.1335) with a null hypothesis which is the same means of the different groups (rejection of the hypothesis if *p-value* > 0.05). The analyses were carried out by calculating the number of species per sample (i.e. by date and site). The averages were then calculated either by month (time scale) or by site (spatial scale) and compared.

Moreover, DNA metabarcoding data are encoding on Rstudio software for Phyloseq analysis R library (Vaulot 2018). Identification can give some results with implausible data explained by several different factors (spurious false-positive BLAST alignments, bees regurgitating honey stomach contents, contact between pollen foragers and stored bee bread within the hive before foraging (Richardson *et al.* 2015)). Therefore, a clean-up before analysis is necessary.
A cut-off at 97% of identity and a number of reads at less than 10 are determined (Stackebrandt & Goebel 1994). In this study, 58% of the data are not reliable data.
Three tables are created:

- The OTU table is a matrix that gives the number of reads per sample per OTU.

- The taxonomy table includes the list of OTUs and their corresponding taxonomic rank. Moreover, three other information are added to the table:
    - the state of herbaceous species (including herbaceous and creeper species) and tree species (including tree and shrubs species) (GBIF website)
    - the state of native, non-native and cultivar species of Japan (information extracted from Appendix 1)
    - the information of the nectar source for honeybees (Sasaki 2010)
- The sample table includes the list of the 69 samples, dates and site information related.

Through the use of this matrix, alpha diversity is assessed and represented by a boxplot, in order to define the species richness by site. Two different indexes have been implemented: Chao1 and Shannon indexes. Concerning Chao 1, the index informs us about the number of observed OTUs and estimate the number of unobserved OTUs from those observed 1 or 2 times given by the following formula:

$$S_{chao} = S^{\geq 1} + \frac{(S^1)^2}{2S^2}$$

with S_{chao} the number of classes (in this case, number of species) that we want to know, S the number of species observed in a sample, S^1 the number of singletons and S^2 the number of doubletons. On the other hand, the Shannon index assesses the width of the OTU relative abundance distribution. According to Mariadassou *et al.* (2016), Shannon entropy reflects our (in)ability to predict the OTU of a randomly picked sample. Shannon index is given by the formula:

$$H = -\sum_{i=1}^{S} p_i \, ln \, p_i$$

with H, Shannon's diversity index, S, the total number of species in the community (richness) and p_i, the proportion of S made up of the i^{th} species.

As mentioned above, the use of metabarcoding to quantify the relative abundances of pollen collected by bees is not recommended (Bell *et al*. 2018). However, phyloseq graphs are created in this study and will inform us about trends of honeybee's exploitation depending on herbaceous and tree species, or depending on native, non-native and cultivar species. This aspect of the study must be nuanced and does not allow any real conclusions to be reached in terms of quantification of pollen collected by honeybees. Moreover, in order to compare the most commonly used species by honeybees, two related top lists species will be used: one based on the sum of the number of reads and one based on the presence of species in the samples, also considered as the frequency of species occurrence. The reliability of metabarcoding, in terms of presence of species in samples, will offset the fact that we are also using the deficient part of this technique (i.e. lack of representation of species abundance by direct relationship with the number of gene sequences). Thus, the two comparative lists will allow us to conclude on the most used species. It should be noted that during this study, most of the species on the top lists are found in both lists.

All phyloseq and non-phyloseq analyses will be interpreted together to address the research questions.

b. Field analysis

i. Preparation of top species list by site

We will focus now on some specific species that honeybees are using the most throughout the year and depending on the site.
As mentioned before, two lists are created to select the most commonly used species by bees in order to identify them in the field. On the one hand, species are classified by their sum of number of reads, calculated by summing the number of reads for each species and all of the samples (all of the dates). On the other hand, the calculated frequency is the number of times the species is present in the collected samples. Species with the highest rate will be those with a long flowering time. This data will allow us to identify the most useful species for bees over a very long period. Finally, a list of the top ten for these two parameters are set up. The following table gives us the information on the four sites. Note that species can be found in both the top lists species (Table 4).

Table 4: List of the top species by the sum of ITS1 reads and by frequency for both herbaceous and tree species for each site (2017 and 2018 data for Yaesu and Kiba sites, and 2018 data for Kashiwanoha and Nishichiba sites).

	Herbaceous species		**Tree species**	
Site	**Sum**	**Frequency**	**Sum**	**Frequency**
Nishichiba	*Trifolium pratense*	*Trifolium pratense*	*Prunus pseudocerasus*	*Dichroa febrifuga*
	Trifolium repens	*Trifolium repens*	*Mallotus barbatus*	*Lagerstroemia indica*
	Centaurea cyanus	*Plantago lanceolata*	*Triadica sebifera*	*Ligustrum lucidum*
	Helianthus annuus	*Phyla canescens*	*Dichroa febrifuga*	*Triadica sebifera*
	Rosa hybrid	*Lolium perenne*	*Erythrina crista-galli*	*Hypericum lancasteri*
	Vicia villosa	*Oenothera rosea*	*Ternstroemia gymnanthera*	*Erythrina crista-galli*
	Brassica napus	*Brassica napus*	*Hypericum lancasteri*	*Mallotus barbatus*
	Trifolium incarnatum	*Helianthus annuus*	*Prunus szechuanica*	*Punica granatum*
	Clematis terniflora	*Trifolium tomentosum*	*Acer buergerianum*	*Rhaphiolepis indica*
	Rubus columellaris	*Allium fistulosum*	*Lagerstroemia indica*	*Acer buergerianum*
Yaesu	*Trifolium repens*	*Trifolium repens*	*Mallotus barbatus*	*Mallotus barbatus*
	Trifolium pratense	*Trifolium pratense*	*Lagerstroemia indica*	*Lagerstroemia indica*
	Cicer arietinum	*Plantago lanceolata*	*Acer buergerianum*	*Styphnolobium japonicum*
	Plantago lanceolata	*Plantago asiatica*	*Schefflera heptaphylla*	*Schefflera heptaphylla*
	Plantago asiatica	*Cicer arietinum*	*Aralia elata*	*Ternstroemia gymnanthera*
	Cosmos sulphureus	*Nelumbo nucifera*	*Ternstroemia gymnanthera*	*Hypericum lancasteri*
	Nelumbo nucifera	*Oenothera rosea*	*Styrax grandiflorus*	*Ligustrum lucidum*
	Artemisia argyi	*Helianthus annuus*	*Stewartia sinensis*	*Erythrina crista-galli*
	Oenothera rosea	*Lolium perenne*	*Castanopsis fargesii*	*Acer buergerianum*
	Helianthus annuus	*Verbena hispida*	*Hypericum lancasteri*	*Castanopsis fargesii*
Kiba	*Trifolium pratense*	*Trifolium repens*	*Eucalyptus vicina*	*Eucalyptus vicina*
	Trifolium repens	*Trifolium pratense*	*Lagerstroemia indica*	*Eucalyptus grandis*
	Rosa hybrid	*Verbena hispida*	*Mallotus barbatus*	*Lagerstroemia indica*
	Solidago canadensis	*Solidago canadensis*	*Eucalyptus grandis*	*Erythrina crista-galli*
	Verbena hispida	*Verbena incompta*	*Erythrina crista-galli*	*Callistemon comboynensis*
	Orobanche pancicii	*Plantago lanceolata*	*Callistemon comboynensis*	*Mallotus barbatus*
	Ampelopsis japonica	*Ampelopsis japonica*	*Pittosporum glabratum*	*Rhus chinensis*
	Verbena incompta	*Melilotus_officinalis*	*Rhus chinensis*	*Triadica sebifera*
	Bidens pilosa	*Rosa hybrid*	*Triadica sebifera*	*Pittosporum glabratum*
	Plantago lanceolata	*Bidens pilosa*	*Dendropanax morbifer*	*Dendropanax morbifer*

Kashiwanoha	*Trifolium pratense*	*Trifolium pratense*	*Lagerstroemia indica*	*Ligustrum lucidum*
	Trifolium repens	*Trifolium repens*	*Dichroa febrifuga*	*Dichroa febrifuga*
	Centaurea cyanus	*Plantago lanceolata*	*Actinidia deliciosa*	*Citrus sinensis*
	Brassica napus	*Centaurea cyanus*	*Mallotus barbatus*	*Lagerstroemia indica*
	Rosa hybrid	*Brassica napus*	*Actinidia chinensis*	*Mallotus barbatus*
	Brassica carinata	*Phyla canescens*	*Dendropanax morbifer*	*Actinidia chinensis*
	Taraxacum obtusifrons	*Lolium perenne*	*Punica granatum*	*Aralia elata*
	Plantago lanceolata	*Rosa hybrid*	*Triadica sebifera*	*Actinidia deliciosa*
	Cirsium setidens	*Brassica carinata*	*Prunus grayana*	*Dendropanax morbifer*
	Vicia villosa	*Cirsium setidens*	*Callistemon comboynensis*	*Punica granatum*

ii. Creation of transects

The map of floral species used by honeybees will be created according to QGIS software (version 2.18.13). The four different locations of the beehives were described above (see Figure 1).

Different ways to realize this map were possible for this research plan. Either we could conduct the study at a very large scale of at least 2.2 km mean distance covered by honeybees between the beehive and the foraging sites (Seeley *et al.* 1995), but in this case, due to the vastness of the zone and for practical reasons, we would have to focus on a very low number of species. Or, the study could be conducted on a smaller area and we would be able to localize more species.

We finally opted to conduct the study within a 500 meters perimeter around the location of the beehive. This decision was based on the local field information: two of the sites (Nishichiba and Kashiwanoha) are surrounded by large green areas, which suggests that bees will not preferentially travel too long distances considering the available resources (Wilson-Rich 2014). In the cases of Yaesu and Kiba, the situation is different with a much higher rate of urbanization. The field data will then provide us with information on the presence or absence of species used by bees in the study area.

50 transects of 100 meters each are created in the study area. Given the urban context of the study, the 50 transects were chosen from the Google Earth Pro software and hand-drawn on practicable roads that do not require traffic permits. After the transect plot was completed, Excel software was used in order to randomly select 15 transects. The 15 selected transects were then recorded on QGIS software. 10-meter buffer zones were created from the selected transect and will mark the study areas for the floristic survey (Figure 5).

Figure 5: Beehive and transects location of Nishichiba, Kiba, Kashiwanoha and Yaesu sites

The same methodology was used for the four sites. However, in the case of Nishichiba, a map of the tree species on the entire campus area was available. As a result, a map for seven tree species had been realized: *Mallotus barbatus, Triadica sebifera, Ternstroemia gymanthera, Acer buergerianum, Lagerstroemia indica, Punica granatum* and *Rhaphiolepsis indica.* The other trees and shrubs species in the top list were either absent of the campus localization or not listed in the supplementary document.

The analysis on Nishichiba campus is detailed thanks to this tool for these specific species not only in the transects but on the entire study campus area. Moreover, 8 transects off-campus have undergone the same methodology as previously explained.

iii. Data collection and encoding

The map of Nishichiba campus is used for a second purpose. Due to the lack of floristic botanical reference, the observation on the field was based on photographs from GBIF website and based on local observations too; indeed, if the studied species was found and located on the map of Nishichiba campus, the specific species were photographed and compared with species on the field and used as a floral reference. The species concerned are *Dendropanax morbifer, Prunus grayana, Triadica sebifera, Ternstroemia gymnanthera, Acer buergerianum, Styphnolobium japonicum and Mallotus barbatus.*

For Nishichiba site, transects were carried out on May 8, 2019, for Yaesu site, on May 30, 2019, for Kashiwanoha site, on May 23, 2019, and finally for Kiba site, on May 14 and May 29, 2019.

Regarding the encoding step, herbaceous species are listed in three categories: category “a” for a number of 1 to 10 included individuals of the same species on the transect; “b” for 11 to 50 included species and “c” for more than 50 individuals on the transect. This information is grouped in Appendices 4 to 7, for each site.

iv. Delimitation of the study area based on a typology of urban green spaces.

In order to assess the limits of my sampling, a typology of urban green spaces will be described. According to Braquinho *et al.* (2015), urban green space elements can be grouped in many ways and are all legitimate (physical appearance, spatial extent and spatial complexity, social function…). For this study, urban design typology will be divided into three different categories, depending on the ownership and management (Barchetta & Chiodelli 2016).

The first one is managed by the city as traffic lanes, parks, ornamental plantings, urban forests, agricultural areas and so on. The second one is composed of urban wastelands (brownfields or vacant lot), formerly managed places. Finally, the third category is private interior gardens, managed by city dwellers.

Furthermore, I will use a second and different typology, the one of Rupprecht and Byrne (2014) (1), regrouping all of the non-managed surfaces as IGS, informal urban greenspace, divided into subtypes of IGS: street verge, lot, gap, railway, brownfield, waterside, structural, microsite and power line IGS (Table 5). This study was partly carried out in Japan and thus corresponds to the typology of green spaces that can be found in this country.

Table 5: Example of the Informal urban greenspace typology (Rupprecht and Byrne, 2014 (1)).

IGS	Examples	Description	Management	From	Substrates
Street verges	Roadside verges, roundabouts, tree rings, informal trails and footpaths	Vegetated area within 5 m from street not in another IGS category; mostly maintained to prevent high and dense vegetation growth other than street trees; public access unrestricted, use restricted.	Regular vegetation removal (>= once per month); governmental and private stewardship	Small: <100m^2, linear	Soil, gravel, stone, concrete, asphalt
Lots	Vacant lots, abandoned lots	Vegetated lot presently not used for residential or commercial purposes; if maintained, usually vegetation removed to ground cover; public access and use restricted	Irregular veg. removal, medium to long removal intervals; private stewardship	Small-medium: <1 ha, block	Soil, gravel
Gap	Gap between walls or fences	Vegetated area between two walls, fences or at their base; maintenance can be absent or intense; public access and use often restricted	Irregular veg. removal; variable removal intervals; private stewardship	Small: <100 m2, linear	Soil, gravel, stone
…	…	…	…	…	…

According to Rupprecht & Byrne (2014) (2), "a small brownfield and a vacant lot may be similar in appearance and size, but their different land-use history, vegetation removal periods and urban context distinguish them". However, in the results part, as I do not have sufficient data to distinguish these two types of IGS, my analysis will group the brownfields and vacant lot into a single group: urban wastelands.

Thus, out of the number of designated urban designs, how many were sampled in the transects? We will then be able to characterize and identify the contribution of the urban road network to the plant diversity used by *Apis mellifera* populations. Besides, assumptions will be made about which species are not included in the surveys.

c. Comparison transect & metabarcoding methods

To compare the two techniques used in this study and to determine if DNA metabarcoding and transects in the fields are two complementary techniques, a Principal Coordinate Analysis (PCoA) has been realized.

A presence/absence matrix was carried out; the 12 samples of the DNA metabarcoding technique from May 2018 were summarized in a presence-absence matrix of the species, following by the data of the transects method. The 12 samples from barcoding method were selected given the time scale of the transect technique (only in May 2019). Then a dissimilarity matrix is built, based on the Raup–Crick dissimilarity index. Raup-Crick index is a probabilistic index based on presence/absence data, as in the Milferstedt *et al.* 2013 study. The two first axes, explaining 63.70% and 36.46% of the data, are chosen (variance explained by the third one was too low). The PCoA is given in the result part and attempts to represent the distances between samples in a low-dimensional, Euclidean space.

RESULTS

1. VEGETATION COMMUNITY ANALYSIS

To identify vegetation communities used by bees in four sites located in the Tokyo urban area using pollen collected from individuals in 2017 and 2018, several analyses will be implemented.

Note that, according to the list of designated invasive alien species (2018), no species belong to the IAS domain in Japan.

a. By pollen collection site in 2018

i. Taxonomic analysis

Table 6 records the number of floral species used by honeybees depending on the site in 2018. A total of 162 species has been identified. The largest number of floral species used by honeybees is in Kashiwanoha, followed by Yaesu, Nishichiba and finally, Kiba with 43 species.

Table 6: Number of floral species used by honeybees by site (Yaesu, Kiba, Nishichiba and Kashiwanoha) and in total in 2018.

Site	Yaesu	Kiba	Nishichiba	Kashiwanoha	Total
Number of floral species used	76	43	72	83	162

Moreover, a boxplot will inform us about the species richness depending on the four sites (Figure 6). The boxes denote interquartile ranges, with the median as a black line and whiskers extending up to the most extreme points. Alpha diversity measure is the highest for Kashiwanoha site, followed by Yaesu and Nishichiba sites and finally Kiba site. There is a significant effect of the site on richness (*p-value* higly significant for Chao 1). Moreover, the effect of the beehive localization on Shannon diversity is also higly significant (*p-value* < 0,001). Thus, effective diversities are not similar from one place to another. For example, Kashiwanoha is dominated by a large number of abundant taxa and, at the opposite, Kiba is dominated by a few abundant taxa.

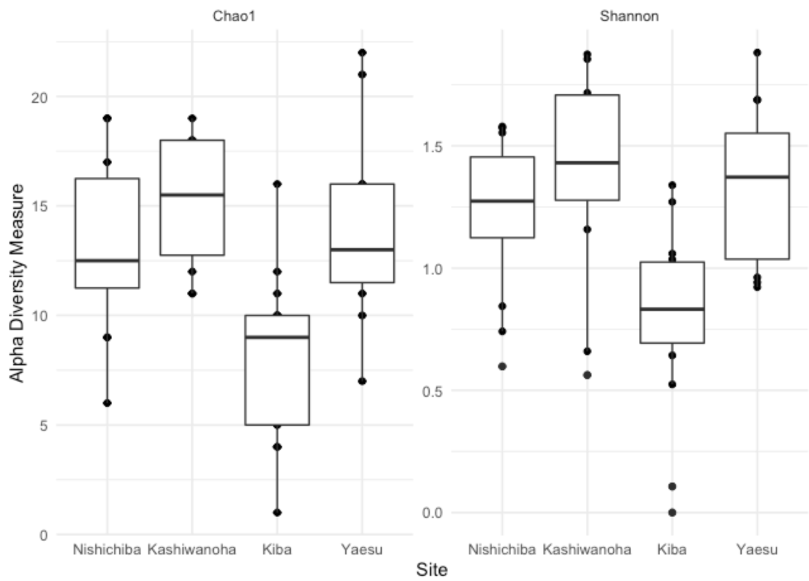

Figure 6: Alpha Diversity Measure (Chao1 richness estimator and Shannon Index) by site (Nishichiba, Kashiwanoha, Kiba and Yaesu sites) in 2018. A point represents a sample (i.e. one date in one site).

ii. Herbaceous and tree species analysis

In 2018, the combined number of trees and herbaceous plants shows a proportion of 54% of herbaceous species and 40% of tree species. The 6 other per cents represents the non-floral species of the database as well as floral species with no-information (Figure 7). We note that to simplify, in this proportion shrubs have been classified as tree species and climbing plants as herbaceous species.

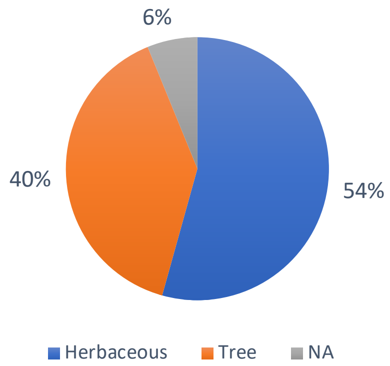

Figure 7: Percentage of herbaceous and tree species for all of the sites in 2018. NA includes species with no information about their herbaceous or tree species state and the non-floral species used by honeybees for all of the sites.

For Yaesu site, globally, 47% of the used species are herbaceous species and 46% are tree species (the rest being data from non-floral species or unidentified species up to the taxonomic level of the species). The two clover species and the tree species of *Lagerstroemia indica* being in the top 3 of the most abundant species. Kiba site is characterized by 49% of herbaceous species and 44% of tree species. At Nishichiba site, 51 % are herbaceous species and 43% of the used species are tree species. For Kashiwanoha site, 51% are herbaceous species and 46% are tree species. Figure 6 includes both of the top 10 species lists by site in 2018.

Table 7: Top list species for the four sites (Yaesu, Kiba, Nishichiba and Kashiwanoha) in 2018. The Sum list is the sum of the DNA number of reads for all of the samples per month and per species. The frequency list is calculated by summing the number of times the species appears in total on all sites per month. The number of samples used in this analysis is different for each site: Yaesu (11), Kiba (13), Nishichiba (12) and Kashiwanoha (12).

Yaesu Sum	Yaesu Frequency	Kiba Sum	Kiba Frequency
Trifolium_repens	*Trifolium_repens*	*Eucalyptus_vicina*	*Eucalyptus_vicina*
Trifolium_pratense	*Plantago_lanceolata*	*Lagerstroemia_indica*	*Eucalyptus_grandis*
Lagerstroemia_indica	*Trifolium_pratense*	*Trifolium_pratense*	*Trifolium_repens*
Plantago_lanceolata	*Lagerstroemia_indica*	*Rosa_hybrid*	*Verbena_hispida*
Acer_buergerianum	*Oenothera_rosea*	*Mallotus_barbatus*	*Verbena_incompta*
Mallotus_barbatus	*Plantago_asiatica*	*Trifolium_repens*	*Lagerstroemia_indica*
Cosmos_sulphureus	*Lolium_perenne*	*Eucalyptus_grandis*	*Trifolium_pratense*
Nelumbo_nucifera	*Verbena_hispida*	*Erythrina_crista-galli*	*Verbena_incompta*
Aralia_elata	*Nelumbo_nucifera*	*Solidago_canadensis*	*Mallotus_barbatus*
Embryophyte_environmental	*Ligustrum_lucidum*	*Pittosporum_glabratum*	*Erythrina_crista-galli*
Nishichiba Sum	**Nishichiba Frequency**	**Kashiwanoha Sum**	**Kashiwanoha Frequency**
Trifolium_pratense	*Trifolium_pratense*	*Trifolium_pratense*	*Trifolium_pratense*
Trifolium_repens	*Trifolium_repens*	*Trifolium_repens*	*Trifolium_repens*
Centaurea_cyanus	Plantago_lanceolata	*Lagerstroemia_indica*	*Plantago_lanceolata*
Prunus_pseudocerasus	*Triadica_sebifera*	*Centaurea_cyanus*	*Centaurea_cyanus*
Helianthus_annuus	*Phyla_canescens*	*Dichroa_febrifuga*	*Brassica_napus*
Mallotus_barbatus	*Ligustrum_lucidum*	*Actinidia_deliciosa*	*Ligustrum_lucidum*
Triadica_sebifera	*Dichroa_febrifuga*	*Mallotus_barbatus*	*Phyla_canescens*
Dichroa_febrifuga	*Hypericum_lancasteri*	*Actinidia_chinensis*	*Lolium_perenne*
Rosa_hybrid	*Lolium_perenne*	*Dendropanax_morbifer*	*Dichroa_febrifuga*
Vicia_villosa	*Oenothera_rosea*	*Punica_granatum*	*Rosa_hybrid*

For the four sites, the percentage between herbaceous and tree species (without the proportion of species with no-information) reaches for the four different sites a ratio of about 50/50. Moreover, the two clover species are found in the top species used by honeybees in each site. However, the numbers of reads are different depending on the site. Indeed, bees will sometimes exploit more one group of species than another. Figure 8 shows that for the two sites of Nishichiba and Yaesu, the number of reads of herbaceous species is highly superior to the one of tree species. At the opposite, at Kiba site, tree species are favored despite herbaceous species. Moreover, we can observe a ratio almost at 50/50 between the number of reads from herbaceous and tree species found in Kashiwanoha.

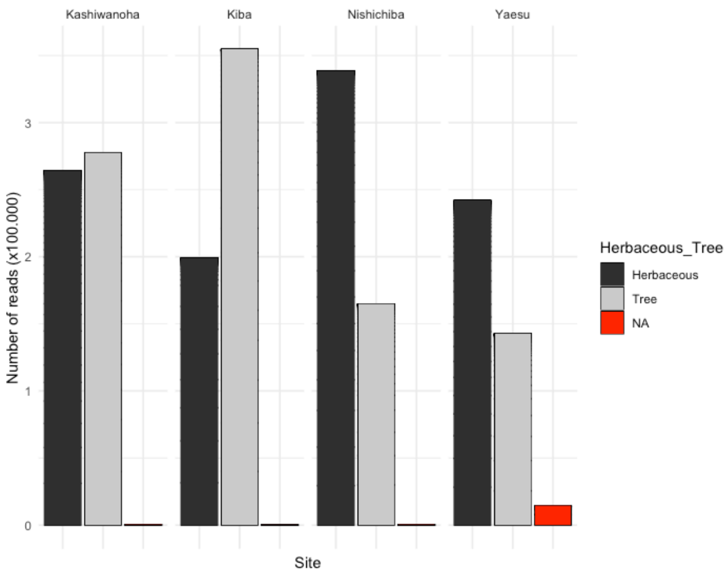

Figure 8: Number of reads depending on herbaceous and tree species in four different sites (Kashiwanoha, Kiba, Nishichiba and Yaesu) in 2018. NA regroups the non-floral species and the unidentified species up to the species' taxonomic level. The number of samples used in this analysis is different for each site: Kashiwanoha (12), Kiba (13), Nishichiba (12) and Yaesu (11).

iii. Native, non-native and cultivar species analysis

In 2018, the proportion of non-native species by honeybees was about 52%, while the other half of the data is divided between native species (25%), cultivar species (20%) and the other non-floral species identified in the honeybee's pollen (3%) (Figure 9).

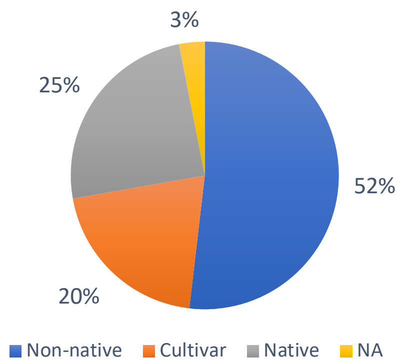

Figure 9: Percentage of non-native, cultivar, native species for all of the sites in 2018. NA includes species with no information about their native or non-native state and the non-floral species used by honeybees for all of the sites.

Regarding the site, an ANOVA analysis allows highlighting if the number of species used by honeybees is different depending on the beehive localization. The three variables are significative depending on the site. Moreover, a more in-depth analysis allows us to determine the most significant number of species on average per site (Table 8).

Table 8: Summary of Anova test and average number of native, non-native and cultivar species per site in 2018

Anova Test			Average number of species by site			
Response variable	Explanatory variable	*p-value*	Kashiwanoha	Kiba	Nishichiba	Yaesu
Number of non-native species	Site	< 0,05	8.42	5.60	8.09	8.18
Number of native species		< 0,001	2.42	0.60	1.72	1.91
Number of cultivar species		< 0,001	3.67	0.80	2.82	2.63

Table 8 shows the effect of the site on the number of non-native, native and cultivar species used by honeybees. Globally, the three categories are impacted by the factor “site". It appears that the number of non-native species is the highest for Kashiwanoha site, followed by Nishichiba site. Regarding native and cultivar species, the effect of the site is highly significant on the number of species used by honeybees. For the three categories, Kashiwanoha remains the site where bees use the highest diversity (use the highest number of species).

Figure 10 shows that non-native species are much more exploited than native species, given the high number of reads found in the hives of the four sites. There is also very high exploitation of cultivar species at Kashiwanoha site, a fact that is explained by the location of the hives at the Kashiwanoha University Campus site and the surrounding horticultural context.

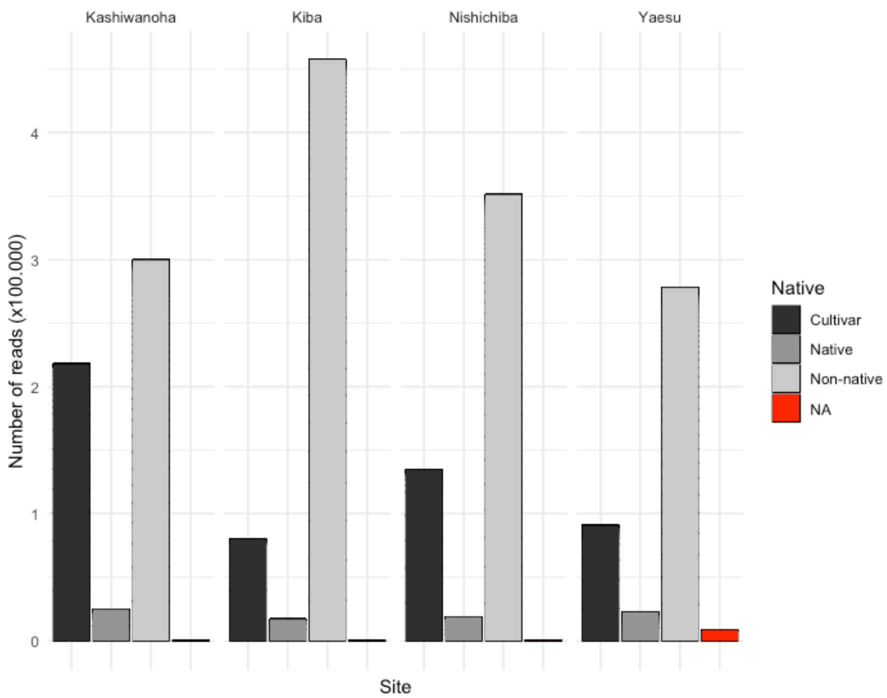

Figure 10: Number of reads depending on cultivar, native and non-native species for the four different sites (Kashiwanoha, Kiba, Nishichiba and Yaesu) in 2018. NA regroups the non-floral species and the unidentified species up to the species' taxonomic level. The number of samples used in this analysis is different for each site: Kashiwanoha (12), Kiba (13), Nishichiba (12) and Yaesu (11)).

b. Comparison of years 2017 and 2018 in Yaesu and Kiba

In Kiba, 26 floral species have been used in 2017 and 43 in 2018. In total, 19 botanical species are common to both years, and 24 new species appeared in 2018. Most of the same species are found in the top of the most commonly used species in both years. The most significant increase highlighted is *Eucalyptus vicina*, with a 2.5 increase in the amount of the number of reads. Concerning herbaceous species, DNA number of reads is 3 times higher for the two clover species in 2018 than in 2017. However, the number of reads of *Solidago canadensis* species is two times lower in 2018 than in 2017, species not even present in the 2018 top list (Table 9). One interesting piece of information is the presence of *Rosa hybrid*, which was exploited very heavily by bees in 2018 (but not present in the top list species based on the frequency), whereas it remains completely absent from the list of species used in 2017. The last interesting information is the presence of the two species of *Verbena* genus in 2018, very frequently throughout the year; in 2017, only *Verbena hispida* in present in one unique sample out of the 11. Species contributing to the bees' food resources remain mostly non-native species.

Table 9: Top list species in Kiba in 2017 and 2018 with (1) their sum of the DNA number of reads for all of the samples throughout the year per species (Sum) and (2) the top list species in Kiba in 2017 and 2018 with the frequency calculated by summing the number of times the species appears in total on all samples (Freq). The number of samples used in this analysis is different for each year: in 2017, Kiba (11) and in 2018, Kiba (13).

2017				2018			
(1) Top species	**Sum**	**(2) Top species**	**Freq**	**(1) Top species**	**Sum**	**(2) Top species**	**Freq**
Lagerstroemia indica	51768	*Lagerstroemia_indica*	5	*Eucalyptus vicina*	105582	*Eucalyptus_vicina*	10
Eucalyptus vicina	39417	*Solidago_canadensis*	5	*Lagerstroemia indica*	77099	*Eucalyptus_grandis*	10
Eucalyptus grandis	31595	*Eucalyptus_vicina*	4	*Trifolium pratense*	67819	*Trifolium_repens*	8
Solidago canadensis	35124	*Erythrina_crista-galli*	3	*Rosa hybrid*	55885	*Verbena_hispida*	6
Trifolium repens	25376	*Eucalyptus_grandis*	3	*Mallotus barbatus*	47713	*Verbena_incompta*	5
Erythrina crista-galli	15399	*Trifolium_pratense*	2	*Trifolium repens*	42345	*Trifolium_pratense*	4

In Yaesu, between 2017 and 2018, 40 more floral species are used by honeybees, also highlighted by a sharp increase in the amount of ITS1 reads, and more particularly in the collection of pollen from herbaceous species. Indeed, the quantity of the two clover species identified are 6 times higher in 2018 than in 2017 and are also present in most samples during 2018. At the opposite, the tree species of *Mallotus barbatus* is more exploited in 2017 than in 2018. However, in 2018, other tree species are highly used, such as *Lagerstroemia indica* and *Acer buergerianum,* also responsible for the significant increase of cultivar species (Figure 12). Another important information is the presence of *Cicer arietinum,* only used in 2017 and absent of the floral species used in 2018. Finally, we found two species of *Plantago* genus in 2018 in the top list species, present abundantly during this year.

Table 10: Top list species in Yaesu in 2017 and 2018 with (1) their sum of the DNA number of reads for all of the samples throughout the year per species (Sum) and (2) the top list species in Yaesu in 2017 and 2018 with the frequency calculated by summing the number of times the species appears in total on all samples (Freq). The number of samples used in this analysis is different for each year: in 2017, Yaesu (10) and in 2018, Yaesu (11).

2017				2018			
(1) Top species	**Sum**	**(2) Top species**	**Freq**	**(1) Top species**	**Sum**	**(2) Top species**	**Freq**
Mallotus barbatus	38737	*Mallotus barbatus*	4	*Trifolium repens*	117669	*Trifolium repens*	11
Cicer arietinum	38548	*Cicer arietinum*	4	*Trifolium pratense*	54833	*Plantago lanceolata*	8
Trifolium repens	26894	*Trifolium repens*	4	*Lagerstroemia indica*	52514	*Trifolium pratense*	7
Lantana sp.	19748	*Schefflera heptaphylla*	3	*Plantago lanceolata*	29081	*Lagerstroemia indica*	4
Schefflera heptaphylla	11838	*Plantago asiatica*	3	*Acer buergerianum*	25552	*Verbena hispida*	4
Artemisia argyi	10827	*Erythrina crista-galli*	3	*Mallotus barbatus*	19124	*Plantago asiatica*	4

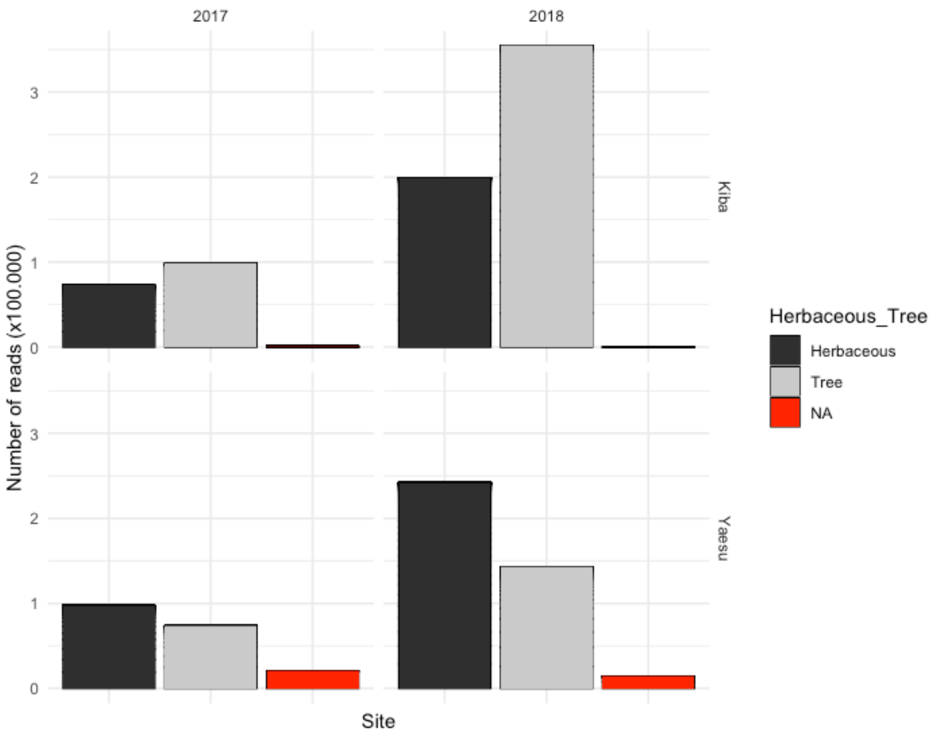

Figure 11: Number of reads depending on herbaceous and tree species for the two sites, Kiba and Yaesu in 2017 and 2018. NA regroups the non-floral species and the unidentified species up to the species' taxonomic level. The number of samples used in this analysis is different for each site and each year: in 2017, Kiba (11), Yaesu (10) and in 2018, Kiba (13) and Yaesu (11).

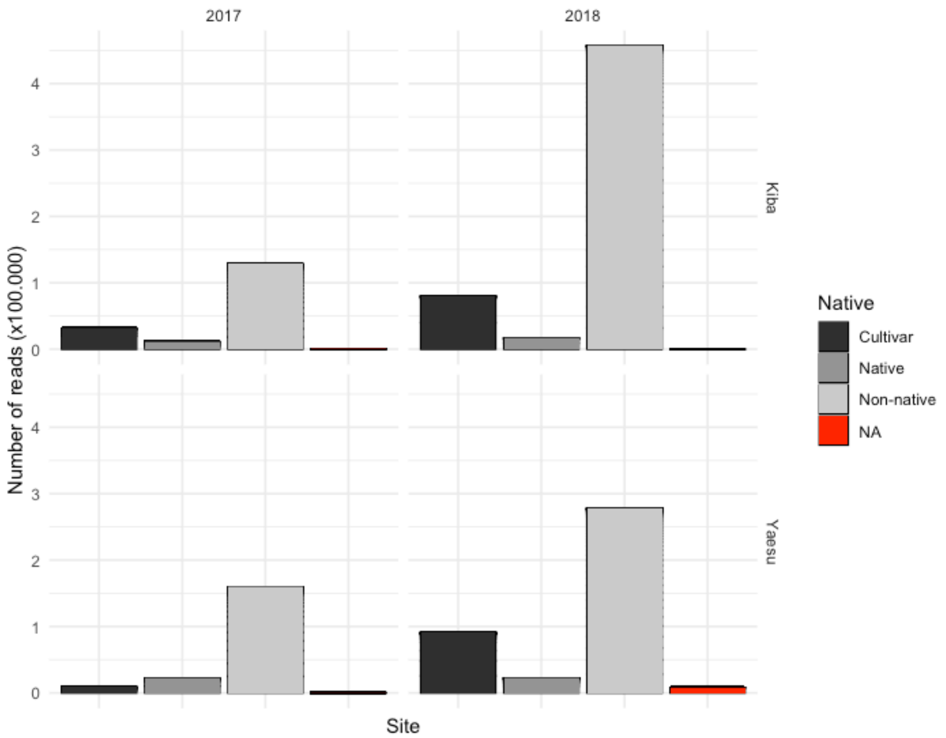

Figure 12: Number of reads depending on cultivar, native and non-native species for the two sites, Kiba and Yaesu in 2017 and 2018. NA regroups the non-floral species and the unidentified species up to the species' taxonomic level. The number of samples used in this analysis is different for each site and each year: in 2017, Kiba (11), Yaesu (10) and in 2018, Kiba (13) and Yaesu (11).

c. By time in 2017 and 2018

In 2017 and 2018, 57 floral families were identified in the pollen database and the largest number of honey plant species are found in *Fabaceae* (15 spp.) followed by *Asteraceae* (14spp.) and *Rosaceae* (8spp.). Other families can be mentioned with four or five pollen species as *Myrtaceae*, *Brassicaceae, Fagaceae, Poaceae, Primulaceae or Verbanaceae*.

i. Taxonomic description

A taxonomic description from March to October of floral species used by honeybees has been conducted and all of the top list species can be found in the appendix 3 (based from the sum of the number of reads and on the frequency).

Firstly, March has only one sample taken from Nishichiba site. The most used species in March were not compared with the frequency method since only one sample was used to identify the top list species. A high quantity of number of reads belongs to the two species of Prunus, *Prunus pseudocerasus* and *Prunus szechuanica*. Indeed, the flowering of cherries in bloom is at the end of March and, therefore, participate as an important resource at the beginning of the pollen and nectar season. The third most commonly used species is the herbaceous *Brassica napus*. In total, 9 species are used in March at Nishichiba site.

April is characterized by a high abundance of number of reads from *Actinidia chinensis* and *Prunus grayana* species for Kashiwanoha site, *Pittosporum glabratum* and *Rosa hybrida* for Kiba site, *Vicia villosa* for Nishichiba and *Acer tataricum* for Yaesu. Other species such as *Taraxacum obtusifrons* and *Brassica napus* at Kashiwanoha site, *Acer buergerianum* at Nishichiba and *Acer palmatum* at both sites are also very important sources of pollen and nectar for bees in April. The list of species used by bees in April is dominated by the presence of maples. 55 species are used in this month.

For May, the same number of species is used than April, but the pollen collection seems increasing at this period. A very high quantity of number of reads of *Centaurus cyanus* was collected from both Nishichiba and Kashiwanoha sites. Then comes the two species of clover, *Trifolium repens* and *Trifolium pratense*, which are abundant and very frequent on the 4 sites but less abundant on Kiba site. Next comes the *Euphorbiaceae* with *Mallotus barbatus* in Nishichiba, Kashiwanoha and Kiba sites.

In June, we find on the podium the previous species (*Trifolium repens*, *Mallotus barbatus* and *Trifolium pratense*) but also the species *Dichroa febrifuga*, especially in great importance in the samples of Nishichiba and Kashiwanoha but also in smaller quantities in Yaesu. Other species are to be mentioned such as *Erythrina crista-galli* (especially in Nishichiba and Kiba), *Punica granatum* (very abundant in Kashiwanoha), *Plantago lanceolata* present on all sites and so on. 55 species are used in June.

In July, 42 species were collected from pollen samples. For both top list species (sum and frequency), the two clover species remain the most common species found in July pollen. Then comes the species *Triadica sebifera* on the three sites of Kashiwanoha, Nishichiba and Kiba. On Nishichiba, a considerable abundance of DNA reads is identified from *Helianthus annuus* species but is not present in the frequency top list. It is also found in very fine quantities on Yaesu site. Other tree species are found such as *Dendropanax morbier*, *Lagerstroemia indica* and *Aralia elata*.

In August, the species *Lagerstroemia indica* dominates the top list species for all of the 4 sites. This is followed by the two clover species, and the two tree species *Eucalyptus vicina* and *Eucalyptus grandis* only at Kiba site. Species such as *Erythrina crysta-galli* or *Plantago lanceolata* are still present. 55 species are used during this month.

In September and October, the two *Eucalyptus* species and *Solidago canadensisis* are the most commonly used species by honeybees in Kiba. *Lagerstroemia indica* is still an essential resource for honeybees in Yaesu and Kiba in September and Kiba in October. In September, on Yaesu site, it's the species of *Cirer arietinum* that is most in demand. It should also be noted that no information on Nishichiba and Kashiwanoha sites is available in September and October and for Nishichiba, Kiba and Yaesu in October. 34 species are used in September and 9 in October.

Table 11: Number of species and samples in 2017 and 2018 for the four sites

Month	Number of species	Number of samples
March	9	1
April	55	6
May	55	13
June	55	13
July	42	13
August	55	12
September	34	7
October	9	4

ii. Herbaceous and tree species analysis

Figure 13 shows the percentage of herbaceous and tree species throughout the year (from March to October). Overall, the number of herbaceous species is higher than the number of tree species used by honeybees throughout the year. However, at the end of April and in May, it appears that the number of tree species is higher. Indeed, in April, 25 tree species are used and in May, 28 species. As already mentioned, April is characterized by the use of *Sapindaceae* as maple tree species, *Rosaceae* as *Prunus* species, *Myrtaceae* as *Eucalyptus* species, *Fagaceae* as *Quercus* species and *Pittosporum glabratum or Styrax_grandiflorus* species. In May, *Acer* and *Prunus* genus are replaced by other species such as *Actinidia deliciosa, Callistemon comboynensis, Styrax grandiflorus, Hypericum lancasteri, Castanopsis fargesii, Rhaphiolepis indica or Acca sellowia*. May is therefore represented by more diversity at the level of the taxonomic families listed. The pick between September and October is explained by the fact that only 9 species are using in October and out of the 9 species, 5 are tree species (*Myrtaceae, Lythraceae, Myrtaceae, Asteraceae* and *Rosaceae* tree species).

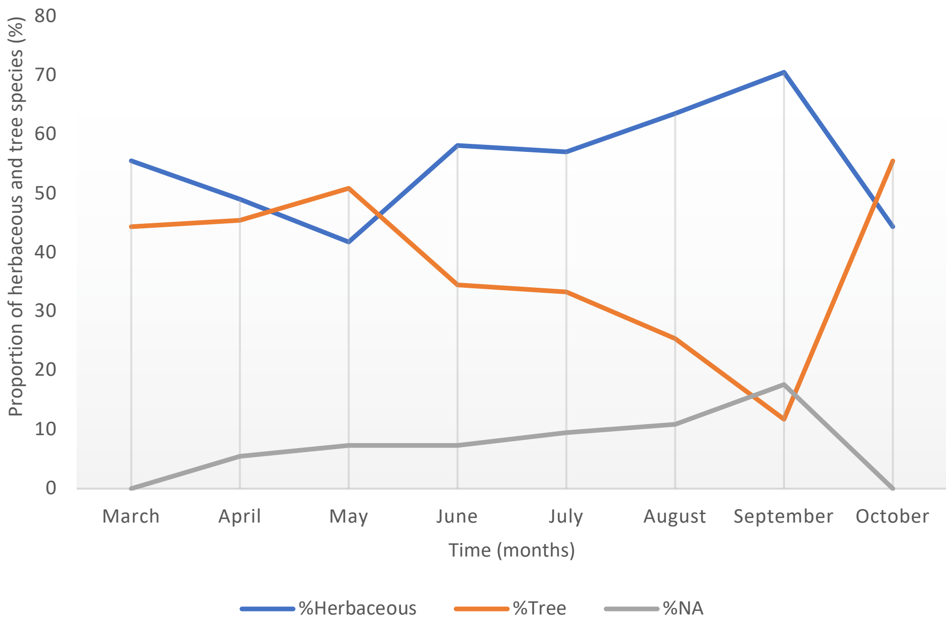

Figure 13: Graph of the proportion of herbaceous and tree species used by honeybees from March to October. NA regroups the non-floral species and the unidentified species up to the species' taxonomic level. The number of samples used in this analysis is different for each month: March (1), April (6), May (13), June (13), July (13), August (12), September (7), October (4).

Regarding the abundance of the species used by honeybees, it appears that in July, the number of ITS1 reads from herbaceous species is identified in a more significant quantity than in June and August (Figure 14). Indeed, July is characterized by the peak season for clover species and sunflowers, which could explain this increase.

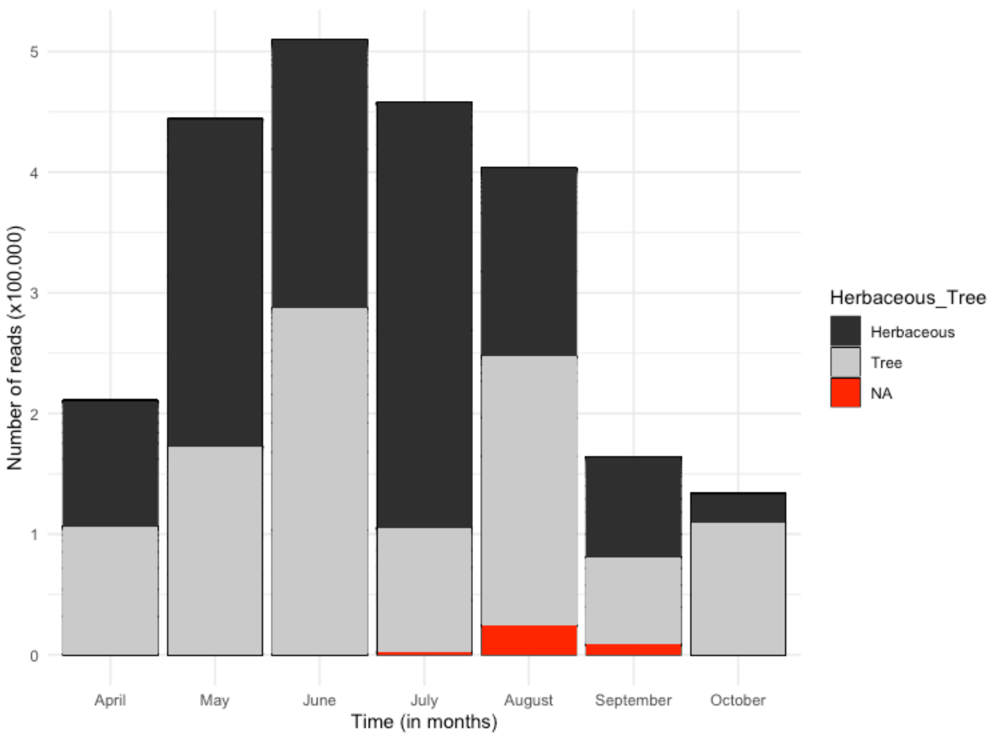

Figure 14: Number of reads depending on herbaceous and tree species by months (from April to October). NA regroups the non-floral species and the unidentified species up to the species' taxonomic level. The number of samples used in this analysis is different for each month: April (6), May (13), June (13), July (13), August (12), September (7), October (4).

iii. Native, non-native and cultivar species analysis

During all of the year, the proportion of the number of non-native species used by honeybees is superior to the ratio of the number of native species used. Moreover, a significant increase in the proportion of the number of non-native species is observed after August.

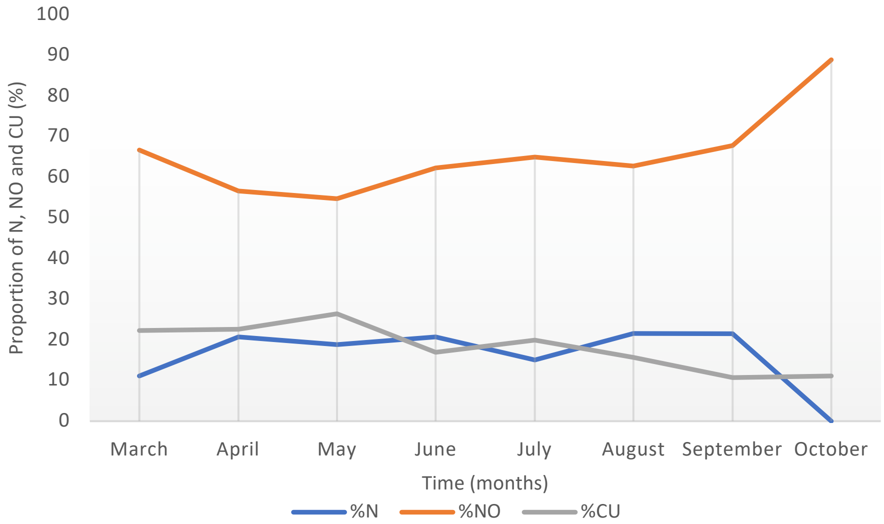

Figure 15: Graph of the proportion of Native (N), Non-native (NO) and Cultivar (CU) species used by honeybees from March to October. The number of samples used in this analysis is different for each month: March (1), April (6), May (13), June (13), July (13), August (12), September (7), October (4).

However, another information shows that unlike non-native and cultivar species, the number of native species used by honeybees according to the months is not significantly different (*p-value* > 0,05). It seems that honeybees are using the same number of native species on average all year round. The native list species used by honeybees throughout the year is composed of 21 herbaceous species and 22 tree species. We find in the most represented taxonomic families three species of *Aquifoliaceae* (*Ilex* genus), three species of *Rosaceae* (as *Prunus grayana, Rhaphiolepis indica* or *Spiranthes sinensis*), or three other species in the *Theaceae* family (*Camellia japonica* and two species of *Stewaria* genus).

Regarding the rate of the number of reads identified throughout the year, Figure 16 shows that cultivar species are highly exploited in August. This increase could be explained by the flowering of the species *Lagerstroemia indica*, highly exploited by honeybees in the four different sites.

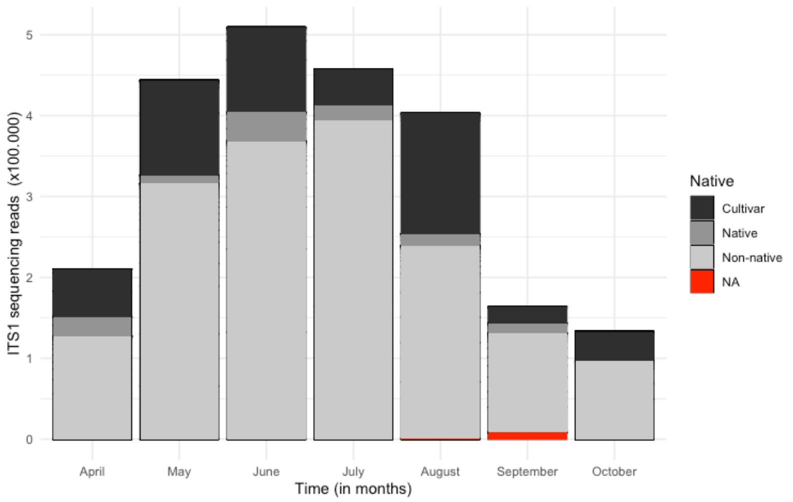

Figure 16: Abundance of cultivar, native and non-native species by months. NA includes species with no information about their native or non-native state and the non-floral species. The number of samples used in this analysis is different for each month: April (6), May (13), June (13), July (13), August (12), September (7), October (4).

2. TRANSECTS

In the top list of species, established in Table 4, the three most represented families are the *Fabaceae* (9 species), the *Asteraceae* (8 species) and the *Rosaceae* (6 species). In total 29 taxonomic families are represented in the most common species used by honeybees in the four different urban sites with 62% of non-native species, 15% of native species and 23% of cultivar species. However, out of these 60 species recorded, only 24 species have been identified in the field (only transects, without Nishichiba study on the campus).

Table 12: top list species classified by families and with their pollen or nectar resources information, their state as herbaceous or tree species and their state of native, non-native or cultivar species in Japan. "NA" means no information. Species in bold are species found in the field (only transects, without Nishichiba study on the campus). For the nectar/pollen column, the significations are: Nectar-source (N), both nectar and pollen provided (NP), Pollen-source (P), Mainly pollen but nectar also (P(n)), mainly nectar but pollen also (N(p)). The second part expresses their ability to exploit the species: excellent, good (well exploited), temporary (temporarily exploited) and rarely (rarely exploited) (Sasaki 2010)

Taxonomy	Species	Nectar/Pollen sources	Herbaceous or Tree species	Native state
Actinidiaceae	***Actinidia_chinensis***	P_good	Tree	Cultivar
	Actinidia_deliciosa	P_good	Tree	Cultivar
Amaryllidaceae	*Allium_fistulosum*	N(P)_good	Herbaceous	Cultivar
Anacardiaceae	***Rhus_chinensis***	NP_good	Tree	Native
Araliaceae	*Aralia_elata*	NP_temporary	Tree	Native
	Dendropanax_morbifer	N(p)_good	Tree	Non-native
	Schefflera_heptaphylla	NA	Tree	Native
Asteraceae	*Artemisia_argyi*	P_temporary	Herbaceous	Non-native
	Bidens_pilosa	NP_excellent	Herbaceous	Non-native
	Centaurea_cyanus	P(n)_temporary_or_good	Herbaceous	Cultivar
	Cirsium_setidens	NP_good	Herbaceous	Non-native
	Cosmos_sulphureus	NP_good	Herbaceous	Non-native
	Helianthus_annuus	NP_excellent	Herbaceous	Cultivar
	Solidago_canadensis	NA	Herbaceous	Non-native
	Taraxacum_obtusifrons	NP_good	Herbaceous	Non-native
Brassicaceae	*Brassica_carinata*	NP_excellent	Herbaceous	Non-native
	Brassica_napus	NA	Herbaceous	Non-native
Euphorbiaceae	***Mallotus_barbatus***	NA	Tree	Non-native
	Triadica_sebifera	P(n)_temporary	Tree	Non-native
Fabaceae	*Cicer_arietinum*	NA	Herbaceous	Non-native
	Erythrina_crista-galli	NP_temporary	Tree	Non-native
	Melilotus_officinalis	NA	Herbaceous	Non-native
	Styphnolobium_japonicum	N(p)_temporary	Tree	Cultivar
	Trifolium_incarnatum	NP_excellent	Herbaceous	Non-native
	Trifolium_pratense	NP_temporary	Herbaceous	Non-native
	Trifolium_repens	NP_excellent	Herbaceous	Non-native
	Trifolium_tomentosum	NA	Herbaceous	Non-native
	Vicia_villosa	NP_excellent	Herbaceous	Non-native
Fagaceae	*Castanopsis_fargesii*	NA	Tree	Non-native
Hydrangeaceae	***Dichroa_febrifuga***	NA	Tree	Cultivar
Hypericaceae	***Hypericum_lancasteri***	NA	Tree	Non-native
Lythraceae	***Lagerstroemia_indica***	P_excellent	Tree	Cultivar
	Punica_granatum	P_temporary	Tree	Cultivar
Myrtaceae	*Callistemon_comboynensis*	NP_good	Tree	Non-native
	Eucalyptus_grandis	NP_good	Tree	Non-native
	Eucalyptus_vicina	NP_good	Tree	Non-native
Nelumbonaceae	*Nelumbo_nucifera*	P(n)_good	Herbaceous	Non-native
Oleaceae	***Ligustrum_lucidum***	NP_good	Tree	Cultivar
Onagraceae	***Oenothera_rosea***	NP_temporary	Herbaceous	Non-native

Orobanchaceae	***Orobanche_pancicii***	NA	Herbaceous	Non-native
Pentaphylacaceae	***Ternstroemia_gymnanthera***	P(n)_rarely	Tree	Native
Pittosporaceae	***Pittosporum_glabratum***	N(p)_temporary	Tree	Non-native
Plantaginaceae	*Plantago_asiatica*	NA	Herbaceous	Non-native
	Plantago_lanceolata	P(n)_rarely	Herbaceous	Non-native
Poaceae	***Lolium_perenne***	NA	Herbaceous	Non-native
Ranunculaceae	***Clematis_terniflora***	P_incidentally	Herbaceous	Native
Rosaceae	*Prunus_grayana*	N(p)_good	Tree	Native
	Prunus_pseudocerasus	NA	Tree	Cultivar
	Prunus_szechuanica	P(n)_temporary	Tree	Non-native
	Rhaphiolepis_indica	NP_temporary	Tree	Native
	Rosa_hybrid	P(n)_temporary	Herbaceous	Non-native
	Rubus_columellaris	NP_good	Herbaceous	Non-native
Rutaceae	*Citrus_sinensis*	N(p)_excellent	Tree	Cultivar
Sapindaceae	***Acer_buergerianum***	N(P)_good	Tree	Cultivar
Styracaceae	*Styrax_grandiflorus*	NP_excellent	Tree	Non-native
Theaceae	*Stewartia_sinensis*	P(n)_good	Tree	Native
Verbenaceae	*Phyla_canescens*	NP_temporary_or_good	Herbaceous	Native
	Verbena_hispida	NA	Herbaceous	Non-native
	Verbena_incompta	NA	Herbaceous	Non-native
Vitaceae	*Ampelopsis_japonica*	NA	Herbaceous	Cultivar

Out of the 30 herbaceous species sought, 11 have been identified and out of the 30 tree species, 13 have been identified in the field. Out of these 24 species found in the traffic lanes, 63% are non-native species, 25% are cultivar species and finally, 13% are native species.

a. Urban green design

According to a spatial analysis using google earth software, the different urban elements recorded in the four selected areas are as follows:

- managed areas: traffic lane, parks, ornamental plantings, agricultural fields and schools.
- urban wastelands for the formerly managed places
- private interior gardens

Out of the 60 transects completed, 46 were along the streets, 10 on Nishichiba and Kashiwanoha university campuses and 3 in parks. Moreover, 3 transects crossed urban wastelands but no transects sampled any floral species from private interior gardens.

The following global analysis will detail the floral species found, depending on the Rupprecht and Byrne (2014) (1) typology, on three different managed spaces: streets, parks and university campus.

Out of the 46 transects along the streets and its close dependency (private gardens in front of passable streets), the majority of the floral species sought are found in street verges (16 transects). The different species found in large quantities in the field in this type of urban green design are the following (all herbaceous species): *Oenothera rosea, Lolium perenne, Trifolium pratense, Trifolium repens, Plantago lanceolata, Vicia villosa, Orobranche pancicii, Solidago canadensis and Clematis terniflora* on vertical structures (fences). Moreover, 7 transects crossed streets with observable private gardens near traffic lanes, but only one species has been recorded, *Rosa hydrida* (on 2 transects). This same species has been observed along 4 transects as ornamental plantings, therefore, managed by the city. Moreover, urban wastelands were sampled through three transects. The species recorded were *Solidago canadensis, Trifolium repens* and *Trifolium pratense*. Finally, in the streets, microsites can host the species *Oenothera rosea* (1 transect).

Regarding the tree species, all of the species found in the streets are only ornamental plantings. The species recorded in all of the sites are *Dichroa febrifuga, Lagerstroemia indica, Dendropanax morbifer, Ligustrum lucidum, Hypericum lancasteri and Ternstroemia gymnanthera.*
One transect in the streets crossed a green wall (structural urban design typology) but no species have been detected.

Out of the three transects in parks, four herbaceous species were recorded: *Trifolium pratense, Solidago canadensis, Orobranche pancicii and Plantago lanceolata.* About the tree species, *Lagerstroemia indica, Pittosporum glabratum, Dendropanax morbifer* and finally, *Mallotus barbatus* were observed.

On Nishichiba and Kashiwanoha campus, herbaceous species were observed in street verges, microsites, and urban gap designs. Out of the 10 transects on universities campus and regarding herbaceous species, *Trifolium pratense*, *Trifolium repens, Lolium perenne, Vicia villosa, Clematis terniflora, Plantago lanceolata, Oenothera rosea* and only one individual of *Taraxacum obtusifrons* has been detected in streets. We can also find *Plantago lanceolata* on microsites (1 transect) and *Vicia villosa* on gaps (1 transect). About tree species, *Triadica sebifera, Dichroa febrifuga* were recorded as ornamental plantings (3 transects). Furthermore, one transect crossed *Actinidia deliciosa* and *Actinidia chinensis* plantings (agricultural areas).

A more detailed analysis is carried out in a second step, differentiating the sampling by site.
The complete data analysis on the field is present in the appendices, detailed by site (Appendix 4 to 7).

b. Nishichiba

The beehives located at Nishichiba campus are surrounded by an ample green managed space, Chiba University campus, and a more urbanized area around the campus. Out of 16 herbaceous species, the half has not been recognized on the field. Moreover, only 2 out of 13 tree species have been recorded on the transects.
However, a global analysis on the entire campus shows that seven tree species of the top list (*Acer buergerianum, Lagertroemia lancasteri, Mallotus barbatus, Rhaphiolepsis indica, Ternstroemia gymanthera, Triadica sebifera, Punica granatum)* have been planting on the campus (Appendix 2). Given the context of this adding study, it seems that for all of these species, the main purpose was ornamental plantings. It should be noted that all individuals of *Acer buergerianum* species are planted in alignment on Nischichiba university campus. Regarding *Punica granatum,* only one individual is recorded thanks to the entire tree species analysis on the campus.

As far as herbaceous species are concerned, some of them are found only in specific urban designs exclusively. For instance, the species of *Lolium perenne* has been found in traffic lanes, and more precisely on street verges, where ornamental and spontaneous species share this green space (Figure 17, (a)).

On the campus, *Vicia villosa* can be observed in a huge number of places and some gap between two walls (stony ground and unmanaged area) (Figure 17, (b)).

Finally, the species *Oenothera rosea is* present in a limited number in the transects visited (at the foot of trees, along hedges, microsites…) (Figure 17, (c)). However, behind administrative buildings, a place out of transect has been observed where *Oenothera rosea* is found in a considerable quantity (Figure 17, (d)).

Figure 17: (a) Lolium perenne (transect 28) in street verges: herbal vegetation between street and sidewalk; (b) Vicia villosa (transect 11) in a gap between two walls; (c) Oenothera rosea (transect 38) at the foot of trees; (d) Oenothera rosea (out of transect) in lots behind buildings on Nishichiba campus

c. Kiba

Out of 11 herbaceous species, 5 species have been found in the field: *Trifolium pratense, Solidago canadensis, Rosa hybrid, Orobranche pancicii* and *Plantago lanceolata.* About tree species, 5 species have been recognized out of 10: *Lagerstroemia vicina, Pittosporum glabratum, Rhus chinensis, Triadica sebifera* and *Dendropanax morbifer.*
Kiba site is an industrialized area where, according to a spatial analysis by Google Earth Pro, very few dwellings are identified. On the 15 transects, one could not be realized (impassable street) (transect 12).

Regarding herbaceous species, *Solidago canadensis* has been identified on 10 transects out of 15 and in a great abundance. This species has been found most of the time in street verges, as a spontaneous herbaceous species. *Solidago canadensis* and *Orobranche pancicii* are two species found exclusively in the top list of Kiba site. *Orobranche pancicii* has also been found on 3 transects. The picture (a) (Figure 18) illustrates the presence of these two species in street verges.
Only one of the two species of *Trifolium* is found. From my field observations, the four species *Plantago lanceolata*, *Trifolium pratense*, *Orobranche pancicii*, and *Solidago canadensis* have been found in the same urban design, unmanaged space in street verges. An example can be found on the transect number 2 (Figure 18, (b)), on a flat wooded strip between two lanes of traffic. Finally, *Rosa hybrida* has been identified only in one transect as ornamental plantings.

About tree species, *Lagerstroemia indica* (Figure 18, (c)), and *Pittosporum glabratum* (Figure 18, (d)) are respectively present as ornamental plantings in streets and the park sampled. Moreover, *Lagerstroemia indica* has been found on 7 transects out of 15 transects. The other species identified were found in the park (transects 19 and 20).

Figure 18: (a) Solidago canadensis & Orobranche pancicii (transect 44) in street verges; (b) Plantago lanceolata, Trifolium pratense, Orobranche pancicii & Solidago canadensis (transect 2) on a flat wooded strip between two lanes of traffic; (c) Lagerstroemia indica (transect 44) as ornamental plantings; (d) Pittosporum glabratum (out transect but just next to transect 20) as ornamental plantings in a park

A transect crossed an urban wasteland (Figure 19, (e)). However, except *Solidago solensis*, no other species have been identified on the site.

After a more global analysis of the urban design of the study area, it turned out that there were no private gardens in this area (since there were no dwellings). However, a large number of species were not found in the roadway samples. A first hypothesis could be the presence of a large green zone on the heliport area in the study zone; this could be a potential resource of floral herbaceous species (Figure 19, (f)). Moreover, a radius of 2.2 km (mean travel for honeybees (Seeley *et al.* 1995)) was achieved around the position of the hives on google earth pro. Many green areas are included, in particular, the presence of several parks (Figure 20).

Figure 19: (e) urban wasteland (transect 34); (f) Tokyo heliport area next to the transect number 24

Figure 20: Aerial view of Kiba site with Google Earth Pro software. The red circle represents a buffer of 2.2 km from the hive position and the white circle represents 500 meters around the hive. A tree icon corresponds to a park.

d. Kashiwanoha

Beehives on Kashiwanoha site are surrounding by Kashiwanoha campus, some houses, high buildings and a park. Out of 12 herbaceous species, the half has been identified on the field: *Trifolium pratense, Trifolium repens, Taraxacum obtusifrons, Plantago lanceolata, Vicia villosa* and *Lolium perenne*. About tree species, out of the 12 top list species, only 5 have been recognized in the study area: *Dichroa febrifuga, Actinidia deliciosa* and *Actinia chinensis, Mallotus barbatus* and finally *Ligustrum lucidum.*

The species *Lolium perenne* has been identified on the foot of trees in the street (Figure 21, (a)) and on 6 transects. Out of transects, the species *Dendropanax morbifer* is found in shaft alignment as ornamental plantings (Figure 21, (b)). The two species of *Trifolium* have been identified: on 3 transects for *Trifolium pratense* and 6 transects for *Trifolium repens*. Moreover, the four species *Plantago lanceolata*, *Trifolium repens, Taraxacum obtusifrons* and *Vicia villosa* are found in large green areas on the campus (Figure 21, (c)). Finally, I noticed some hedges of *Lucidum lucidum* next to residential areas as ornamental plantings and in order to delimit some areas.

Along the two transects 11 and 47, the exotic species cultivated on Kashiwanoha campus, *Actinidia deliciosa, Actinidia chinensis* were observed. We supposed that other cultivar species could be found on the campus as *Citrus sinensi*s or *Punica granatum.*

Two transects crossed an urban wasteland where *Trifolium pretense* and *Trifolium repens* are found in great abundance (transects 15 and 16) (Figure 21, (d)).

Figure 21: (a) Lolium perenne (transects 39 & 26) in street verges at the foot of the trees (b) Dendropanax morbifer (out of transects) as ornamental plantings (c) Plantago lanceolata & Trifolium repens & Taraxacum obtusifrons & Vicia villosa (transect 24) on Kashiwanoha campus (d) Trifolim pratense & Trifolium repens & Lolium perenne (transects 15 &16) on the urban wasteland.

e. Yaesu

Beehives located in Yaesu site are surrounded by a dense urban area. Moreover, private gardens are almost absent. According to google earth pro software, some green roofs are also in the study area but were not sampled.

The main observation that can be made at this study site is the absence of herbaceous species in the samples taken. About the tree species, only 3 species out of 13 have been identified in Yaesu: *Lagerstoemia indica, Ternstroemia gymnanthera* and *Hypericum lancasteri.* In this area, green places are highly maintained and only the alignment of shelters and shrubs are found. Moreover, the same species are found in the entire city, as *Ginko biloba* for example (Figure 22, (a)). The species, *Hypericum lancasteri* has been recorded on 5 transects as ornamental species (Figure 22, (b)).

Figure 22: (a) Ginko biloba in the streets as ornamental plantings (not in the top list of species used by honeybees); (b) Hypericum lancasteri (transect 15) as ornamental plantings

On 2.2 kilometers of radius around beehives location, very large green areas are present (including the gardens of the imperial palace) (Figure 23). These green places suggest that honeybees are moving far away from their close perimeter to find pollen and nectar sources from herbaceous species among others.

Figure 23: Aerial view of Yaesu site with Google Earth Pro software. The red circle represents a buffer of 2.2 km from the hive position (mean travel for honeybees (Seeley et al. 1995)) and the white circle represents 500 meters around the hive.

3. COMPARAISON TRANSECTS & METABARCODING METHODS

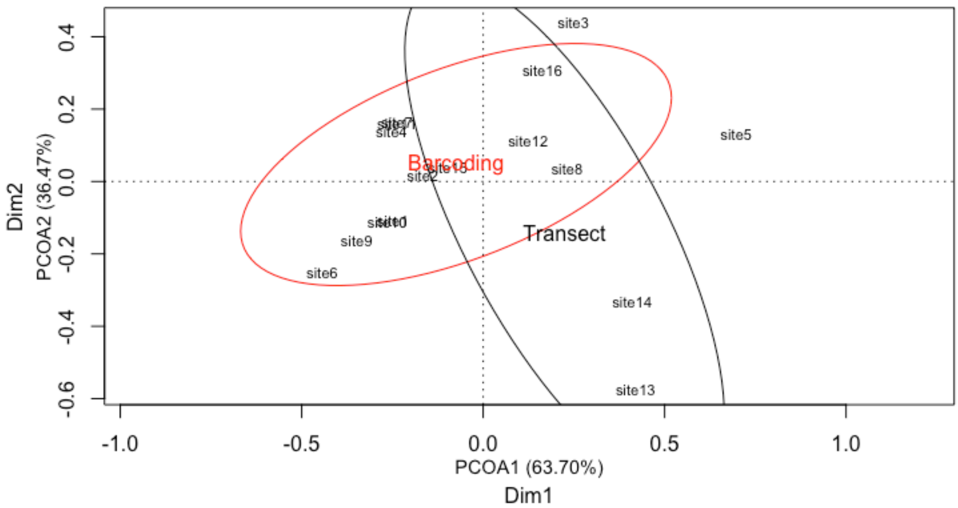

Figure 24: PCoA ordination (unconstrained) of the two techniques used in this study: DNA metabarcoding and transects in the field. Sample 1 to sample 12 are related to DNA metabarcoding from May 2018: site 1 to site 3 for Yaesu, sites 4 and 5 for Kiba, site 6 to site 8 for Nishichiba and site 9 to site 12 for Kashiwanoha. Site 13 (Yaesu), site 14 (Kiba), site 15 (Nishichiba) and site 16 (Kashiwanoha) are the four samples realized in the field in May 2019.

The two techniques are not complementary (ellipses overlap). Both sites of Yaesu and Kiba, from the transect method, force the ellipse of the transects to extend. Both of them are very urbanized sites. In the same case, sites 3 (from Yaesu site) and site 5 (from Kiba site) are outside the ellipse's DNA metabarcoding technique.

DISCUSSION

1. PREFERRED VEGETATION COMMUNITY'S ANALYSIS

Kashiwanoha is the site with the highest richness. This conclusion is explained by the localization of the beehive, surrounding by cultivar species. Moreover, the richness is higher for the two sites located in the peri-urbanization area of Tokyo City, Nishichiba and Kashiwanoha than for the two sites in the downtown area of the capital.

Even if we have a ratio of about 50/50 herbaceous trees species, it seems that one group can be more exploited than the other depending on the site. There are a higher number of reads identified from herbaceous species than from tree species for Nishichiba and Yaesu sites, and more from tree species for Kiba and Kashiwanoha sites. Indeed, for Kiba site, this is explained by the highly industrialized context and the presence of a very high abundance of the species *Lagertroemia indica*, especially in August. Besides, field results confirm this hypothesis (presence of the species in 7 out of 15 samples). The two herbaceous clover species are also significant sources of nectar and are always present in the species most commonly used by honeybees from April to August included for the four sites.

The number of non-native species is all the year higher than the use of native species. But we can see that between September and October, the number of native species used drops to zero. Honeybees can continue to collect pollen and nectar from exclusively non-native and cultivar species. Indeed, in August, 11 native species are used by honeybees while only 6 native species are identified in September (*Rhus chinensis, Clematis terniflora, Paederia foetida, Pueraria montana, Boehmeria nivea* and*Lactuca indica*) and none during October. It is therefore attractive for these honeybees to have species that bloom longer or more focused in late summer to have access to pollen and nectar sources until the end of the season.
Indeed, it has been well established that some species found in the top of the most abundant species are actually species used at the end of the season and are therefore more heavily exploited given the lack of floral diversity available at the end of the season. Several examples can be mentioned, such as the species *Eucalyptus vicina*, which is exploited from August and pollen collected is increasing until October. This species is an excellent resource of pollen and nectar. Another example is given by *Cicer arietinum* in Yaesu (top list species in September) or *Solidago canadensis* also in Kiba (top list species in September and October). No information regarding the two sites of Nishichiba and Kashiwanoha are available. It could be very interesting in the project of 2019 to collect more samples from months at the end of the year to be able to have more data to compare.

The comparative study between the years 2017 and 2018 also leads us to other interesting conclusions. For the two study sites, it appears that the number of species and the number of reads is much higher in 2018 than in 2017. For Kiba site, as mentioned above, *Solidago canadensis* is considered as an essential resource at the end of the pollen collection season. However, it should be noted that the exploitation of this species has decreased significantly between these two years. This leads us to conclude that bees have used other species with long flowering times to compensate for this food resource. In addition, this is confirmed by the fact that 24 new species are found in 2018 compared to 2017.

For Yaesu, the study shows that *Cicer aritenium* is only present in 2017 and not in 2018. In 2017, fewer species are using but with a very high number of reads identified, especially for *Malotus barbatus* and *Cicer arietinum* species. On the other hand, the following year, *Cicer arietinum* is absent from the species used by bees, and we supposed that this species was planted for food purposes and that the plantation was not renewed the following year. As a result of this absence, bees had to adapt their food resources differently and may have sought their food further away. Several facts correlate this hypothesis : (1) field analysis reveal that no transect indicates the presence of herbaceous plants on green spaces of the traffic lanes, and (2) through google earth analysis, very few other urban designs include green spaces near the hives but indicate a large green surface to the northwest of the hives. This resource area could be the new foraging sites sought by bees and could explain the substantial increase in herbaceous species observed in 2018 (especially clover species).

2. TRANSECTS

The following discussion is based on the list of species most used by bees, previously established in Table 4, and the list of species concretely identified through transects carried out in the field.

a. By flower family

As two other studies realized in northeastern Nigeria by Dukku (2014), and northern Egypt by Taha *et al.* (2017), the largest number of species identified by metabarcoding method was recorded in the same family, the *Fabaceae*. According to the referent document by Sasaki (2010), Erythrina crista-galli and three of *Trifolium* species are great sources of nectar and pollen for honeybees. Out of the 9 *Fabaceae* species, 8 are non-native species and one, *Styphnolobium japonicum* is a cultivar species (but not found in the field). Regarding the herbaceous species, several species were not found on the transects as *Trifolium incarnatum*, *Trifolium tomentosum*, *Melilotus officinalis* and *Cicer arietinum*. In Yaesu, only green roofs and parks are recorded on the spatial analysis. Thus, one hypothesis is that chickpea could be planted on green roofs for consumption purposes. The other three species, not found on the field, were exclusively identified as top species list in the frequency category; this fact may explain their non-identification in the field (frequent identification during the year but less abundantly).

Trifolium pratense and Trifolium repens are found in great abundance and very frequently in the four different sites according to metabarcoding method. Both of the species are a source of nectar and pollen for honeybees and can be found in spontaneous vegetation in urban areas as gaps or street verges in the field. Depending on the site, these two species can be found in the same green spaces as other herbaceous species such as: *Plantago lanceolata (Plantaginaceae)*, *Vicia villosa* (*Fabaceae*), *Lolium perenne* (*Poaceae), Orobranche pancicii* (*Orobranchaceae) or Solidago canadensis (Asteraceae).* All of the species are non-native species. To conclude, herbaceous floral species used by honeybees and found mainly in the traffic lanes are non-native species exclusively and very low in diversity (only 7 species).

It has to be noticed that the domination of alien grasses as *Lolium perenne* can be a source of hay fever and social issues (Koizumi 1998) as a study has shown in schools localized in the center of Tokyo (Parent group learning on grass pollinosis, 1999). In our research, it appears that Kashiwanoha is the site where the species has been recorded most often in field transects (6 transects out of 15). However, no information about the source of pollen and nectar has been found in the referent document (Sasaki 2010).

Concerning one of the most used families by honeybees, the *Asteraceae* family, only herbaceous species are found in the most commonly used species list based on metabarcoding method: *Artemisia argyi, Bidens Pilosa, Centaurea cyanus, Cirsium setidens, Cosmos sulphureus, Helianthus annuus, Solidago canadensis* and *Taraxacum obtusifrons*. Except for *Helianthus annuus* and *Centaurea cyanus* (cultivar species), all of these *Asteraceae* species are spontaneous alien species. Moreover, this family includes species that can be essential resources of pollen and nectar for bees (Table 12).
In this list, only *Solidago canadensis* (Kiba site) and *Taraxacum obtusifrons* (Kashiwanoha site) have been identified in the field which involves that other species are found in different foraging sites than the transects realized. By hypothesis, we can assume that the two cultivar species could be identified in agricultural areas. Indeed, both are in Nishichiba top list species and no transects have been sampled in agricultural transects. About the four other species (not found on the field et not cultivar species), we suppose to find them at different urban green spots such as private gardens or wastelands.

In the *Rosacea*e family, 6 species are identified as the most abundant or most frequently species used by bees according to metabarcoding method: 4 tree species (*Prunus grayana, Prunus pseudocerasus, Prunus szechuanica* and *Rhaphiolepsis indica)* and 2 herbaceous species *(Rosa hybrid* and *Rubus* columellaris).
At Nishichiba site, the two species of *Prunus* genus, *Rubus columellaris* and *Rhaphiolepis indica* were not identified in the field. However, thanks to the global analysis on the campus, it appears that 14 hedging plants of ornamental *Rhaphiolepis indica* species have been planted on Chiba university campus. This species is considered as a temporary exploited resource for pollen and nectar by honeybees and is a native species. For both *Prunus* species in Nishichiba site, a lack of precision in the reference document of the location of campus trees did not allow identification up to the species. However, since the species *Prunus pseudocerasus* is very abundant in pollen surveys of honeybees, it is easily assumed that most of the *Prunus* on campus belong to this species. Indeed, the main alleys of this campus are famous in the region during the flowering of *Sakura* (cherry trees' names in Japanese) in April and May. Finally, based on the previous results, *Rosa hybrida* species seems to be often used in private gardens around traffic lanes as well as ornamental plants within these traffic lanes. Besides, roses are present in 3 sites (no Yaesu site) and are a temporary exploited resource of manly pollen but also nectar for honeybees.
Other taxonomic families that are not very well represented but very important will be described.

Regarding the family of *Brassicaceae,* the two species of *Brassica napus* (rapeseed) and *Brassica carinata* were not found on the field. These two species are in the list of the most commonly used species in both sites of Kashiwanoha and Nishichiba and offer a flowering from the end of February to the end of May. The question remains as to the location of these two species in urban areas. Indeed, the sampling context of the traffic lanes did not allow the presence of these species to be highlighted. The hypotheses that can be raised are therefore as follows: the presence of species on different green urban designs (notably in the agricultural zone) or the presence of these spontaneous species in other non-sampled urban areas. Rapeseed plants have been introduced in Japan in the 19^{th} century throughout the country but *Brassica napus* farming stays very low; indeed, according to Nishizawa *et al.* (2010), "more than 99% of domestic demand is satisfied by imports". Rapeseed plants are used for many purposes as crops, animal feed and ornamental plants. However, new environmental concerns are arising regarding these species: spontaneous individuals form massive populations along riverbanks in Japan and disrupt natural ecosystems due to their rapid growth potential. It is therefore important to keep in mind that, despite their important pollen and nectar resource for bees, imported exotic species of *Brassicaceae* must remain controlled species to avoid any competition with natural ecosystems.

In the *Lythraceae,* there are also two tree and cultivar species identified in the top list species: *Lagerstroemia indica* and *Punica granatum*, found for the first one in great abundance in the field in different sites, as ornamental plantings in streets, and for the second one, found only on the campus of Nishichiba but also present in the list of Kashiwanoha site. The presence of *Punica granatum* (pomegranate tree) in the top list of the two campus sites suggests a cultivar purpose of the species but where only one individual was found on Nishichiba. Moreover, both of them are a source of pollen for honeybees (Table 12).

About *Verbenaceae*, 3 herbaceous species are found in the most commonly used species list: *Phyla canescens, Verbena hispida* and *Verbena incompta*. Phyla canescens, species native to South America, is an aggressive agricultural and environmental weed in many parts of the world (Gross 2010). Indeed, according to this same study, *Apis mellifera* "is the primary floral visitor and pollinator" of *Phyla canescens* and the abundance of honeybees' population is also positively correlated with the abundance of the floral species. Even if the list of designated IAS doesn't mention this species in Japan, It is important to notice that the relationship between *Apis mellifera* and *Phyla canescens* could have negative impacts on natural ecosystems as it already happens in Australian agricultural areas. In the field, this species has not been identified, either in Nishichiba or Kashiwanoha which suggests their presence either in agricultural areas (Gross 2010 in Australia) or as ornamental plantings in private gardens (used as an alternative to lawns (Pépinière filippi)). Moreover, no observation in the field and no information as regards of nectar or source state of the two *Verbena* species were recorded.

As mentioned in the results part, the two species of *Eucalyptus grandis* and *Eucalyptus vicina* in the *Myrtaceae* family are listed in the top list of Kiba site and present in very high abundance but were not identified in the field. The high abundance of this species found in pollen samples suggests that this species could be valued as eucalyptus wood products. However, no information can confirm this hypothesis. Finally, in the same family, the tree species of *Callistemon comboynensis,* also knew as Bottlebrush plant, was identified as common species used both in Kiba and Kashiwanoha sites by honeybees but were not recorded in the field either.

In the *Euphorbiaceae* family, two tree species *Mallotus barbatus* and *Triadica sebifera* are well represented in the field.

b. By pollen collection site

At Nishichiba site, out of the 13 tree species sought, only two species were found in the transects, but 6 other species can be added via the complete study located on the Nishichiba University Campus (Appendix 2). Based on this added analysis, transects sampling only city and campus traffic lanes have little species diversity. We can, therefore, find more diversity within the campus but outside the traffic lanes and especially in less maintained spaces, between buildings, or areas with ornamental species... Moreover, the species *Helianthus annuus* is found in very high abundance in collected samples from Nishichiba but was not identified in the field. The time analysis revealed that *Helianthus annuus* marker genes are only found in July in high quantity. Thus, the spatial scale (only in traffic lanes) and temporal context (fieldwork carried out in May) therefore probably explains the fact that the species has not been found in the field.

An example of a more industrialized site is Kiba site. In this place, tree species are more used than herbaceous species by honeybees which could be explained by the great resources of the *Eucalyptus* species as already mentioned but also by the non-presence of private gardens or parks in the close perimeter. Moreover, in several transects (24 and 36), the green spaces are arranged on large surfaces but do not contain any species useful to bees. One solution to diversify the pollen resources of domestic and wild bees would, therefore, be to reflect these areas in a more ecological dynamic and more attractive way to pollinating species.

A final remark was made along transect 34 where a small wasteland zone was observed but only one species was recorded (Figure 19, (e)). These areas could also be considered as a temporary potential biodiversity area.

On Kashiwanoha site, there are cultivar species (explained by the university horticultural context) and a certain omnipresence of *Lolium perenne* species. In addition, as at Kiba site, a large green area has been observed along transects 15 and 16, yet only 3 herbaceous species are present. These areas should be reconsidered as a source of biodiversity.

At Yaesu site, no herbaceous species were found on the 15 different transects while the phyloseq analysis indicated that more pollen collected from hives belonged to herbaceous species than to tree species. This could be explained by the fact that the only type of vegetation found in the study area is tree lines and some highly managed green areas. However, many herbaceous species are also found in the surveys, suggesting that bees will look for these species in other more distant green spaces, particularly in the gardens of the Imperial Palace to the north of the study area.

c. conclusion on the field surveys

To conclude this part, urban traffic lanes and its close dependency contribute partly as pollen and nectar resources for honeybees (24 species out of 60). Depending on the site, we found different principal species in the transects (S*olidago canadensis* for Kiba, *Lolium perenne* for Kashiwanoha). Moreover, all of the species identified in the field are not native species which leads to the conclusion that in urban traffic lanes non-native and cultivar species are promoted, to the detriment of native species.

To increase biodiversity and promote green space in urban japan, it could be interesting to favor more native species. However, it appears that native species are less exploited as pollen or nectar sources by honeybees than non-native species. Then, it seems appropriate to combine native with non-native species, but always in a reflection of diversity and not to promote one plant over another.

3. BARCODIND & TRANSECTS TECHNIQUES

The PCoA analysis allows us to affirm that DNA metabarcoding and transects realized in the field are not two complementary methods in order to identify the preferred vegetation communities of honeybees.

For the transect technique, it seems that the two sites of Kashiwanoha and Nishichiba are well explained by this technique and, at the opposite, Yaesu and Kiba sites are far away from the other sites and extend the ellipsoid. For the metabarcoding method, the majority of the sites are well explained by this technique, except for two sites, one from Yaesu and one from Kiba.

Firstly, we can conclude that very urbanized sites are not well explained by the field technique; this could be explained by the fact that species were recorded for the transect method only in the traffic lanes and in a small perimeter. Thus, transect method does not represent well the diversity of the pollen resources from honeybees, explained by two facts: (1) in Yaesu, no herbaceous species were found in the field while metabarcoding technique indicates that herbaceous species are highly exploited by honeybees, and (2) the downtown is composed in majority of green areas as parks, green roofs and private gardens, which were not sampled in this field study. Secondly, results observed in the suburbs are well explained by both DNA metabarcoding and transects techniques. Indeed, suburbs could be more explained by transect methodology because of the diversity present in the streets.

DNA metabarcoding is the best method for identification of resources used by honeybees. However, the localization information about the available species stays unknown. Transects method can be used for this purpose, as proposed in this study, but will not reveal the whole possibility of available resources. This technique, therefore, must be combined with DNA metabarcoding. In order to improve the study of honeybee's foraging ecology, new techniques are used, such as harmonic radar technique, referenced for bumblebees in Woodgate *et al.* study (2016), or decoding bee dances in the study of Steffan-Dewenter & Kuhn (2003).

4. COMPARISON WITH LITERATURE

In the majority of investigated studies, bee populations (wild and domesticated) prefer to forage native plants than exotic ones (Morandin *et al.* 2013; Salisbury *et al.* 2015; De Vere *et al.* 2017; Memmott & Waser 2002). Salisbury *et al.* (2015) affirmed the fact that gardens would provide more resources by planting both of native species and near-native species, extending the flowering season and potentially providing resources for specialist pollinators groups. Indeed, only native species from one single region are not optimal resources for pollinators. Outside the urban area context, the conservation of native plants is essential to participate in the conservation of native bees in intensive agricultural landscapes (Morandin *et al.* 2013). Sensitizing to the conservation of native plants contributes then to the conservation of native bees and increase the biodiversity.

However, exotic species provide also pollen and nectar resources, but mostly for generalist pollinators (Memmott & Waser 2002) and so, for managed honeybees. Note that alien species must be carefully selected.

This study therefore provides new information about pollen resources used by honeybees in urban areas and concludes that the majority of the species used by these individuals are exotic ones. But do they use more exotic species in cities because the available resources are mainly composed of exotic species? To complete this study, it would be interesting to identify all plant species in the study area and compare the ratios of species used to the total number of available species (already carried out in De Vere *et al. 2017*'s study in the National Botanic Garden of Wales). In addition, the field study shows us that the road network consists mainly of exotic species, and that most of them are used for ornamental purposes. What about other urban designs?

5. LIMITS

The study is very representative of the expected urban environment, given the context of a large metropolitan area and increasing urbanization around the world. Moreover, the 4 apiaries are very different from each other and therefore reflect the different urban areas that can be found in Tokyo (downtown and suburbs, very industrialized...). In addition, a large amount of data has been collected over the past two years, which makes it possible to provide several quality points of view.

However, several drawbacks induce certain limits of the study. Firstly, during data collection, samples of the different hives are grouped together. Some data is therefore lost during this process. It would have been interesting to compare beehives present on the same site in order to assess the strength of each colony (brood combs, stored food, and adult bee population) (Taha *et al.* 2017).

Secondly, during the first step of data analysis, a cut-off at 97% of identity and a number of reads at less than 10 had been determined, thus deleting 58% of the data. In the case of bacteria, a cut-off of 97% has been identified as the concordance threshold between the element to be identified and the reference element, allowing the identification of the bacterium. If this threshold is not reached, the unknown specimen belongs to a different species (Stackebrandt and Goebel 1994). However, in the case of plants, according to Chen *et al.* (2010), several marker genes are possible, as explained above, and different cut-off success can be considered. This choice is still at the user's decision.

Still in metabarcoding techniques, as mentioned several times in this study, too much contradictory information in the literature do not allow us to draw firm conclusions about fluctuations in the abundance of pollen collected by bees. Since this information is very important in this type of study, it would be very interesting to provide new data during pollen surveys, such as the weight collected from samples by hive, date and place (Taha *et al.* 2017). This would allow us to draw real conclusions about bee activity during the year. On the other hand, specific abundances of the species are still unknown. Very recent techniques are carried out to address this drawback (Peel *et al.* 2019).

Concerning the temporality of pollen collection, little data has been provided to conclude that exotic species are more exploited than native ones at the end of the flowering season. It would therefore be really interesting to have more samples at the beginning of the flower season and, above all, at the end of the season, in September and October, at all sites. Thus, the conclusions drawn in this study could be continued, particularly in the two sites in peri-urban areas where no data are recorded.

About the fieldwork carried out, several remarks can be made. Firstly, the work done in the field does not correspond to the spatial scale of honeybees. Indeed, bees browsing on average 2.2 km (and more if necessary) while fieldwork method records only 500 meters, a small part of the floral species available for bees. In addition, at the time scale, only one survey was carried out per site, at different dates for each site. In conclusion, this study is therefore spatially limited and at the level of recognizable flowering of floral species. Moreover, as mentioned several times, only traffic lanes have been sampled, which limits considerably the opportunity to localize all of the floral species used by honeybees (including private gardens, green roofs, agricultural lands and wastelands). In Nishichiba, beehives are located on a green roof and bees have access to pollen and nectar resources directly near the hive. This feature was not taken into account in the field study. It would therefore be interesting to incorporate floral species chosen on Nishichiba green roof in order to study the impact of distances made by bees if there is a direct available food resource. In addition, 3 of the 4 apiaries have not been visited.

CONCLUSION

The four different sites studied have a different vegetation diversity that is specific to each site and each urban context.

As for the question of the use of exotic species by bees, it appears that only 25% of the species recorded are native to Japan. In particular, the proportion of spontaneous non-native species used in large quantities by bees includes species such as *Trifolium pratense* and *Trifolium repens*, a source of nectar and pollen over more than 5 months. In cultivar species category, there are chronologically important species used by honeybees such as *Prunus pseudocerasus*, *Actinidia chinensis*, *Centaurea cyanus*, *Dichroa febrifuga*, *Helianthus annuus* or *Lagerstroemia indica.*

Should some species be promoted more than others? We have seen that in the case of the most urbanized site, Yaesu, it appears that the urban context does not allow bees to benefit from a great source of pollen in traffic lanes. Indeed, as suggested by Geslin *et al.* (2000), the majority of ornamental plants in urban areas are poorly or totally unattractive to pollinators. In this context, it would be preferable to favor certain plants, and specifically herbaceous species. Indeed, many flower beds are initiated in cities but are not sources of pollen. Some greening projects should, therefore, consider pollinators' needs.

For generalist pollinators as *Apis mellifera,* alien species represent a food alternative, especially at the end of the year, after the flowering of most native species. Indeed, some species are used by bee populations only from the end of August, such as *Eucalyptus vicina* in Kiba and *Solidago canadensis* or *Cicer arietinum* in Yaesu.

It is very important to note that these movements of initiatives, to promote green spaces through urban beekeeping, must also take into account the interactions of specialist pollinators. Indeed, we now know that it is crucial to maintain a diversity of pollinators to preserve our sustainable ecosystems, but also to ensure our food security and our daily well-being (Potts *et al.* 2016 (2)). A diversity of pollinators implies a diversity of plants and thus helps to limit the potential negative effects of the introduction of alien species (Traveset & Richardson 2014).

Regarding the contribution of the Japanese urban road network and its close dependence (private gardens in front of houses) in the diversity of floral resources useful for bees, the study shows that 24 out of 60 top list species have been identified in the field, so less than half of the species sought. Depending on the site, most species have been identified at the foot of trees, on roadsides, on more or less managed lawns, between buildings etc. However, surveys of private (but observable) gardens have been very poor (unique presence of *Rosa hybrid* species). It can be concluded that in front of urban Japanese gardens, very few species participate in the pollen and nectar resources of bees.

Other interesting studies would be to sample other urban designs such as private gardens. It would also be interesting to increase the field area of study given the large surface of action of honeybees, or to carry out sampling at several periods during the year to be able to locate certain species with shorter flowering times.

In 2019, 40 new sites will be added to Tokyo, Chiba and Toronto (Canada) to improve data quality. Moreover, another study with the collaboration of the two universities of Chiba and Gembloux Agro-Bio Tech will be carried out as part of a master thesis.

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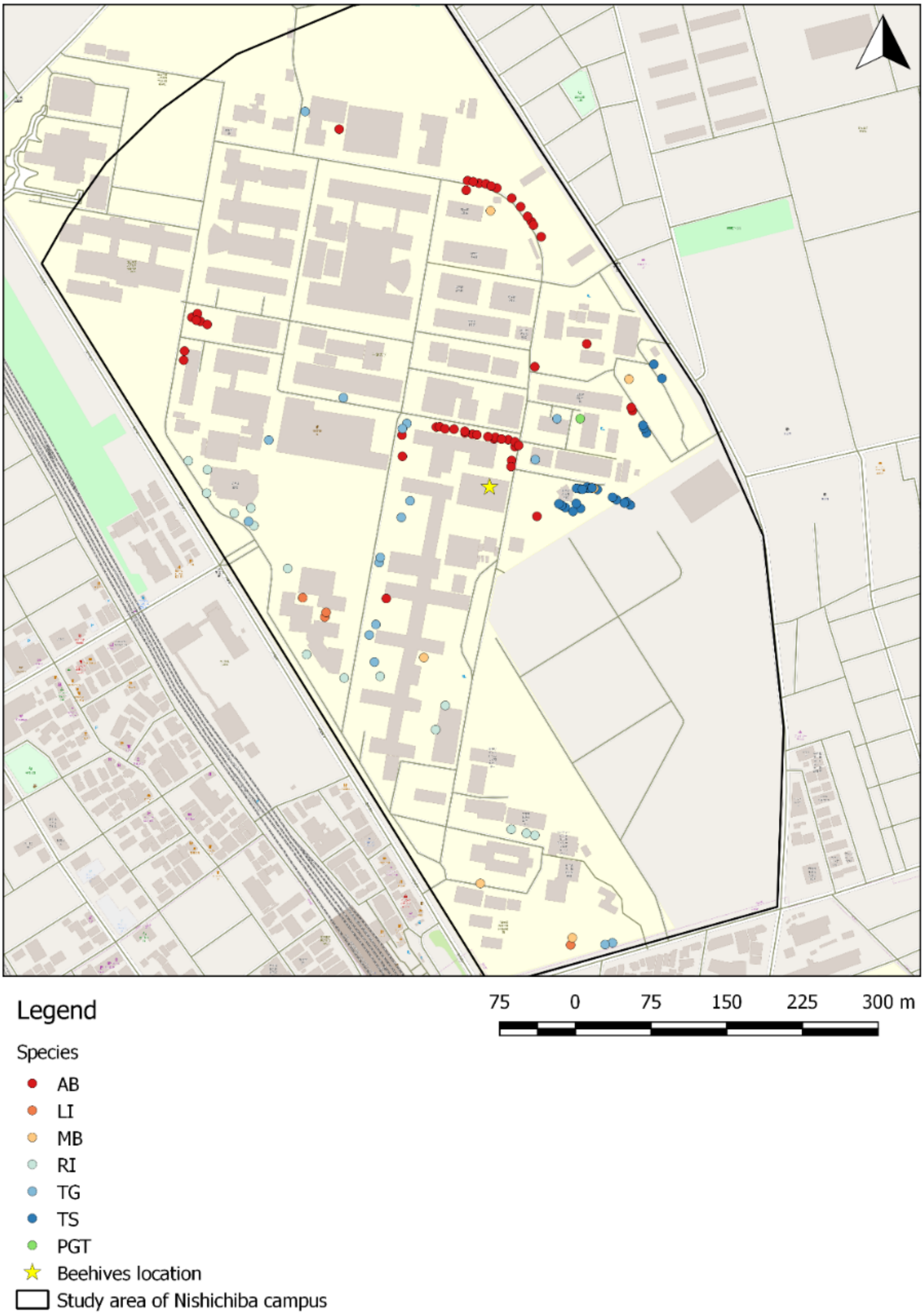
APPENDIX

Appendix 1: Native (N), non-native (NO) and cultivar (CU) state of each species. The difference between non-native and naturalized species is not considered for the analysis (both are regrouped in the “non-native” category).

Species	NO/N/CU	Species	NO/N/CU	Species	NO/N/CU
Acalypha_australis	N	Daphniphyllum_oldhamii	NO	Phyla_canescens	NO
Acca_sellowiana	CU	Daphniphyllum_paxianum	NO	Phyla_nodiflora	N
Acer_albopurpurascens	NO	Datura_stramonium	NO	Phytolacca_americana	NO
Acer_buergerianum	CU	Daucus_carota	NO	Picris_hieracioides	NO
Acer_caesium	NO	Dendropanax_morbifer	NO	Piper_kadsura	N
Acer_campbellii	NO	Desmodium_tortuosum	NO	Pittosporum_fairchildii	NO
Acer_davidii	NO	Deutzia_x	NO	Pittosporum_glabratum	NO
Acer_erianthum	NO	Dichroa_febrifuga	CU	Pittosporum_undulatum	CU
Acer_negundo	CU	Diospyros_kaki	NO	Plantago_asiatica	NO
Acer_nigrum	NO	Duchesnea_chrysantha	N	Plantago_lanceolata	NO
Acer_obtusifolium	NO	Echinops_sp.	NO	Plantago_sparsiflora	NO
Acer_oliverianum	NO	Elaeagnus_oldhamii	NO	Platanus_orientalis	CU
Acer_palmatum	N	Elaeocarpus_sylvestris	N	Plectocomiopsis_geminiflora	NO
Acer_pseudoplatanus	CU	Eleusine_coracana	CU	Poa_pratensis	NO
Acer_pycnanthum	N	Eragrostis_ferruginea	N	Poaceae_sp.	NO
Acer_tataricum	NO	Erigeron_annuus	NO	Portulaca_grandiflora	CU
Acer_tschonoskii	N	Erigeron_morrisonensis	NO	Pourouma_bicolor	NO
Acer_x	NO	Erigeron_sumatrensis	NO	Primula_duclouxii	NO
Acnistus_arborescens	NO	Eriobotrya_japonica	NO	Prunus_conradinae	NO
Actinidia_arguta	N	Erythrina_crista-galli	NO	Prunus_grayana	N
Actinidia_callosa	NO	Erythrina_fusca	NO	Prunus_lusitanica	NO
Actinidia_chinensis	CU	Erythrina_vespertilio	NO	Prunus_pseudocerasus	CU
Actinidia_deliciosa	CU	Eucalyptus_grandis	NO	Prunus_szechuanica	NO
Actinidia_rufa	N	Eucalyptus_quadrangulata	NO	Pueraria_candollei	NO
Actinidia_sp.	NO	Eucalyptus_siderophloia	NO	Pueraria_montana	N
Adinandra_millettii	NO	Eucalyptus_vicina	NO	Pulmonaria_filarszkyana	NO
Aesculus_hippocastanum	CU	Euphorbia_maculata	NO	Punica_granatum	CU
Aesculus_x	NO	Euphorbia_nutans	NO	Quercus_coccifera	NO
Allium_fistulosum	CU	Eurya_acuminatissima	NO	Quercus_dentata	N
Allium_giganteum	N	Eurya_chinensis	NO	Quercus_dilatata	NO
Allium_tuberosum	NO	Fagus_japonica	N	Quercus_frainetto	NO
Alnus_maximowiczii	N	Fatoua_villosa	N	Quercus_hartwissiana	NO
Ambrosia_artemisiifolia	NO	Firmiana_platanifolia	N	Quercus_ilex	NO
Ambrosia_trifida	NO	Foeniculum_vulgare	CU	Quercus_infectoria	NO
Amorpha_nana	NO	Fraxinus_chinensis	NO	Quercus_ithaburensis	NO
Ampelopsis_japonica	CU	Gaillardia_pulchella	NO	Quercus_macranthera	NO
Anethum_graveolens	CU	Gaura_lindheimeri	NO	Quercus_petraea	NO
Anisodontea_malvastroides	NO	Gentiana_scabra	CU	Quercus_pontica	NO
Antirrhinum_majus	CU	Gladiolus_hybrid	CU	Quercus_pubescens	NO
Aralia_elata	N	Gladiolus_palustris	NO	Quercus_pyrenaica	NO
Archontophoenix_cunninghamiana	NO	Glebionis_coronaria	CU	Quercus_suber	CU
Ardisia_crenata	N	Glycine_max	CU	Quercus_trojana	NO
Artemisia_argyi	NO	Gomphrena_sonorae	NO	Raphanus_sativus	CU
Asparagus_falcatus	NO	Gossypium_hirsutum	CU	Rhaphiolepis_indica	N
Asparagus_oligoclonos	N	Hedera_helix	CU	Rhaponticum_uniflorum	NO
Aster_indicus	N	Helenium_autumnale	CU	Rhododendron_nakaharae	NO
Astragalus_sinicus	NO	Helianthus_annuus	CU	Rhus_chinensis	N
Atriplex_australasica	NO	Helleborus_orientalis	CU	Robinia_pseudoacacia	NO
Begonia_foliosa	NO	Hibiscus_syriacus	NO	Rorippa_indica	N
Begonia_sp.	NO	Houttuynia_cordata	N	Rosa_hybrid	NO
Betula_pumila	NO	Hovenia_acerba	NO	Rubus_cf.	NO
Bidens_frondosa	NO	Humulus_lupulus	CU	Rubus_columellaris	NO
Bidens_pilosa	NO	Hypericum_acmosepalum	NO	Rudbeckia_hirta	NO
Boehmeria_nivea	N	Hypericum_calycinum	CU	Rytidosperma_aff.	NO
Borago_officinalis	CU	Hypericum_humifusum	NO	Rytidosperma_sp.	NO
Brandzeia_filicifolia	NO	Hypericum_lancasteri	NO	Saccharomyces_paradoxus	NO

Brassica_carinata	NO	Hypericum_pseudohenryi	NO	Sagina_procumbens	NO
Brassica_napus	NO	Hypochaeris_radicata	NO	Salacca_affinis	NO
Brassica_oleracea	NO	Idesia_polycarpa	N	Salvia_rosmarinus	NO
Briza_minor	NO	Ilex_cornuta	CU	Sapindus_mukorossi	N
Bussea_sakalava	NO	Ilex_crenata	N	Schefflera_heptaphylla	N
Bussea_sakalava	NO	Ilex_latifolia	N	Scilla_scilloides	N
Callicarpa_dichotoma	N	Ilex_mucronulata	NO	Sedum_bulbiferum	N
Callicarpa_kochiana	N	Ilex_rotunda	N	Sedum_lineare	NO
Callistemon_comboynensis	NO	Indigofera_heterantha	NO	Sedum_pallidum	NO
Calystegia_pulchra	NO	Jasminum_didymum	NO	Serissa_japonica	N
Camellia_japonica	N	Kerriodoxa_elegans	NO	Sherardia_arvensis	NO
Camellia_oleifera	NO	Lactuca_indica	N	Sicyos_davilae	NO
Camellia_reticulata	CU	Lagenaria_siceraria	NO	Sloanea_sinensis	NO
Camellia_szechuanensis	NO	Lagerstroemia_indica	CU	Solanum_lyratum	N
Carpinus_fangiana	NO	Lantana_sp.	NO	Solanum_physalifolium	NO
Carpinus_laxiflora	N	Leucanthemum_vulgare	NO	Solidago_canadensis	NO
Castanopsis_calathiformis	NO	Ligustrum_lucidum	CU	Solidago_houghtonii	NO
Castanopsis_fargesii	NO	Lilium_tsingtauense	NO	Sonchus_asper	NO
Castanopsis_fissa	NO	Linaria_fastigiata	NO	Sorghum_halepense	NO
Castanopsis_sp.	NO	Liquidambar_styraciflua	CU	Spiraea_chamaedryfolia	NO
Celastrus_gemmatus	NO	Liriodendron_tulipifera	CU	Spiraea_chartacea	NO
Celastrus_scandens	N	Lithocarpus_skanianus	NO	Spiraea_japonica	N
Celastrus_sp.	NO	Lithospermum_erythrorhizon	N	Spiranthes_sinensis	N
Celtis_sinensis	N	Lolium_perenne	NO	Stewartia_pseudocamellia	N
Centaurea_cyanus	CU	Lunaria_annua	NO	Stewartia_sinensis	N
Cerastium_glomeratum	NO	Lythrum_salicaria	N	Styphnolobium_japonicum	CU
Chelidonium_majus	N	Maackia_amurensis	N	Styrax_confusus	NO
Chenopodium_ficifolium	NO	Maclura_pubescens	NO	Styrax_grandiflorus	NO
Chenopodium_ficifolium	NO	Magnolia_sp.	NO	Swida_controversa	N
Chenopodium_serotinum	NO	Mallotus_barbatus	NO	Symphytum_x	NO
Cirsium_setidens	NO	Matthiola_longipetala	CU	Symplocos_paniculata	N
Citrus_aurantiifolia	NO	Medicago_sativa	NO	Syringa_josikaea	NO
Citrus_maxima	CU	Melampodium_paludosum	N	Tanacetum_coccineum	N
Citrus_sinensis	CU	Melilotus_officinalis	NO	Taraxacum_obtusifrons	NO
Clematis_apiifolia	N	Melissa_officinalis	NO	Tarenaya_hassleriana	NO
Clematis_brachyura	NO	Mentha_spicata	NO	Ternstroemia_gymnanthera	N
Clematis_fasciculiflora	NO	Miscanthus_floridulus	N	Ternstroemia_microphylla	NO
Clematis_fusca	N	Momordica_charantia	CU	Toxicodendron_succedaneum	N
Clematis_terniflora	N	Myrtus_communis	N	Toxicodendron_sylvestre	N
Clematis_texensis	NO	Nelumbo_nucifera	NO	Toxicodendron_wallichii	NO
Clerodendrum_trichotomum	N	Nelumbo_pentapetala	NO	Trachycarpus_martianus	NO
Coleogyne_ramosissima	NO	Nelumbo_sp.	NO	Triadica_sebifera	NO
Commelina_communis	N	Neviusia_cliftonii	NO	Trichosanthes_pilosa	N
Convolvulus_lanatus	NO	Nuphar_variegata	NO	Trifolium_incarnatum	NO
Conyza_canadensis	N	Oenothera_heterophylla	NO	Trifolium_pratense	NO
Coreopsis_grandiflora	CU	Oenothera_rosea	NO	Trifolium_repens	NO
Coriandrum_sativum	NO	Oenothera_speciosa	NO	Trifolium_strictum	NO
Cornus_disciflora	NO	Orlaya_daucoides	NO	Trifolium_tomentosum	NO
Cornus_florida	CU	Orobanche_pancicii	NO	Tristagma_sp.	NO
Cornus_hongkongensis	N	Oryza_meridionalis	NO	Verbena_hispida	NO
Cornus_macrophylla	N	Oxalis_articulata	NO	Verbena_incompta	NO
Cortaderia_araucana	NO	Paederia_foetida	N	Veronica_agrestis	NO
Cortaderia_jubata	NO	Paeonia_obovata	N	Veronica_persica	NO
Cortaderia_rudiuscula	NO	Paeonia_suffruticosa	N	Viburnum_awabuki	N
Corydalis_aurea	NO	Papaver_bracteatum	NO	Viburnum_plicatum	CU
Cosmos_bipinnatus	NO	Papaver_nudicaule	CU	Vicia_kurdica	NO
Cosmos_sulphureus	NO	Papaver_rhoeas	NO	Vicia_villosa	NO
Cucumis_sativus	CU	Papaver_somniferum	CU	Zanthoxylum_piperitum	N
Cuscuta_campestris	NO	Parthenocissus_himalayana	NO	Zanthoxylum_schinifolium	N
Cuscuta_chinensis	N	Paspalum_dilatatum	NO	Zinnia_angustifolia	NO
Cydonia_oblonga	CU	Pentas_lanceolata	NO	Zinnia_haageana	NO
Dactyliandra_welwitschii	NO	Petunia_axillaris	CU	Zoysia_japonica	N

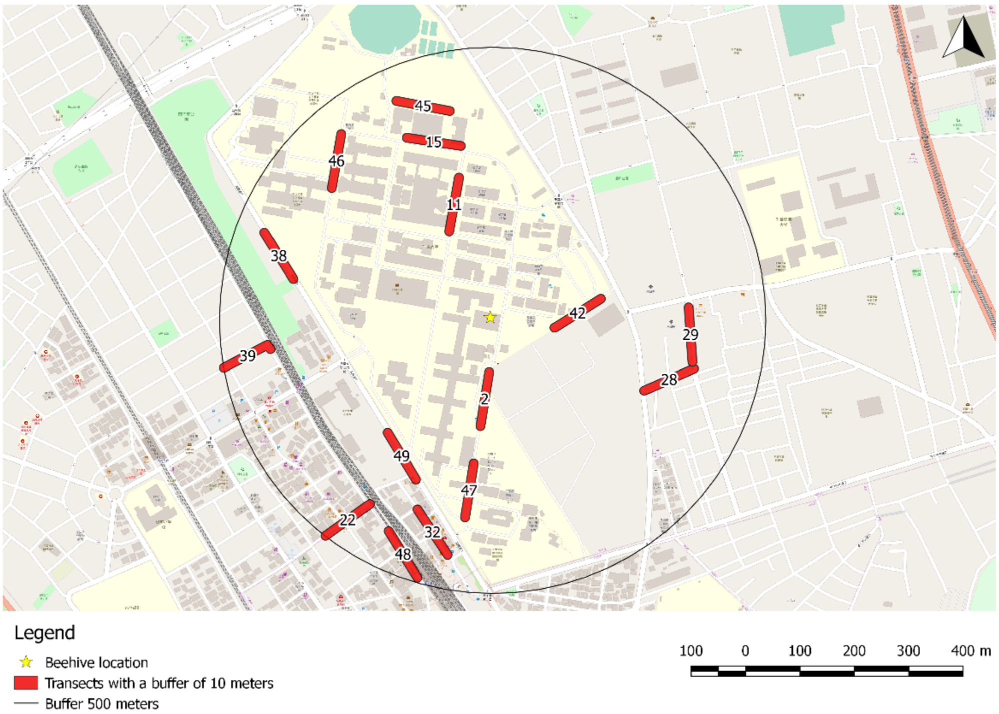
Appendix 2 : Nishichiba campus map of seven tree top species, Acer buergerianum (AB), Lagertroemia lancasteri (LI), Mallotus barbatus (MB), Rhaphiolepsis indica (RI), Ternstroemia gymanthera (TG), Triadica sebifera (TS), Punica granatum (PGT).



Appendix 3: Top list species from March to October in 2017 and 2018 for the four sites. The Sum list is the sum of the DNA number of reads for all of the samples per month and per species. The frequency list is calculated by summing the number of times the species appears in total on all sites per month. The number of samples used in this analysis is different for each month: March (1), April (6), May (13), June (13), July (13), August (12), September (7), October (4).

March Sum	March Frequency	April Sum	April Frequency	May Sum	May Frequency	June Sum	June Frequency
Prunus pseudocerasus	/	*Rosa hybrid*	*Rosa hybrid*	*Centaurea cyanus*	*Trifolium repens*	*Trifolium repens*	*Trifolium repens*
Prunus szechuanica		*Actinidia chinensis*	*Brassica napus*	*Mallotus barbatus*	*Trifolium pratense*	*Mallotus barbatus*	*Mallotus barbatus*
Brassica napus		*Acer tataricum*	*Acer palmatum*	*Trifolium pratense*	*Centaurea cyanus*	*Dichroa febrifuga*	*Trifolium pratense*
Carpinus laxiflora		*Pittosporum glabratum*	*Trifolium repens*	*Trifolium repens*	*Rosa hybrid*	*Trifolium pratense*	*Erythrina crista-galli*
Eurya chinensis		*Prunus grayana*	*Prunus pseudocerasus*	*Actinidia deliciosa*	*Plantago lanceolata*	*Erythrina crista-galli*	*Dichroa febrifuga*
Primula duclouxii		*Vicia villosa*	*Actinidia chinensis*	*Callistemon comboynensis*	*Lolium perenne*	*Punica granatum*	*Plantago lanceolata*
Salvia rosmarinus		*Taraxacum obtusifrons*	*Acer tataricum*	*Rosa hybrid*	*Ligustrum lucidum*	*Plantago lanceolata*	*Ligustrum lucidum*
Helleborus orientalis		*Brassica napus*	*Pittosporum glabratum*	*Cicer arietinum*	*Callistemon comboynensis*	*Ternstroemia gymnanthera*	*Hypericum lancasteri*
Artemisia argyi		*Acer palmatum*	*Prunus grayana*	*Brassica napus*	*Mallotus barbatus*	*Schefflera heptaphylla*	*Triadica sebifera*
Trifolium pratense		*Acer buergerianum*	*Vicia villosa*	*Styrax grandiflorus*	*Brassica napus*	*Ligustrum lucidum*	*Plantago asiatica*
July Sum	**July Frequency**	**August Sum**	**August Frequency**	**September Sum**	**September Frequency**	**October Sum**	**October Frequency**
Trifolium pratense	*Trifolium pratense*	*Lagerstroemia indica*	*Lagerstroemia indica*	*Eucalyptus vicina*	*Lagerstroemia indica*	*Eucalyptus vicina*	*Eucalyptus vicina*
Trifolium repens	*Trifolium repens*	*Trifolium pratense*	*Trifolium repens*	*Cicer arietinum*	*Verbena hispida*	*Lagerstroemia indica*	*Eucalyptus grandis*
Triadica sebifera	*Plantago lanceolata*	*Trifolium repens*	*Lagerstroemia indica*	*Solidago canadensis*	*Eucalyptus vicina*	*Eucalyptus grandis*	*Solidago canadensis*
Helianthus annuus	*Lagerstroemia indica*	*Eucalyptus vicina*	*Trifolium pratense*	*Lagerstroemia indica*	*Eucalyptus grandis*	*Solidago canadensis*	*Bidens pilosa*
Dendropanax morbifer	*Phyla canescens*	*Erythrina crista-galli*	*Plantago lanceolata*	*Eucalyptus grandis*	*Artemisia argyi*	*Bidens pilosa*	*Solanum physalifolium*
Lagerstroemia indica	*Aralia elata*	*Lantana sp.*	*Helianthus annuus*	*Cosmos sulphureus*	*Clematis terniflora*	*Callistemon comboynensis*	*Lagerstroemia indica*
Aralia elata	*Triadica sebifera*	*Plantago lanceolata*	*Verbena hispida*	*Artemisia argyi*	*Allium tuberosum*	*Eriobotrya japonica*	*Callistemon comboynensis*
Plantago lanceolata	*Dendropanax morbifer*	*Eucalyptus grandis*	*Styphnolobium japonicum*	*Rhus chinensis*	*Pueraria montana*	*Solanum physalifolium*	*Eriobotrya japonica*
Nelumbo nucifera	*Cirsium setidens*	*Rhus chinensis*	*Melilotus officinalis*	*Plantago asiatica*	*Glycine max*	*Lagerstroemia indica*	*Lagerstroemia indica*
Orobanche pancicii	*Cucumis sativus*	*Verbena hispida*	*Verbena incompta*	*Embryo_environmental*	*Cicer arietinum*	*Bidens pilosa*	*Bidens pilosa*

Appendix 4: Nishichiba site. Map and table of the transect divided into herbaceous and tree species. The column A/F (Abundance/Frequency) gives additional information about the species' membership in the top list in the abundance category only (A), frequency only (F) or both abundance and frequency (AF). This information is listed in table 4. Moreover, if the species is present in the transect, an abundance information has been reported: category "a" for a number of 1 to 10 included individuals of the same species on the transect; "b" for 11 to 50 included species and "c" for more than 50 individuals on the transect. Tree species are counted individually if possible. Otherwise, a cross is noted in the cell.



Species	A/F	38	11	15	32	46	29	39	28	47	48	42	49	22	45	2
Herbaceous species																
Trifolium pratense	*AF*		c	a		a				c					a	b
Trifolium repens	*AF*														b	a
Centaurea cyanus	*A*															
Helianthus annuus	*AF*															
Rosa hybrid	*A*						a	a	a					a		
Vicia villosa	*A*		c	c		c				c		c			c	a
Brassica napus	*AF*															
Trifolium incarnatum	*A*															
Clematis terniflora	*A*				x					x					x	x
Rubus columellaris	*A*															
Trifolium tomentosum	*F*															
Allium fistulosum	*F*															
Plantago lanceolata	*F*		c			a				a					c	b
Phyla canescens	*F*															
Lolium perenne	*F*								c							
Oenothera rosea	*F*	a				b	a			a						
Tree species																
Prunus pseudocerasus	*A*															
Mallotus barbatus	*AF*															
Triadica sebifera	*AF*											5				
Dichroa febrifuga	*AF*									8	5	9	1	5		10
Erythrina crista-galli	*AF*															
Ternstroemia gymnanthera	*A*															
Hypericum lancasteri	*AF*															
Prunus szechuanica	*A*															
Acer buergerianum	*AF*															
Lagerstroemia indica	*AF*															
Ligustrum lucidum	*F*															
Punica granatum	*F*															
Rhaphiolepis indica	*F*															

Appendix 5: Kiba site. Map and table of the transect divided into herbaceous and tree species. The column A/F (Abundance/Frequency) gives additional information about the species' membership in the top list in the abundance category only (A), frequency only (F) or both abundance and frequency (AF). This information is listed in table 4. Moreover, if the species is present in the transect, an abundance information has been reported: category "a" for a number of 1 to 10 included individuals of the same species on the transect; "b" for 11 to 50 included species and "c" for more than 50 individuals on the transect. Tree species are counted individually if possible. Otherwise, a cross is noted in the cell.

Legend

☆ Beehive location

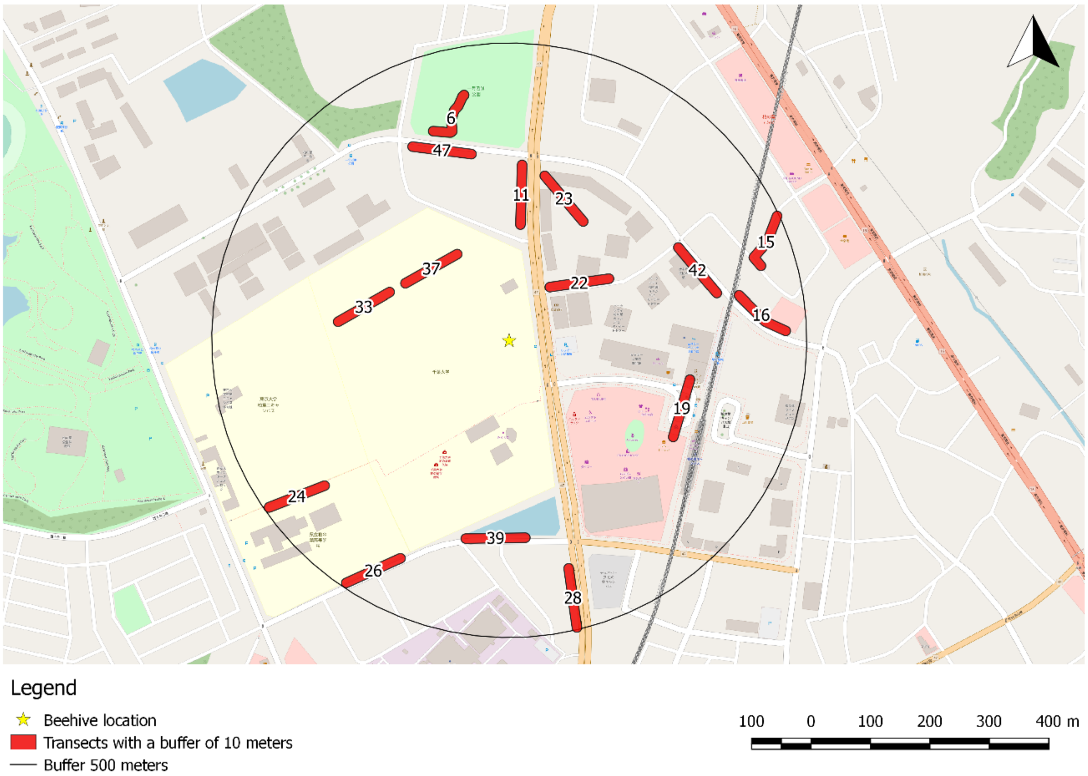
Transects with a buffer of 10 meters

Buffer 500 meters

100 0 100 200 300 400 m

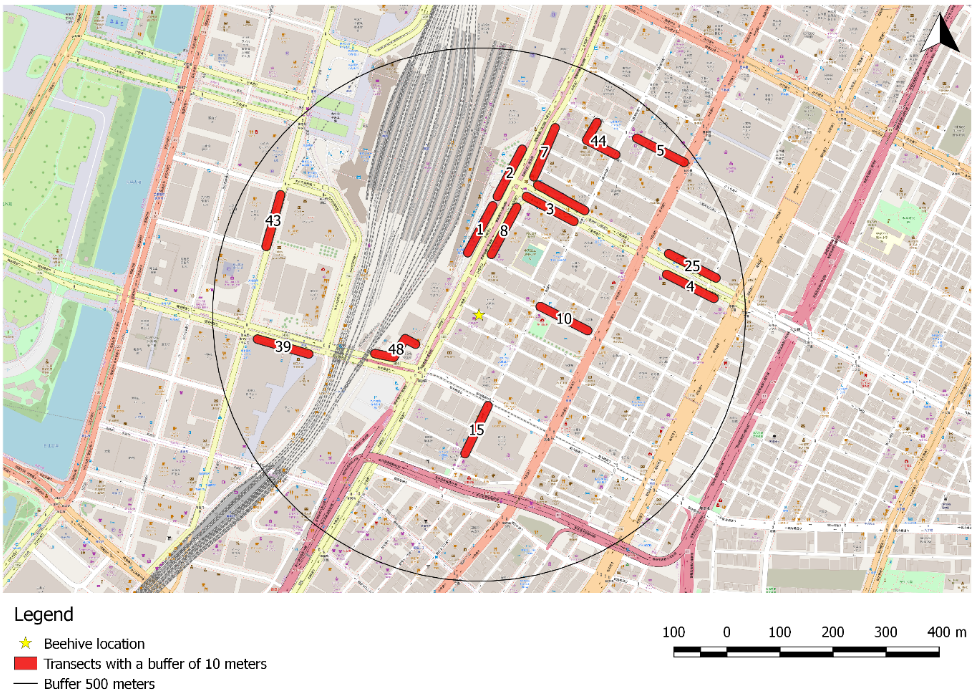
Species	A/F	2	6	12	17	19	20	24	28	31	33	34	36	43	44	45
Herbaceous species																
Trifolium pratense	*AF*	c				c	c									
Trifolium repens	*AF*															
Rosa hybrid	*AF*													a	a	
Solidago canadensis	*AF*	c				c	c		c	c	a	c		c	c	b
Verbena hispida	*AF*															
Orobanche pancicii	*AF*	a				b	b								b	
Ampelopsis japonica	*AF*															
Verbena incompta	*AF*															
Bidens pilosa	*AF*															
Plantago lanceolata	*AF*	c	a			c	c									
Melilotus officinalis	*F*															
Tree species																
Eucalyptus vicina	*AF*															
Lagerstroemia indica	*AF*		1		6	2			4					1	9	4
Mallotus barbatus	*AF*															
Eucalyptus grandis	*AF*															
Erythrina crista-galli	*AF*															
Callistemon comboynensis	*AF*															
Pittosporum glabratum	*AF*					1	2									
Rhus chinensis	*AF*														2	
Triadica sebifera	*AF*						1									
Dendropanax morbifer	*AF*						7		13							

Appendix 6: Kashiwanoha site. Map and table of the transect divided into herbaceous and tree species. The column A/F (Abundance/Frequency) gives additional information about the species' membership in the top list in the abundance category only (A), frequency only (F) or both abundance and frequency (AF). This information is listed in table 4. Moreover, if the species is present in the transect, an abundance information has been reported: category "a" for a number of 1 to 10 included individuals of the same species on the transect; "b" for 11 to 50 included species and "c" for more than 50 individuals on the transect. Tree species are counted individually if possible. Otherwise, a cross is noted in the cell.



Species	A/F	11	23	33	16	6	22	28	15	37	47	39	19	42	24	26
Herbaceous species																
Trifolium pratense	AF				c			c	c							
Trifolium repens	AF			b	a				c	a		c			c	
Centaurea cyanus	AF															
Brassica napus	AF															
Rosa hybrid	AF															
Brassica carinata	A															
Taraxacum obtusifrons	AF														a	a
Plantago lanceolata	AF											a			c	
Cirsium setidens	AF															
Vicia villosa	A														c	
Phyla canescens	F															
Lolium perenne	F	a			c			c	c			c				c
Tree species																
Lagerstroemia indica	AF															
Dichroa febrifuga	AF		2													
Actinidia deliciosa	AF	x									x					
Mallotus barbatus	AF					1										
Actinidia chinensis	AF	x									x					
Dendropanax morbifer	AF															
Punica granatum	AF															
Triadica sebifera	A															
Prunus grayana	A															
Callistemon comboynensis	A															
Ligustrum lucidum	F		x											x		
Citrus sinensis	F															
Aralia elata	F															

Appendix 7: Yaesu site. Map and table of the transect divided into herbaceous and tree species. The column A/F (Abundance/Frequency) gives additional information about the species' membership in the top list in the abundance category only (A), frequency only (F) or both abundance and frequency (AF). This information is listed in table 4. Moreover, if the species is present in the transect, an abundance information has been reported: category "a" for a number of 1 to 10 included individuals of the same species on the transect; "b" for 11 to 50 included species and "c" for more than 50 individuals on the transect. Tree species are counted individually if possible. Otherwise, a cross is noted in the cell.



Species	A/F	4	15	2	48	10	3	28	39	8	5	43	7	25	1	44
Tree species																
Mallotus barbatus	*AF*															
Lagerstroemia indica	*AF*		1													
Acer buergerianum	*AF*															
Schefflera heptaphylla	*AF*															
Aralia elata	*A*															
Ternstroemia gymnanthera	*AF*								1		1					
Styrax grandiflorus	*A*															
Stewartia sinensis	*A*															
Castanopsis fargesii	*AF*															
Hypericum lancasteri	*AF*	x	x	x		x									x	
Styphnolobium japonicum	*F*															
Ligustrum lucidum	*F*															
Erythrina crista-galli	*F*															
Herbaceous species																
Trifolium repens	*AF*															
Trifolium pratense	*AF*															
Cicer arietinum	*AF*															
Plantago lanceolata	*AF*															
Plantago asiatica	*AF*															
Cosmos sulphureus	*A*															
Nelumbo nucifera	*AF*															
Artemisia argyi	*A*															
Oenothera rosea	*AF*															
Helianthus annuus	*AF*															
Lolium perenne	*F*															
Verbena hispida	*F*															