# Functional structure of foraged plants by the honeybee (Apis mellifera L.) through pollen metabarcoding in Japan

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**In collaboration with Chiba University (Japan)**

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# Abstract

Biodiversity is declining world-widely and this decrease also impacts pollinators. The causes are multiples and one of them is due to urbanisation. In a context of a rapidly growing urbanisation around the world, an understanding and management of the biodiversity is crucial. Therefore, *Apis mellifera* is known to be one of the major pollinators and is globally distributed on the globe. To address these challenges, it is necessary to acknowledge the diet of honeybees in urban areas.

Plenty of investigations are already made to comprehend the taxonomic diversity of plants foraged by bees. However there is a need to go further into details and gain a better understanding of which plant functional traits bees prefer to forage on.

Indeed, taxonomic and functional diversity can be influenced by various factors such as location, month, and year. While general studies often assess locations and occasionally sample specific months, they rarely consider year-long gradients and gradients across different years.

Nonetheless, this type of analysis requires specific tools. Indeed, metabarcoding techniques and tools are now permitting a relatively cheap, time-efficient and trustful method to assess this foraged-plants diversity by analysing bee pollen balls. Furthermore, many plant functional traits are readily available through reliable and well-organised online databases.

In order to address this issue, this study analysed the floral diversity of flower species that were identified through pollen metabarcoding in 2018 and 2023 between three different sites located in urban areas from the Kanto region in Japan. The maximum range analysed included samples from March to December. Each pollen samples were then clustered along months and their DNA content was analysed through metabarcoding. The resulting data was then cleaned and used to assess taxonomic diversity of foraged plant by bees at species level. Finally, this output was used to extract functional trait information of each foraged species on online plant databases.

All of the extracted data were then analysed using multiple significance test. Indeed, a NMDS ordination followed by a PERMANOVA test was done to analyse the community compositions of foraged plants. The rest of the time, GLMs followed by a deviance analysis were employed to assess the significance of the computed indexes and measures.

Predictive factors such as month, year, and location significantly impacted the diversity and taxonomic/functional richness of plants foraged by *A. mellifera*. These results highlight the dynamic nature of honeybee foraging behavior and the importance of context in shaping environmental pollinator communities.

# Résumé

La biodiversité est en déclin dans le monde entier, et cette diminution affecte également les pollinisateurs. Les causes sont multiples, et l'une d'elle est due à l'urbanisation. Dans un contexte d'urbanisation rapide à travers le monde, il est crucial de comprendre et de gérer cette biodiversité. Ainsi, *Apis mellifera* est connue pour être l'un des principaux pollinisateurs et elle est répartie à l'échelle mondiale. Pour relever ces défis, il est nécessaire de connaître le régime alimentaire des abeilles mellifères en milieu urbain.

De nombreuses études ont déjà été réalisées pour comprendre la diversité taxonomique des plantes butinées par les abeilles. Toutefois, il est essentiel d'approfondir ces recherches pour mieux comprendre les traits fonctionnels des plantes préférées par les abeilles.

En effet, cette diversité taxonomique et fonctionnelle peut être influencée par divers facteurs tels que la localisation, le mois et l'année. Bien que les études générales évaluent souvent les localisations et échantillonnent parfois des mois spécifiques, elles prennent rarement en compte les gradients sur une année complète, et les gradients entre différentes années.

Néanmoins, ce type d'analyse nécessite des outils spécifiques. En effet, les techniques et outils de metabarcoding permettent désormais une méthode relativement économique, rapide et fiable pour évaluer la diversité des plantes butinées en analysant les pelotes de pollen des abeilles. De plus, de nombreux traits fonctionnels des plantes sont facilement disponibles via des bases de données en ligne fiables et bien organisées.

Afin de répondre à cette problématique, cette étude a analysé la diversité florale des espèces de fleurs identifiées grâce au metabarcoding de pollen en 2018 et 2023 sur trois sites différents situés dans des zones urbaines de la région du Kanto au Japon. La période d'analyse maximale comprenait des échantillons de mars à décembre. Les échantillons de pollen ont ensuite été regroupés par mois et leur contenu en ADN a été analysé par metabarcoding. Les données obtenues ont ensuite été nettoyées et utilisées pour évaluer la diversité taxonomique des plantes butinées par les abeilles (au niveau des espèces). Enfin, ces résultats ont été utilisés pour extraire les informations sur les traits fonctionnels de chaque espèce butinée à partir de données en ligne sur les plantes.

Toutes les données extraites ont ensuite été analysées à l'aide de tests de significativité. En effet, une ordination NMDS suivie d'un test PERMANOVA ont été réalisées pour analyser les compositions des communautés de plantes butinées. Le reste du temps, des GLM suivis d'une analyse de déviance ont été employés pour évaluer la significativité des indices et mesures calculés.

Des facteurs prédictifs tels que le mois, l'année et la localisation ont eu un impact significatif sur la diversité et la richesse taxonomique/fonctionnelle des plantes butinées par *A. mellifera*. Ces résultats soulignent la nature dynamique du comportement de butinage des abeilles mellifères et l'importance du contexte environnemental dans la formation des communautés de pollinisateurs.

*"Science is made up of so many things that appear obvious after they are explained."*

— Pardot Kynes, *Dune Messiah*

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# 1 Introduction

## 1.1 Decline of pollinators

### 1.1.1 Pollinators and pollination importance

The reproductive success of 87,5% of flowering plants is closely linked to animal pollination (Ollerton et al., 2011). Pollination increases the harvest size, quality and stability of more than 70% of global crops (Klein et al., 2007). Furthermore, this pollination contributes to approximately 10% of the economic value of cultivated plants in 2005 due to the numerous services provided (Aryal et al., 2020; Gallai et al., 2009). Indeed, pollinators play an essential functional role in most terrestrial ecosystems, ensuring the maintenance of wild and cultivated plants (Aguilar et al., 2006; Ashman et al., 2004; Ricketts et al., 2008).

However, it is now evident that the numbers of these pollinators are declining (Dicks et al., 2021; Koh et al., 2016; Potts et al., 2010; Powney et al., 2019), leading to significant biodiversity loss and economic consequences, particularly in agricultural production (Reilly et al., 2020). Without pollinators, it is estimated that one-third of plants would not produce seeds, and more than 80% of plants would experience a loss in fertility (Rodger et al., 2021).

### 1.1.2 Honeybee focus (*Apis spp.*)

The European honeybee (*Apis mellifera* Linnaeus, 1758), is commonly considered the reference pollinator by the general public (Papa et al., 2022). Known for its extensive food range, this species is distributed globally (Purdy, 2024; Sentil et al., 2022). However despite its prominence, quantifying the pollination services provided by honeybees remains challenging, especially for wild plants, (Klein et al., 2007).

Honeybees are present worldwide, with over 100 million managed hives (Visick et al., 2023). Unfortunately, like other pollinators, modern honeybees are experiencing a 50% reduction in longevity compared to individuals from the 1970s (Nearman et al., 2024), leading to a decline in hive populations (Becher et al., 2014; Betti et al., 2016; Martin, 2001). Indeed, between 1985 and 2005, 25% of bee colonies in Central Europe were lost (Potts et al., 2010). The decline of bees and pollinators in general results from various significant factors, with urbanisation and the intensification of agriculture identified as primary causes (Outhwaite et al., 2022; Potts et al., 2010). These factors reduce the availability and diversity of floral resources (Millard et al., 2023; Outhwaite et al., 2022).

In addition to urbanisation and agricultural intensification, several other detrimental factors specifically impacting insects and bees must be addressed. These factors include the use of pyrethroid and neonicotinoid pesticides, the presence of invasive predator species like the Asian hornet (*Vespa velutina*) (Abudulai et al., 2022; Monceau et al., 2014), and parasites such as *Varroa* spp. (Michez, 2019).

However, several solutions are available to counter this decline. Indeed, restoration and diversification of pollinator habitats, as well as grazing management, have been shown to have a positive impact on pollinators, whether wild or domesticated (Carvell et al., 2017; Tonietto et al. al., 2018).

### 1.1.3   Honeybee context in Japan

In Japan, both wild and domesticated bees exist (Chantawannakul et al., 2018). The diversity of foraged plants varies depending on whether the bee is domestic or wild, with domesticated honeybees more likely to be negatively affected by the removal of non-native plants compared to wild bees, which prefer native species (Urbanowicz et al., 2020; Sarot, 2023).

As in the rest of Asia, *A. mellifera* has been introduced in Japan (Dogantzis et al., 2021), and is now coexisting with native bees. The Asian honeybee (*Apis Cerana* J.Fabr.), is an East-Asia native bee species present in Japan (Theisen-Jones et al., 2016). It is very similar to *A. mellifera*, in fact it also lives in colonies and can be domesticated, but the colony is smaller then *A. mellifera* (2,000–20,000 vs. 30,000–50,000 individuals) (Koetz, 2013; Theisen-Jones et al., 2016). So unfortunately, this native species is now in competition with the non-native *A. mellifera* which is more productive (Theisen-Jones et al., 2016)

## 1.2   European honeybee

### 1.2.1   Description

**Taxonomy**

*A. mellifera* belongs to the order Hymenoptera and the family Apidae (Purdy, 2024). These bees form colonies in which individuals cooperate to build nests, where they store honey and pollen as food sources (Purdy, 2024).

**Social organisation**

Honeybees are eusocial insects, exhibiting the highest level of social organisation among animals (Costa et al., 2005; Wilson et al., 2005). This eusocial structure is characterised by overlapping generations, cooperative care of larvae, and a division of reproductive labor, with sterile workers performing various tasks (Costa et al., 2005; Wilson et al., 2005). The bee colony functions as a "superorganism," where members operate similarly to cells in a multicellular organism, with distinct reproductive and somatic components (Page, 2012; Rueppell et al., 2016; Boomsma et al., 2018). This collective functioning involves groups of individuals sharing selected genes, thus relying on their relatedness (Rautiala et al., 2019; Boomsma et al., 2018).

However, the superorganism concept has its limitations. A bee colony lacks two fundamental traits of an animal organism: a central brain or equivalent control center, and a physically connected communication system akin to a nervous system (Rueppell et al., 2024). Within

the bee colony, communication occurs through temporary connections between individuals rather than a permanent, unified system (Rueppell et al., 2024).

### Lifespan

The lifespan of honeybees varies significantly with the seasons. In winter, they can live from a few weeks to several months (Fluri et al., 1982). This longevity is influenced by seasonal changes and the effectiveness of provision stocks rather than a simple aging process (Alaux et al., 2018; Wild et al., 2021). Notably, about half of the bees die before their first nectar harvest, primarily during pre-foraging exploratory flights (Nearman et al., 2024).

*A. mellifera* colonies are most active during the spring and summer months when flowers are abundant and nectar flow is high (Purdy, 2024). During this period, the hive population can increase to over 50,000 individuals, whereas in winter, the number typically drops to below 20,000 (Purdy, 2024).

### Task organisation

The tasks of worker bees are largely determined by their age (Crailsheim, 1998; Robinson, 1987, Rueppell et al., 2016). Significant physical, neurological, metabolic, and genomic changes occur throughout most of the worker's life, primarily controlled by genes (Robinson, 1987). This progression is orchestrated by a complex interplay of regulatory genes involved in development, hormone signaling, and behavior, which dictate the worker's transition through various tasks as it ages (Crailsheim, 1998; Robinson, 1987; Rueppell et al., 2016).

### Senses and signals

Bees have a very wide variety of senses. For example, regarding chemical signals, these include taste, olfactory, pheromonal and hormonal signals (de Bruyne et al., 2008; Robertson et al., 2006). They can, for example, use and learn different sensory information such as the scents left by other bees on flowers, the specific scent of a flower or even their own scent placed passively at the entrance to their nest (Reinhard et al., 2004; Stout et al., 2001). Bees have approximately 2-3 times more genes encoding olfactory receptors compared to other highly studied diptera (de Bruyne et al., 2008). This olfactory information can also be used for tasks such as detecting the entrance to the nest or following a route inside the nest (Chittka et al., 1999).

Regarding physical signals, vision remains the main information channel used by bees for navigation (Srinivasan, 2010). Hymenoptera, like humans, are equipped with three types of spectral photoreceptors. Their sensitivity peaks around 340, 430 and 535 nm, corresponding respectively to the ultraviolet, blue and green sections of the colour spectrum (Hempel de Ibarra et al., 2014; Backhaus et al., 1992). Furthermore, honeybees are capable of selecting plants based on the colour of their flowers (Brunet et al., 2021; Kulbaba et al., 2012).

### Information channels

Thus, when it comes to information sharing, there are five main information channels (Rueppell et al., 2024):

1. Inter-individual patrolling and sensory perceptions.

2. Trophallaxis, a process by which individuals exchange food or other liquid substances by regurgitating and ingesting the liquid from the other individual's stomach.

3. Waiting delays between workers being assigned to different tasks.

4. Inspection and inter-individual antennation.

5. Acoustic, tactile, and visual signals, such as bee dances (waggle dance).

These information channels include both positive and negative signals, which can be indirect, incidental, direct, or mixed, as exemplified by waggle dancing (Rueppell et al., 2024).

Indeed, in 1973, Karl von Frisch received a Nobel Prize for his work on honeybee communication; notably for his discovery that bees can inform each other of the location of food resources through a "danced" body language, the "waggle dance" (von Frisch, 1974). Indeed, honeybees transmit information to their peers in the hive via "round dances", thus signalling to dancing "follower" bees that the food resource is approximately 100 metres from the hive (Rueppell et al., 2024).

### 1.2.2   Foraging behaviour

**Food sources**

From flowers' plant, honeybees collect nectar, which is their main source of carbohydrates, and also pollen, which provides essential proteins and lipids (Brunet et al., 2024; Jeffree et al., 1957; Winston, 1987; Wykes, 1953) . In addition, they gather materials such as plant resins, mineral salts, and water to meet the needs of their colony (Bonoan et al., 2017; Winston, 1987).

The primary source of sugar, digestible protein, and other nutrients for foragers is trophallactic provisioning (Winston, 1987). Trophallaxy ensures efficient food distribution within the colony, allowing bees to maintain a diet that minimizes residue (The food is soft and energy concentrated), which is beneficial for flight and reduces the need for excretion (Crailsheim, 1998; Wright et al., 2018).

The main sugars found in most nectars are glucose, fructose, and sucrose, which are found in solutions that prevent crystallisation (Kostryco et al., 2022; Wykes, 1953). It should be noted that the majority of foragers choose to collect nectar, while only some available members of the workforce focus on other resources (Bonoan et al., 2017; Klein et al., 2019).

**Food intake behaviour**

Regarding food intake, honeybees choose between sucking and licking the liquid based on its viscosity (Wei et al., 2020). Licking preference is linked to sugar concentration, which allows bees to efficiently collect liquid from different sources (Wei et al., 2020). A honeybee

forager can store up to 60 $\mu$L of nectar in its crop, although the typical amount is 10–40 $\mu$L (Doussot et al., 2024). Nectar foraging is primarily oriented toward maximising the rate of energy intake, although foragers may also consider overall efficiency (Schmid-Hempel et al., 1985). Indeed, although energy intake is the priority, other aspects of efficiency can also be taken into account, such as the distance travelled or the time needed to find and collect nectar (Stabentheiner et al., 2016 ; Robinson et al., 2022). Pollen collection, unlike nectar collection, continues depending on available reserves until the capacity of the hive is reached (Ohlinger et al., 2022). Indeed, pollen collection is regulated to maintain reserves around an optimal homeostatic level (Fewell et al., 1992; Schmickl et al., 2004).

**Secondary metabolites**

Pollen often contains significant amounts of secondary metabolites, which can be toxic (Palmer-Young et al., 2019). To counteract this, bees possess various catalytic proteins, such as cytochrome oxidases and carboxylases, which are adapted to detoxify these secondary metabolites in pollen and nectar (du Rand et al., 2017; Lucchetti et al., 2018). The major secondary compounds present in pollen (and to a lesser extent in nectar), whether from cultivated or wild plants, include flavonoids, terpenoids, alkaloids, amines, and chlorogenic acids (Palmer-Young et al., 2019). Some of these compounds serve dual functions, acting both as defences against herbivores and as beneficial or at least non-harmful substances to pollinating insects (Stevenson, 2020).

**Foraging mediation**

Regarding the colony, the current consensus is that optimal foraging behavior is the combined result of individual bee behaviors (Purdy, 2024; Cook et al., 2020). For bees, the primary goal of foraging is not to meet their own dietary needs but to gather resources such as food, water, minerals, and plant resins for the colony (Purdy, 2024; Seeley et al., 2003).

Foraging behavior is influenced by multiple factors. The age at which bees begin their first nectar harvest is a classic example of a complex trait controlled by various genetic factors (Rueppell et al., 2004). Several genes play crucial roles in regulating and facilitating this behavior, particularly by affecting the transition of bees into foragers (Nelson et al., 2007).

Furthermore, foraging behaviour in bees is regulated by complex hormonal mechanisms. The key hormone that initiates the desire to forage is juvenile hormone, also known as JH (Robinson, 1987). Once the bee begins foraging, the act of nectar collection triggers the release of octopamine, often considered a "satisfaction hormone" in insects (Schulz et al., 2001).

**Foraging journey**

Regarding the foraging trip, a "journey" represents the time between the departure and return of a bee to the hive. *A. mellifera* complete an average of 4.6 ± 0.2 foraging trips per day in a suburban-agricultural landscape context (Minahan et al., 2018). Each foraging trip lasts approximately 48.7 ± 0.8 minutes, and a bee brings on average 3.68 ± 0.08 mg of pollen to the hive per trip (Minahan et al., 2018). This corresponds to an average of 19 foraging trips made throughout the life of an individual honeybee (Klein et al. 2019; Minahan and Brunet,

2018). Honeybees undertake their first flights only a few days after emerging from pupae and before their flight muscles are fully developed. These first flights are important for learning and orientation (Prado et al., 2020). However, honeybees do not start foraging until they are around 3 weeks old (Doussot et al., 2024).

At some point in its journey, the bee switches from foraging to returning to the nest and begins navigating toward home. This transition may be driven by a lack of energy or by having collected the expected amount of nectar, pollen or other resources (Schmid-Hempel et al., 1985). Bees use a route-following model based on nodes representing flowers and vectors defining the distance and direction between these flowers (Doussot et al., 2024).

A complete foraging trip, beginning and ending near the nest, involves the bee engaging in complex cognitive processes with long-term impact on its navigation (Doussot et al., 2024; Chittka et al., 1999; Zeil, 2012; Kraft et al., 2011), such as learning visual information (Zeil, 2012) and navigation using a "celestial compass" along familiar paths (Kraft et al., 2011). However, this also requires short-term cognitive abilities such as obstacle avoidance (Ravi et al., 2020), crossing gaps between vegetation (Ravi et al., 2018), and altitude control (Portelli et al., 2017). In addition, honeybees tend to optimise their route geometry (Buatois et al., 2016) and take shortcuts between known sites (Müller et al., 2018; Paffhausen et al., 2021).

Foraging generally constitutes the last phase of bees' lives (Rueppell et al., 2024). However, there is large variability or plasticity in the age at which this transition occurs, and task specialisation among foragers to collect specific resources is considered to be independent of age (Calderone, 1998; Rueppell et al., 2007). There is also high inter-individual variation in performance, where a small percentage of bees, the "elites", carry out the majority of foraging activities in the hive (Brunet et al., 2024; Klein et al., 2019). However, the foraging efficiency of individual bees improves with experience (Brunet et al., 2024; Dukas et al., 1994). Characteristic foraging distances are listed in Table 1.

Table 1: Characteristic distance to forage site in km (Noel, 2023; Seeley et al., 2003)

| Distance characteristics | Measure [km] |
| --- | --- |
| Modal Distance | 0.7 |
| Median Distance | 1.6 |
| Mean Distance | 2.2 |
| Maximum Distance | 10.9 |
| 95th Percentile distance | 6.0 |

Seasonal variations also play a role; for example, in August when resources may be scarce, larger colonies may extend their foraging distance to ensure an adequate supply of resources for colony maintenance (Beekman et al., 2004; Danner et al., 2017). Plus, landscape diversity is another factor influencing foraging distances: honeybees compensate for less diverse landscapes by increasing their foraging range to maintain the amount and diversity of collected pollen (Danner et al., 2017).

**Bee floral fidelity**

The European honeybee maintains a high level of floral fidelity during foraging (Amaya-Márquez, 2009; Grüter et al., 2011; Wilson et al., 2010). This means individual foraging bees are highly flower-constant, limiting their visits to a single flower type (e.g., plant species) even when other types are available (Waser, 1986). For instance, during a foraging trip, 87% to 89% of individual bees collect pollen from a single plant family or plant morphotype (Brunet et al., 2024). This loyalty extends beyond species level to the plot level. Fragoso et al. (2023) observed strong patch fidelity in honeybees, with 76% of bee sightings occurring in the patches where they were initially marked. Furthermore, this patch fidelity did not vary with patch size; bees demonstrated similar degrees of loyalty to both smaller and larger patches (Fragoso et al., 2023; Brunet et al., 2024). Foragers recruited to a specific patch tend to remain attached to it rather than migrating to another equally productive but unfamiliar patch (Beekman et al., 2004; Brunet et al., 2024).

## 1.3   Beekeeping context

Unfortunately, the beekeeping industry is in decline in Europe and the USA (Potts et al., 2010; Bruckner et al., 2023). Despite these losses, the number of *A. mellifera* hives in the world has increased by 45% since 1961 and the proportion of crops dependent on pollinators increased by more than 300%, making the number of bees insufficient (Aizen et al., 2009; Breeze et al., 2014).

### 1.3.1   Beekeeping services

Honeybee crop pollination is often mistaken for a natural ecosystem service, but it is actually performed by domesticated bees, not wild ecosystems (Geldmann et al., 2018). Consequently, domestic bee hives should sometimes be excluded from protected areas to avoid negatively impacting wild pollinators (Geldmann et al., 2018). While honeybees benefit crop pollination, beekeeping with *A. mellifera* may conflict with conservation goals aimed at protecting wild pollinator populations and natural habitats (Geldmann et al., 2018).

### 1.3.2   Urban beekeeping

There are multiple types of urban beekeeping, influenced by the urbanisation gradient and varying definitions of what is considered urban. Assessing an urbanisation gradient is challenging due to the blurred boundaries between rural (extensive or intensive), semi-urban, and urban areas (Udy et al., 2020).

Urban agriculture fosters diverse bee communities and enhances urban pollination services (Normandin et al., 2017). However urban beekeeping questions about resource scarcity and its potential negative impact on urban biodiversity. In many cities, food resources are insufficient to support the high density of hives (Casanelles-Abella et al., 2022; Stevenson et al., 2020). High densities of honeybees can increase competition between wild and domestic bees,

especially when food resources are limited (Herrera, 2020; Magrach et al., 2017; Prendergast et al., 2021). Evidence suggests that artificially high densities of domestic bees due to beekeeping can worsen the decline of wild pollinators (Geldmann et al., 2018; Mallinger et al., 2017). For example, a study in Paris, France, found a negative correlation between the visitation rates of wild pollinators and the density of honeybee colonies in the area (Ropars et al., 2019).

Green infrastructure in cities, such as parks and gardens, provides crucial refuges for pollinating insects, which are declining globally in agricultural areas (Daniels et al., 2020). Urban areas have the potential to support diverse pollinator populations. To achieve this, it is important to cultivate a wide variety of flowering plants and make use of even small green spaces (Daniels et al., 2020). Thus, urban beekeeping can be sustainable if hive densities are managed properly and there are enough foraging plants available.

## 1.4 Plant functional diversity

### 1.4.1 Plant functional traits

Plant functional plant traits can be described as characteristics such as morphology, physiology and phenology that reflect how plants adapt to their environment, influence other organisms and shape ecosystems (Lavorel et al., 2007; Pérez-Harguindeguy et al., 2013; Violle et al., 2007). Studying variation in these traits is crucial for understanding ecological and evolutionary processes. Standardised methods for measuring these traits are necessary to create predictive models and quantify various natural and anthropogenic changes in ecosystems (Pérez-Harguindeguy et al., 2013).

The functional differences of species can be taken into account to assess biodiversity. To measure this diversity, we use functional diversity, which aims to quantify the distribution of traits within a community (Cadotte et al., 2011; Normandin et al., 2017).

### 1.4.2 Plant-bee interactions

Through the lens of honeybee pollination, plant functional traits of interest are combined into a list in Table 2. Notably, flower reflectance (Colour), flower height/area, flower morphology, nectar-sugar concentration, and nectar-amino acid concentration are the most important traits shaping plant–insect interactions (Fornoff et al., 2017).

Table 2: List of plant functional traits of interest through the lens of honeybee pollination (Fornoff et al., 2017, Parachnowitsch et al., 2012, Phillips et al., 2020) N/A were putted when the traits can have different unity modality.

| Trait | Unity |
|---|---|
| Plant size | m |
| Inflorescence diameter/area | m or m² |
| Amount of pollen | g |
| Amino acid content of nectar | g/L |
| Inflorescence nectar volume | mL |
| Availability of flower nectar | Yes/No |
| Sugar concentration of inflorescence nectar | % or g/mL |
| Smell | ng/mL |
| Flowering period | Days or months |
| Inflorescence colour | N/A |
| UV reflectance of the Inflorescence | N/A |
| Flower shape | N/A |
| Position of flower stamens | N/A |
| Number of stamens in the flower | N/A |

It's important to note that variations in floral traits is common in plant populations, even at the genus level, and such variation affects pollinator visitation (Bauer et al., 2017; Brunet, 2009; Zhao et al., 2016).

### 1.4.3 Plant honest signalling

The "honest signalling" of a plant to a pollinator refers to the plant's ability to provide reliable signals about the presence of rewards such as nectar or pollen (Bauer et al., 2017; Knauer et al., 2015). Pollinators use these plant functional traits to select those they will preferentially visit (Bauer et al., 2017; Knauer et al., 2015). This honest signalling is also beneficial for the plant, as receiving more visits from pollinators can increase the reproductive success of plants through both male plants (pollen dispersal) and female plants (pollen establishment and seed production) functions (Bauer et al., 2017; Brunet et al., 2009; Zhao et al., 2016). This relationship between floral traits and reproductive success can lead to a pollinator-mediated selection on floral traits. Bees engage in associative learning, enhancing their ability to locate rewarding flowers (Brunet et al., 2021; Parachnowitsch et al., 2012; Parachnowitsch et al., 2010).

In this context, the term "reward" refers to the four main resources collected from the environment (see 1.2.2) and brought back to the colony, which will be collectively referred to as rewards.

### 1.4.4   Particular functional traits

**Inflorescence traits**

To understand the impact of inflorescence colour on bees, it is crucial to accurately assess this characteristic as a functional trait. This assessment is based on factors such as spectral reflectance, chroma (darkness or saturation), tone , and reflectivity (brightness) of the petals or sepals (Trunschke et al., 2021). Pollinators perceive colours differently from humans. Their colour perception depends on their spectral photoreceptors and nervous system (Chittka, 1992).

Other visual characteristics of flowers, such as the size of the flower display, are important in attracting pollinators. For example, several studies have shown that many bee species prefer to visit plants with larger floral displays, including the size, number and arrangement of flowers on the plant (Harder et al., 1995; Ishii, 2006; Ohashi et al., 2002; Zhao et al. (2016)). Flower display size generally refers to the number of flowers that are simultaneously open on a plant or inflorescence (Goodwillie et al., 2010). The more flowers open, the larger the size of the flower display (Bauer et al., 2017; Mitchell et al., 2004). Plants with larger floral displays tend to receive more visits from pollinators, which can increase their seed set rate (Mitchell et al. , 2004; Zhao et al., 2016). This means that the size of the flower display can serve as an honest signal to indicate the richness of the plant's rewards for pollinators (Brunet et al., 2015; Makino et al., 2007). Interestingly, bees can exhibit innate preferences for flower colours, showing a spontaneous response to flower colour without prior exposure (Giurfa et al., 1995; Lunau et al., 1995). Thus, naive honeybees tend to favour bee-UV-blue flowers (Giurfa et al., 1995; Hempel de Ibarra et al., 2014).

**Floral bouquet**

Each plant has a unique set of olfactory compounds called a "floral bouquet," which can vary between species and within populations of the same species (Delle-Vedove et al., 2017; Knudsen et al., 2006). Pollinators use these distinctive bouquets to differentiate between plant species (Knudsen et al., 2006). Indeed, plants emit VOCs from several chemical classes (Peakall et al., 2014).

Studies have shown that various pollinator groups respond differently to visual and olfactory signals (Balkenius et al., 2006; Burkle et al., 2017; Burger et al., 2010; Omura et al., 2005).

## 1.5   DNA analysis of pollen

### 1.5.1   Pollen identification history

Melissopalynology, the study of pollen collected by bees, has become an essential tool in pollination biology and in understanding the foraging behavior of pollinators (Baum et al., 2011; Louveaux, 1959; Wilson et al., 2010).

Several techniques can be used to assess plant-pollinator interactions, including direct observation of insect visitation to individual flowers (Nagai et al., 2022), capture-mark-recapture (Hinneberg et al., 2022), computational models of foraging behaviour (Gheorghe et al., 2001), electronic tracking devices (Sipos et al., 2020), chemical signatures (Shakoori et al., 2023), optical microscopy (Louveaux et al., 1978) and genetic sequencing (metabarcoding) (Hawkins et al., 2015).

Techniques that involve identifying pollen collected from insect bodies or nests offer a more comprehensive understanding of plant-pollinator interactions compared to merely observing insect visits to flowers (Cornman et al., 2015).

Historically, pollen mixtures were primarily analysed using light microscopy and dichotomous keys. This method, which is laborious and requires expertise in pollen identification, identifying only a fraction of the taxonomic diversity (Cornman et al., 2015; Richardson et al., 2015). It is time-consuming and limited in taxonomic precision, making it impractical for large-scale studies.

### 1.5.2   DNA Metabarcoding breakthrough

There is now a reliable and effective alternative to traditional melissopalynology: DNA metabarcoding (Bell et al., 2023; Richardson et al., 2015). DNA metabarcoding is a technique that identifies and quantifies different species present in a sample using specific DNA sequences (Bell et al., 2023; de Sousa et al., 2019). In the context of melissopalynology, this approach enables the identification of plant species whose pollen has been collected by bees by sequencing a specific region of plant ribosomal DNA called Internal Transcribed Spacer or ITS (Chen et al., 2010). Other commonly used DNA regions include matK and rbcL (Bell et al., 2017).

This method offers several advantages over traditional melissopalynology, including greater taxonomic accuracy, reduced reliance on human expertise, and the ability to process large-scale samples more quickly and efficiently (Keller et al., 2015; Chen et al., 2010; Richardson et al., 2015).

While the technical and biological specifics can differ significantly between metabarcoding studies, the general workflow remains consistent (Deagle et al., 2019). This process involves the extraction of total DNA from the sample, followed by Polymerase Chain Reaction (PCR) amplification of DNA barcode markers for the taxa of interest, and then DNA sequencing using High Throughput Sequencing (HTS) to achieve taxonomic classification sequences (Deagle et al., 2019).

It's important to understand that bees may collect nectar from a flower without collecting its pollen. Therefore, some flowers visited by bees might not be detected in metabarcoding results since, as these flowers do not leave pollen traces despite being foraged.

## 1.6   Objectives

Despite a global distribution and an essential ecological and economic role, honeybee populations are declining due to factors including urbanisation (Udy et al., 2020). In fact, there is an urgent necessity to comprehend the types and dynamics of human-bee interactions in urban settings, in order to effectively manage these interactions (Soulsbury et al., 2015). Thus, knowledge of the ecology of bees in urban environments is crucial to help these insects and consequently maximise the ecosystem services they provide, such as pollination and biodiversity maintenance (Weisser et al., 2023).

Honeybees use a variety of senses to communicate and perform tasks, including chemical, olfactory, pheromonal, hormonal and visual signals, with different information channels to share positive or negative signals. The signals that bees perceive and record are crucial regarding their interaction with the plants on which they forage.

The plant, especially its flower, can be analysed based on several distinct functional traits. This approach helps us understanding the specific characteristics that bees tend to forage more or not. While many studies focus on the taxonomic range of plants foraged by bees, there is limited exploration of this foraging behaviour in terms of functional traits.

Furthermore, In urban environments, such as greater Tokyo in Japan, one of the widest urban areas in the world, a detailed understanding of taxonomic and functional diversity of foraged plants by bees within is highly relevant (Danner et al., 2017). Indeed, our understanding of how bees' preferences for particular taxa and functional traits develop over months, years and locations remains limited (Fornoff et al., 2017; Phillips et al., 2020; Wilson et al., 2010).

Through the analysis of metabarcoding DNA pollen samples from honeybees, this study aims to achieve the following objectives:

- Gain a better understanding of *A. mellifera* ecology and the taxonomic range and richness of foraged plants over months, years and locations.

- Enhance our understanding of how *A. mellifera* adapts its foraging behaviour focusing on functional traits over months, years and locations.

- Improve our understanding of the relation between the species richness and the functional richness of foraged plants by *A. mellifera*

The research hypotheses are :

**H1**: Both plant taxonomic diversity and functional diversity are anticipated to evolve positively over months during *A. mellifera*'s main activity time.

**H2**: Both plant taxonomic diversity and functional diversity are anticipated not to evolve significantly over years, honeybee's environment being considered stable

**H3**: In highly plant-diverse locations, honeybees, being generalist pollinators, are expected to forage across a broader spectrum of species. Therefore, it is anticipated that the diversity and richness of species foraged by honeybees will decrease as urbanisation increases.

**H4**: The diversity of plants foraged by *A. mellifera* varies depending on the plants' functional traits.

**H5**: The plant species richness foraged by *A. mellifera* is positively correlated with plant functional richness

# 2   Material and methods

## 2.1   Study area and experimental setup

Chiba University has been conducting a series of studies on urban beekeeping since 2017. In this study, data from 2018 and 2023 were selected. Three sites were studied during this project : Kashiwanoha, Nichichiba and Sumida. All of those sites are located in Chiba University campuses and are located in the northern part of the Tokyo-bay area. By looking at Figure 1, an arbitrary urban gradient can be attributed to the three analysed locations. Indeed, those locations are either urban or semi-urban (Udy et al., 2020). Kashiwanoha is the most semi-urban, surrounded by some fields, Nishichiba is more urban, while Sumida is totally urbanised, almost in the city center (Figure 1).

### 2.1.1   Sampling design
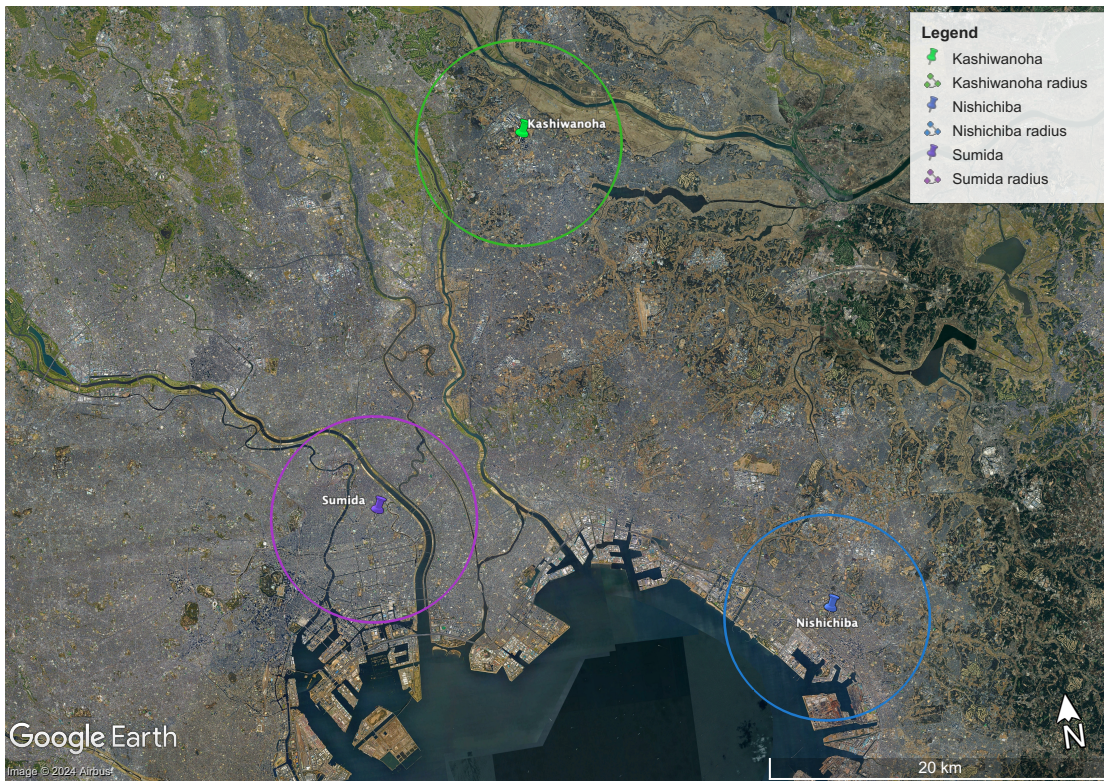


Figure 1: Satellite map of study sites distributed across the northeast Kanto region, Japan. The map displays three study sites: Kashiwanoha, Nishichiba, and Sumida. Each site is marked with a specific colour-coded symbol and a corresponding 6 km radius representing the 95% percentile distance of the maximum distance bees travel (Table 1). Sources: Chiba University, Japan ; Google Earth, 2024 Airbus.

A total of 91 samples of pollen collected by the research team were studied. Samples were collected at approximately weekly intervals from 27 March to 31 August in 2018 and from 5 April to 16 December in 2023. Because pollen sampling depends on weather conditions and the beekeepers' schedules, samples were often collected at inconsistent intervals.

Table 3: Sample information (code and date) by year and location, clustered sample in the same boxes

| 2018 | | | | 2023 | | | | | |
|------|------|------|------|------|------|------|------|------|------|
| Nishichiba | | Kashiwanoha | | Nishichiba | | Kashiwanoha | | Sumida | |
| Date | Code | Date | Code | Date | Code | Date | Code | Date | Code |
| 27/03/2018 | NP-1 | 10/04/2018 | KP-0 | 06/04/2023 | V-N1-A | 05/04/2023 | V-K1-A | 03/04/2023 | V-S1-A |
| 10/04/2018 | NP-2 | 22/04/2018 | KP-1 | 13/04/2023 | V-N1-B | 11/04/2023 | V-K1-B | 10/04/2023 | V-S1-B |
| 02/05/2018 | NP-3 | 01/05/2018 | KP-2 | 19/04/2023 | V-N1-C | 18/04/2023 | V-K1-C | 17/04/2023 | V-S1-C |
| 16/05/2018 | NP-4 | 09/05/2018 | KP-3 | 27/04/2023 | V-N1-D | 28/04/2023 | V-K1-D | 24/04/2023 | V-S1-D |
| 28/05/2018 | NP-5 | 15/05/2018 | KP-4 | 02/05/2023 | V-N2-A | 03/05/2023 | V-K2-A | 01/05/2023 | V-S2-A |
| 13/06/2018 | NP-6 | 29/05/2018 | KP-5 | 11/05/2023 | V-N2-B | 10/05/2023 | V-K2-B | 22/05/2023 | V-S2-B |
| 27/06/2018 | NP-7 | 09/06/2018 | KP-6 | 18/05/2023 | V-N2-C | 17/05/2023 | V-K2-C | 29/05/2023 | V-S2-C |
| 28/06/2018 | NP-8 | 22/06/2018 | KP-7 | 25/05/2023 | V-N2-D | 19/05/2024 | V-K2-D | 05/06/2023 | V-S3-A |
| 10/07/2018 | NP-9 | 07/07/2018 | KP-8 | 01/06/2023 | V-N3-A | 07/06/2023 | V-K3-A | 19/06/2023 | V-S3-B |
| 27/07/2018 | NP-10 | 16/07/2018 | KP-9 | 08/06/2023 | V-N3-B | 15/06/2023 | V-K3-B | 26/06/2023 | V-S3-C |
| 31/07/2018 | NP-11 | 30/07/2018 | KP-10 | 13/06/2023 | V-N3-C | 22/06/2023 | V-K3-C | 03/07/2023 | V-S4-A |
| 31/08/2018 | NP-12 | 17/08/2018 | KP-11 | 21/06/2023 | V-N3-D | 30/06/2023 | V-K3-D | 10/07/2023 | V-S4-B |
| | | | | 29/06/2023 | V-N3-E | 07/07/2023 | V-K4-A | 20/07/2023 | V-S4-C |
| | | | | 05/07/2023 | V-N4-A | 12/07/2023 | V-K4-B | 31/07/2023 | V-S4-D |
| | | | | 15/07/2023 | V-N4-B | 18/07/2023 | V-K4-C | 07/08/2023 | V-S5-A |
| | | | | 19/07/2023 | V-N4-C | 01/08/2023 | V-K5-A | 29/08/2023 | V-S5-B |
| | | | | 02/08/2023 | V-N5-A | 18/08/2023 | V-K5-B | 15/09/2023 | V-S6-A |
| | | | | 08/08/2023 | V-N5-B | | | 28/09/2023 | V-S6-B |
| | | | | 23/08/2023 | V-N5-C | | | 16/10/2023 | V-S7-A |
| | | | | 31/08/2023 | V-N5-D | | | 27/10/2023 | V-S7-B |
| | | | | 14/09/2023 | V-N6-A | | | 27/11/2023 | V-S8-A |
| | | | | 28/09/2023 | V-N6-B | | | 03/12/2023 | V-S9-A |
| | | | | 18/10/2023 | V-N7-A | | | 16/12/2023 | V-S9-B |
| | | | | 30/10/2023 | V-N7-B | | | | |
| | | | | 16/11/2023 | V-N8-A | | | | |
| | | | | 30/11/2023 | V-N8-B | | | | |
| | | | | 07/12/2023 | V-N9-A | | | | |

Metabarcoding reads provide only a semi-quantitative measure of species abundance (Bell et al., 2023). Additionally, due to the study's use of metabarcoding data collected five years apart, the reads were deemed insufficiently reliable for accurately quantifying species presence in a community (Bell et al., 2023). Consequently, all reads equal to or greater than one were converted to 1 (presence) and 0 indicates (absence).

Across all 91 samples collected, data was clustered and merged by month. It means that each same-month samples reads were fusionned and summed together before being converted into presence/absence data. This clustering was done after metabarcoding the 2018 data using R software (ver. 4.3.3, R Core Team, 2024) with the RStudio interface (RStudio, PBC, Boston, MA), and before metabarcoding and DNA extraction by mixing the pollen for the 2023 data. This variability is due to the fact that the 2018 data had already been processed, the high cost

of metabarcoding and the significantly larger number of samples in 2023. However, this should not affect the subsequent results because as explained later it is done using presence/absence data.

In Table 3 we can see the month clustering represented by the lines separating the different months. From the 91 original samples, 32 monthly-groups samples are remaining.

This project will sometimes present data along two major dimensions, especially when detailed information needs to be displayed. Reducing the data along these two dimensions facilitates easier interpretation of the transmitted information. These dimensions are described in the tables below:

| | 2023 | |
|---|---|---|
| | Nishichiba | Sumida |
| Months | April | April |
| | May | May |
| | June | June |
| | July | July |
| | August | August |
| | September | September |
| | October | October |
| | November | November |
| | December | December |

Table 4:   Month-oriented comparison of monthly merged samples data.

| | 2018 | | 2023 | |
|---|---|---|---|---|
| | Nishichiba | Kashiwanoha | Nishichiba | Kashiwanoha |
| Months | April | April | April | April |
| | May | May | May | May |
| | June | June | June | June |
| | July | July | July | July |
| | August | August | August | August |

Table 5:   Year-oriented comparison of monthly merged samples data.

Indeed, some of the harvested data could not be compared across all modalities. For example, Sumida's samples were not collected in 2018, making year-gradient comparisons impossible in this case. Additionally, it was not feasible to compare all of Nishichiba's 2023 data with the 2018 data since the 2018 sampling stopped in August.

Nevertheless, this decision is mostly aesthetic. In fact, monthly clustered samples were consistently tested together to ensure no insights were lost. Whenever year-oriented or month-oriented comparisons are used, it will be specifically mentioned.

### 2.1.2   Sample harvesting

To collect pollen samples, pollen traps were placed at the entrances of beehives. These traps collected the bee corbicula content, also known as pollen balls.

The traps were designed with a mesh sized for bees (Figure 4), which removed the pollen balls from bees entering the hive. A tray below the mesh collected the pollen balls (Figure 2) (Prdun et al., 2021.; Mahmood et al., 2017). The pollen balls were then transferred into 50 ml tubes (Figure 4), labelled with the date and location. Samples were collected monthly and stored in a freezer at -20°C in small plastic bags until further use.

It is important to note that pollen traps tend to have no effect on honeybee colony development (Prdun et al., 2021).



Figure 2: Pollen traps content and pollen temporal storage (location: Nishichiba campus Chiba University, 17 April 2024)



Figure 3: Pollen traps at the hive entrance (location: Nishichiba campus Chiba University, 17 April 2024)

## 2.2   DNA isolation, sequencing and bio-informatics

All the DNA isolation and sequencing described below focus on 2023's samples. However the 2018's samples were already processed along a similar protocol described in Noël, 2023. In fact the 2018 extraction was already conducted by other researchers previously, but the protocol was nearly the same. All samples were mixed by month by merging the contents of the pollen-filled plastic bags. Regarding data from 2018, samples were merged after DNA extraction by combining the metabarcoding results across Operational Taxonomic Unit (OTU).

### 2.2.1   DNA extraction

The 2023 DNA extraction from pollen balls was performed following the protocol outlined in Hawkins et al. (2015), which utilised the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). All of the DNA extraction was released in the Chiba University Matsudo campus (B Building) and $75 \pm 2$ mg of pollen balls were used per plastic bag (N=23).

Using sterilised products, the extraction procedure involved crushing the pollen balls by hands, weighing out 75 mg, freezing with liquid nitrogen (-196 °C), and crushing the content with 4 stainless balls in micro refrigerated centrifuge (Kubota, model 3740, Fujioka, Japan) (4000 rpm, 30 seconds) until powdered (4 times). Buffer AP1 with proteinase K was added to the tube containing the material, followed by incubation at 65°C and mixing. RNase A was then added, mixed, and incubated at room temperature. The subsequent steps followed the DNeasy Plant Mini Kit protocol, including centrifugation, washing, and elution.

The DNA extraction was confirmed by measuring DNA concentration and purity using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, U.S.A) and Qubit(HS) fluorometer (Thermo Fisher Scientific, Singapore, Singapore). Finally, the extracted DNA was stored at -20°C.

### 2.2.2   Library preparation and sequencing

Libraries, alias collections of DNA sequences prepared for sequencing, were generated using a two-step tailed Polymerase Chain Reaction (PCR) method (see 1.5.2). The target region of the primers used is the ITS1 region of the fungal ribosomal RNA gene cluster.

In total, 23 PCR reactions were conducted for each PCR run (first and second).

**First PCR role**

The role of the first PCR is to amplify the target region of interest (in this case, the ITS region) from the sample DNA. This step focuses on increasing the amount of the specific DNA region that will be sequenced.

For the first PCR reaction, a 20 $\mu$L mix was prepared containing 2.6 $\mu$L of water, 10 $\mu$L of 2x KOD FX Neo Buffer, 4 $\mu$L of 2 mM dNTPs, 1 $\mu$L each of Primer1 (10 $\mu$M) and Primer2 (10 $\mu$M), 0.4 $\mu$L of KOD FX Neo (1 u/$\mu$L), and 1 $\mu$L of DNA solution (max 1 ng/$\mu$L) (Masamura et al., 2014). The mix ingredients come from the TOYOBO laboratory (KOD FX Neo,

Toyobo, Japan).

The mix was thoroughly mixed and spun down before adding 1 µL of the DNA solution using a multi-pipetman. The NTC (No Template Control) consisted of water. The reaction was then sealed and mixed by inversion, ensuring no air bubbles were present. The PCR was done using TaKaRa PCR Thermal Cycler Dice TP350 (TAKARA BIO INC, Otsu, Shiga, Japan). The PCR program included an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 57°C for 30 sec, and elongation at 68°C for 30 sec. A final extension step was performed at 68°C for 7 min, followed by holding at 20°C indefinitely (Masamura et al., 2014).

To see if the PCR was right, Agarose gel electrophoresis was then conducted using the following parameters: A 1-1.5% agarose gel prepared with either 0.5x TBE or 1x TAE buffer. Loading mix included 1 µL of 100 bp DNA ladder with Midori green direct and loading dye. First PCR product (3 µL) was mixed with the loading dye. Electrophoresis was carried out at 100-135 V for 20-30 minutes to separate and visualise the PCR products.

**Primers description**

This primer pair was chosen for its superior effectiveness in identifying a broader range of Japanese plant species, as demonstrated by Maeda and Takahashi (2017). Both of the primers are targeting the ITS1 region. In fact, it outperformed the ITS2 region primers developed by Cheng et al. (2016).

Table 6: Description of primers used for the first PCR (Masamura et al., 2014)

| Target-specific primers used for the initial DNA amplification | Sequence description | Components (5'→3') |
|---|---|---|
| 18S-MiseqF (Forward) | Illumina adapter + Variable region + Forward primer specific to 18S rRNA gene | TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGnnnnnGACGTCGCGAGAAGTCCA |
| 5.8S-MiseqR (Reverse) | Illumina adapter + Variable region + Reverse primer specific to 5.8S rRNA gene | GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGnnnnnTCGCATTTCGCTACGTTCTTCATCG |

Illumina sequencing flow cell. This adapter region includes sequences specific to the target regions (18S and 5.8S rRNA genes), with "n" representing the variable nucleotides.

The primers 18S-MiseqF and 5.8S-MiseqR are designed to bind to specific regions within the DNA that encode the 18S ribosomal RNA (rRNA) and 5.8S ribosomal RNA (rRNA) genes, respectively. 18S-MiseqF: Amplifies the 18S rRNA gene region. 5.8S-MiseqR: Amplifies the 5.8S rRNA gene region

These primers were used to ensure that the target regions are effectively amplified for sequencing (Masamura et al., 2014).

In this case, the two primers (18S-MiseqF and 5.8S-MiseqR) are designed to amplify a specific region which must be between 350 and 500 bp in length. Amplification of this region will yield amplicons with an expected total length of between 450 and 600 bp, taking into account

the sequences added by the adapters and primers used in the PCR process (Masamura et al., 2014).

## First PCR products

The first PCR products were sent to the private company Gikenbio Bioengineering Lab. Co., (Sagamihara, Japan; https://www.gikenbio.com/). All the following experimentations are then done by this company. Following the initial amplification, the 1st PCR product was purified by adding an equal volume of VAHTS DNA Clean Beads (Vazyme) to the 1st PCR solution. This step involved binding the PCR products to the beads, followed by washing and elution to obtain purified PCR products. The resulting purified PCR products constituted the first library. The concentration of the purified 1st PCR solution was then accurately measured using the Synergy H1 microplate reader (BioTek, Winooski, VT, USA) and the QuantiFluor dsDNA System (Promega, Madison, WI, USA).

## Second PCR role

The 2nd PCR aims to add the necessary sequences for sequencing, such as Illumina adapters and indexes, to the DNA fragments amplified in the 1st PCR (first library). This step prepares the DNA for high-throughput sequencing (here, NGS metabarcoding).

For the second PCR (indexing), indexing primers were used to add unique indices to the amplified DNA fragments. This facilitates the identification and differentiation of samples after sequencing.

Table 7: Description of primers used for the second PCR (Masamura et al., 2014))

| Target-specific primers used for the initial DNA amplification | Sequence description | Components (5'→3') |
|---|---|---|
| 2ndF (Forward) | Illumina adapter + Index2 + Universal Tail Sequences | AATGATACGGCGACCACCGAGATCTACAC-Index2- TCGTCGGCAGCGTC |
| 2ndR (Reverse) | Illumina adapter + Index1 + Universal Tail Sequences | CAAGCAGAAGACGGCATACGAGAT-Index1-GTCTCGTGGGCTCGG |

For the second PCR, a 10 $\mu$L reaction mix was prepared, which included 5 $\mu$L of 2x KOD FX Neo Buffer, 2 $\mu$L of 2 mM dNTPs, 0.5 $\mu$L each of Primer1 (10 $\mu$M) and Primer2 (10 $\mu$M), 0.2 $\mu$L of KOD FX Neo (1 u/$\mu$L), and 0.5 $\mu$L of DNA solution (max 1 ng/$\mu$L). This second PCR reaction was carried out under specific thermal cycling conditions: an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and elongation at 68°C for 30 seconds, ending with a final extension at 68°C for 2 minutes.

The concentration of the resulting library was measured again using the Synergy H1 (BioTek, Winooski, VT, USA) and QuantiFluor dsDNA System (Promega, Madison, WI, USA). Quality verification of the library was conducted using the Fragment Analyzer and the dsDNA 915 Reagent Kit (Agilent Technologies, Santa Clara, CA, USA).

## Sequencing

The metabarcoding was performed using Next Generation Sequencing (NGS) with the resulting PCR product/library. This sequencing was conducted on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (Illumina) with 500 cycles (Illumina, San Diego, CA, USA). The sequences were analysed using a paired-end sequencing approach (Reads 1 and 2) for the fragment ends, and the indexing method (Index-1 and Index-2 reads) for the indexes. Additionally, the first three bases at the start of the second PCR primers were excluded from Reads 1 and 2 using the "DarkCycle" option in the MiSeq Control Software (Illumina). The "DarkCycle" option allows certain sequencing cycles to be intentionally dark or unrecorded, meaning the sequencer does not collect data for these cycles. This is done because the first few bases may contain non-biological sequences or artifacts from the PCR primers ("Custom recipes for Illumina Stranded libraries on NovaSeq X Series | Illumina Knowledge," 2024).

### 2.2.3 Data sequencing and analysis

**Sequencing output**

Amplicon Sequence Variants (ASVs) offer a more precise classification than Operational Taxonomic Units (OTUs), going below the species level. In this study, the generation of the ASV file commenced with the Gikenbio lab pipeline, which included the collection and sequencing of PCR-generated libraries. This process resulted in raw sequence data in FASTQ format. Subsequent quality control and data preparation steps were carried out to ensure the integrity of the data and proper processing of the samples.

Reads matching the primer sequences were extracted using the 'fastx barcode splitter' tool from the FASTX-Toolkit (ver. 0.0.14, Gordon, 2024). Primer sequences were subsequently removed using 'fastxtrimmer', also part of the FASTX-Toolkit (ver. 0.0.13, "FASTX Barcode Splitter - Discovery Environment Applications List - Confluence," August-4-2024). Quality control was performed with sickle (ver. 1.33, Najoshi, 2024), which removed sequences with a quality score below 20 and discarded pairs where any read was shorter than 40 bases. Following this, paired-end reads were merged using the FLASH tool (ver. 1.2.11, (Magoc et al., 2011)) with a minimum overlap of 10 bases pairs.

For further analysis, QIIME2 (ver. 2024.2, ("QIIME 2," August-1-2024)) was employed. The DADA2 plugin was used to eliminate chimeric and noisy sequences, generating representative sequences compiled in repset.fasta and Amplicon Sequence Variant (ASV) tables in a CSV format. Each ASV was identified by matching its sequence against a database using a BLASTN algorithm (ver. 2.13.0) against the NCBI database for phylogenetic identification, providing detailed species identification. Top matches and detailed BLAST results were recorded, listing species with the highest similarity.

Table 8: Example of a row from the ASV file provided by Gikenbiolab following metabarcoding

| ASV_ID | V-S1 | ... | Accession_top1 | Target_top1 | Identity_top1 | Alignment_length_top1 | Species_top1 | ... |
|---|---|---|---|---|---|---|---|---|
| ASV_214 | 0 | ... | MT610968.1 | Trifolium_pratense | 99,708 | 342 | ムラサキツメクサ | ... |
| ... | ... | ... | ... | ... | ... | ... | ... | ... |

In table 8, the *#ASV_ID* column contains the unique identifier for each Amplicon Sequence Variant (ASV). ASVs are highly resolved genetic sequences used to identify and differentiate species in a sample; nevertheless, the numbers used to identify them are arbitrary and don't have a specific meaning. *V-S1* and similar columns (e.g., *V-S2*, *V-S3*, etc.) represent the sample identifier. Below is located the number of reads associated with this sample for a specific *ASV_ID*. The *Accession_top1* column contains the GenBank accession number of the top matching sequence in the database. It uniquely identifies the sequence entry in GenBank, which is a public database of nucleotide sequences. (Schoch et al., 2020; https://www.ncbi.nlm.nih.gov/genbank/). The *Target_top1* column is for the target organism identified as the closest match to the ASV (species name). The *Identity_top1* column shows the percentage of sequence identity between the ASV and the top matching sequence in the database. Higher percentages indicate closer matches. The *Alignment_length_top1* column represents the length of the sequence alignment used to calculate the identity percentage. It indicates how many base pairs were compared between the ASV and the reference sequence. The *Species_top1* column shows the common or scientific name of the species corresponding to the top matching sequence. Here it is basically the Japanese name of the *Target_top1* identification. Subsequent columns describe the top_2 to top_10 correspondences. However, for this analysis, we are exclusively using the top_1 information.

The Gikenbio lab already performed a data cleaning regarding *Identity_top1* and chose to select ASVs with a minimum 97% identity with evalue $\leq 1 \times 10^{-3}$, query cover 100 (Altschul et al., 1990). Indeed, it is the usual threshold used in metabarcoding (Joos et al., 2020; Knight et al., 2018).

**Output data modifications**

All the following statistics were performed in R ver. 4.3.3 with the Rstudio interface (RStudio, PBC, Boston, MA).

It was chosen to have a minimum Alignment length threshold to ensure the reliability of the identifications. Indeed, rows with an *Alignment_length_top1* lower than 150 were discarded (Noël 2023). Moreover all Genus level identifications OTU and non angiosperm OTU were removed. In fact the analysis focuses on plant species level.

ASV results offer higher resolution than OTU (Operational Taxonomic Unit) results. However, since the 2018 data were processed at the OTU level, the ASV data were converted to OTU data to ensure consistency. This conversion was achieved by merging ASVs based on the same species name in the *Identity_top1* column using the `dplyr` package (Wickham et al., 2023). Reads from same ASV rows were merged were summed together before converting all reads into presence/absence data (Bell et al., 2023; Lamb et al., 2019)

## 2.3   Vegetation community analysis

The subsequent analysis will focus on data across three main gradients: location, month, and year. Specifically, we aim to examine how the vegetation community evolves based on different sites (Kashiwanoha, Nishichiba, and Sumida), seasons (from April to December), and years of data collection (years 2018 and 2023).

### 2.3.1   Taxonomic community composition

**Phylogeny**

The phylogenetic composition of all samples was depicted using a phylogenetic tree to provide insights into the taxonomic distribution. The `Phyloseq` package (McMurdie et al., 2013), was utilised to manage and visualise phylogenetic data. Taxonomic information was extracted from the NCBI database using the `Taxize` package (Chamberlain et al., 2012), which facilitates taxonomic searches and data retrieval. An NCBI API key ("New API Keys for the E-utilities," 2017) was employed to access recent taxonomic information. The V.PhyloMaker package (Jin et al., 2023) was used to construct large-scale phylogenetic trees based on plant species data. Finally, the Phytools package (Revell, 2012) provided functions for manipulating and plotting the phylogenetic tree, allowing for both standard and circular tree visualisations.

**Jaccard dissimilarity**

To analyse the taxonomic composition of samples, the Jaccard dissimilarity metric from the `vegan` R package was used (Oksanen et al., 2012). The use of the Jaccard dissimilarity metric is appropriate because it is based solely on presence/absence data and is insensitive to abundance (in this context, reads)(Chung et al., 2019). Additionally, it allows for the comparison of multiple communities to understand their similarities and differences in species composition, which can be useful for tracking changes over time, such as between different years or months (Chung et al., 2019).

**Permanova**

Differences in plant community composition between months, locations, and landscape gradients were investigated using an Adonis test, also known as PERMANOVA. This was performed using the "adonis" function from the `vegan` R package (Oksanen et al., 2019). Unlike ANOVA, the Adonis test can handle multivariate data. Specifically, the Adonis test evaluates whether there are significant differences in community composition between predefined groups (Anderson, 2001; Chizinski, 2014). Adonis uses a common distance matrix (here Jaccard), to quantify dissimilarity between samples (Chizinski, 2014) (Anderson, 2001.). Additionally, the Adonis test is robust to the assumptions of multivariate normality and homogeneity of variances because it is a non-parametric method (Chizinski, 2014 ; Anderson, 2001.). Due to its use of permutations, the test statistic is recalculated for each permutation to build a distribution of the test statistic under the null hypothesis. Significance was measured using 999 permutations.

**Non-Metric Multidimensional Scaling**

The dissimilarities in plant community structures were visualised using non-metric multidimensional scaling (NMDS) with the Jaccard metric and 999 permutations. NMDS excels in representing relative dissimilarities within a single 2D plot, offering greater flexibility than other techniques like Principal Coordinates Analysis (PCoA) (Legendre et al., 2012).

### 2.3.2 Taxonomic diversity composition

**Venn diagrams**

The visualisation of species richness among sites and years was done using a Venn Diagram thanks to the VennDiagram R package. All the graphics were generated using the `ggplot` package (Wickham (2016)).

**Diversity metrics**

Not only the species richness, but other diversity metrics were computed, such as the Shannon Index, Simpson Index, and Chao1 Index, to have a comprehensive overview of biodiversity by considering different aspects like species richness (Chao1, Species Richness) and evenness (Shannon, Simpson). All these indices were computed with the `vegan` R package (Oksanen et al., 2012).

Species richness ($S$) is a measure of the number of different species present in a given ecological community (Chao, 2006). It provides an indication of biodiversity within that community.It was calculated using the formula: Species richness ($S$) was calculated using the formula:

$$S = \sum_{i=1}^{n} 1$$

where $n$ is the number of different species observed (Chao, 2006).

The Chao1 Index is used to estimate the total species richness in a community. It accounts for the number of rare species observed (i.e., singletons and doubletons) (Chao et al., 2014b).

$$\hat{S}_{\text{Chao1}} = S_{\text{obs}} + \frac{F_1^2}{2F_2}$$

Where:

- $S_{\text{obs}}$ = the number of observed species.

- $F_1$ = the number of species observed exactly once (singletons).

- $F_2$ = the number of species observed exactly twice (doubletons).

The Shannon Index (also known as the Shannon-Wiener or Shannon-Weaver Index) measures the entropy in the species distribution, taking into account both abundance and evenness (Spellerberg et al., 2003).

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

Where:

- $S$ = the total number of species (species richness).

- $p_i$ = the proportion of individuals belonging to the $i$-th species.

The Simpson Index measures the probability that two individuals randomly selected from a sample will belong to the same species (Simpson, 1949). Here is one common form, also known as Simpson's Diversity Index:

$$D = 1 - \sum_{i=1}^{S} p_i^2$$

Where:

- $S$ = the total number of species (species richness).

- $p_i$ = the proportion of individuals belonging to the $i$-th species.

## 2.4   Trait-based community analysis

Concerning the interaction between plants and pollinators, there exists a trade-off in the utilisation of traits (Greenop et al., 2023). On one hand, there are traits that are simple to measure, yet their role in pollination is only minimally supported by evidence (Fornoff et al., 2017; Pérez-Harguindeguy et al., 2013). On the other hand, there are traits that are more challenging to measure but demonstrate a stronger and more reliable connection to the delivery of pollination services (Fornoff et al., 2017; Greenop et al., 2023).

### 2.4.1   Ecological attributes

While status attributes such as plant origin and taxonomy are not functional traits, they are important ecological attributes. These statuses were recorded from reliable sources (primarily Kew Botanical Gardens) to provide context for the functional traits and their potential ecological impact. Ecological attributes are good to have an idea of the place of each plant species in the local environment context.

Table 9: Recorded ecological attributes

| Nr. | Ecological attribute | Definition |
|---|---|---|
| I | Plant origin | Either the plant is native or introduced. Every non-native plant would be considered. |
| II | Taxonomy | The classification of the plant in a hierarchical system. |
| II.A | Genus | The genus to which the species belongs. |
| II.B | Family | The family to which the species belongs. |
| II.C | Species | The specific species name. |

### 2.4.2   Functional trait selection and measurements

It is complicated to address functional traits that are hard to measure, especially chemical traits. As nectar concentration or odours (Phillips et al., 2020). In this context, the functional trait criteria selection is based on : [A] Traits relevant regarding the plant-bee interaction [B] traits that are found abundantly in serious online resources, [C] Easy to measure traits on herbarium or plant pictures. The trait description choice is described in the result part.

First a literature review was done to look at pertinent functional traits regarding the plant-bee interaction. Indeed, the review can be found in Table 2. Therefore, it was chosen to work with traits that are well-represented in online functional trait databases.

TRY database (Fraser, 2020) is the reference in terms of online plant trait database as it re-groups a lot of existing plant functional trait databases. However, there was a clear lack of data on a lot of plant functional traits that were relative to plant-bee interaction on this database. Indeed, TRY focuses mainly on European and North American plants (Fraser, 2020), it was not comprehensive enough for the study needs.

Then a customised database with all the chosen traits was created. A manual completion of the customised database was done with traits respecting the 3 criterias previously mentioned. The sourcing was done with traits that were readily available from serious online resources, such as the Kew Botanical Gardens ("Plants of the World Online | Kew Science," July-17-2024), iPlant ("iPlant" July-17-2024), Flora of China ("Flora of China @ efloras.org," July-17-2024) and World Flora Online (WFO) (https://www.worldfloraonline.org/, July-17-2024). Rarely, information was gathered on Wikipedia ("Wikipedia, the free encyclopedia," July-17-2024) or in Botanical centres websites.

Table 10: Plant functional traits used to calculate overall functional diversity (Fornoff et al., 2017)

| Nr. | Trait | Unit/coding | Levels/range/raw unit | Data structure | Trait type | Ecological importance | Definition |
|---|---|---|---|---|---|---|---|
| 1 | Plant height | m | 0.05 - 50 | Continuous | Quantitative continuous | Plant recognition | General maximum height that the plant will reach during its lifetime |
| 2 | Inflorescence size | mm | 0.7 - 800 | Continuous | Quantitative continuous | Flower recognition | General maximum diameter/size of inflorescence |
| 3 | Inflorescence colour | | | | | Flower recognition | Major colours of the inflorescence using RGB classification with (R/G/B) format |
| 3.1 | R | 0-255 | 0 - 255 | Integer | Quantitative discrete | | Red component of the inflorescence color using RGB classification |
| 3.1 | G | 0-255 | 0 - 255 | Integer | Quantitative discrete | | Green component of the inflorescence color using RGB classification |
| 3.1 | B | 0-255 | 0 - 255 | Integer | Quantitative discrete | | Blue component of the inflorescence color using RGB classification |
| 3.2 | Colour range | - | | | | | Inflorescence colour gradient along the species. Hyphen '-' separation representing the colour gradient along all inflorescence |
| 3.2 | Bi-colour | ; | | | | | Different distinct colours on the same inflorescence. Point-comma ';' separation between all the different colours of one inflorescence |
| 3.3 | Inflorescence colour type | 0-2 | Uni-colour = 0, Colour range = 1, Bi-color = 2 | Integer | Integer | Flower recognition | General attribution of whether the flowers are visible or not |
| 3.4 | Luminosity | 0-255 | 0 - 255 | Integer | Quantitative continuous | Flower recognition | Luminosity gradient based on the RGB value |
| 4 | Flower symmetry majority | 1/0 | Radial = 0, Bilateral = 1 | Integer | Qualitative nominal | Flower recognition | Major flower symmetry (Other symmetry types were replaced by NA) |
| 5 | Flower conspicuous majority | 1/0 | Yes = 1, No = 0 | Integer | Qualitative nominal | Flower recognition | General attribution of whether the flowers are visible or not |
| 6 | Flower reproductive time | 1-12 | January = 1, [...], December = 12 | Integer | Qualitative ordinal | First time of resource availability | General first flowering month |
| 7 | Life form majority | 1-5 | Therophyte = 1, Hemicryptophyte = 2, Geophyte = 3, Liana = 4, Chamaephyte = 5, Phanerophyte = 6 | Integer | Qualitative ordinal | Plant recognition | General raunkiaer life form attributed. Ordinary classed by a height hierarchy approximation |

For plant traits measured in length, the general maximum size recorded was used. This measurement was obtained from online sources or directly from herbarium pictures (e.g., Kew Botanical Gardens, iPlant) using the provided scale. For qualitative ordinal traits, the most common first measure present was chosen. For qualitative nominal traits, the measure with the highest occurrence was selected and attributed to the species' functional trait, sourced from online databases or pictures.

Colour-related traits were handled differently due to their qualitative and quantitative nature. RGB values of each inflorescence were measured for a precise quantitative approach. Those

RGB values were extracted using the Digital Color Meter on macOS Sonoma 14, and colour distribution was represented by gradients (hyphen "-") or distinct colours (semicolon ";"). To compute luminosity, or perceived brightness, the following formula was used, reflecting human visual sensitivity (Poynton, 2012): $Y = 0.2126 \times R + 0.7152 \times G + 0.0722 \times B$ . Colours that needed to be identified were named with the help of ChatGPT 4.0, which was used to name all the different colours extracted ("ChatGPT," August-6-2024). If an object had multiple colours, the first recorded colour was used for luminosity computation. In all cases, if information was unavailable, NA was recorded.

### 2.4.3   Functional diversity indices

Computations on functional diversity (FD) were carried out with the `FD` package (Laliberté et al., 2010). The library calculates a range of multidimensional indices, based on Principal Co-ordinates Analysis (PCoA). This process is done using the presence/absence species matrix per sample and the functional trait matrix that was constructed. However, the package could not handle NA in the functional trait matrix, so mean imputation was chosen for those missing values, replacing missing values (NA) in the dataset with the mean value of the respective trait (column).

Computed indices were functional richness (FRic), functional evenness (FEve), functional divergence (FDiv) (Villéger et al., 2010), and Rao's quadratic entropy (RaoQ) (Botta-Dukát, 2005). Each index independently measures the functional trait space and the distribution of species within this space. With the exception of FRic, all indices incorporate species abundance in the quantification of functional diversity (Festjens et al., 2023).

Functional Richness (FRic) represents the total amount of functional space filled by the community. It is measured as the minimum convex hull volume and has no upper limit (Villéger et al., 2010; Laliberté et al., 2010).

$$\text{FRic} = \text{Volume of the convex hull}$$

Functional Evenness (FEve) measures the evenness of species abundance distributions in functional trait space. It is calculated as the average branch length of the minimum-spanning tree weighted by relative abundance, and ranges between 0 and 1 (Villéger et al., 2010; Laliberté et al., 2010).

$$\text{FEve} = \frac{\sum_{i=1}^{S-1} l_i \left( \frac{p_i + p_{i+1}}{2} \right)}{L_{\text{total}}}$$

where $l_i$ is the branch length between species $i$ and $i+1$, $p_i$ and $p_{i+1}$ are the relative abundances of species $i$ and $i+1$, and $L_{\text{total}}$ is the total branch length of the minimum-spanning tree.

Functional Divergence (FDiv) evaluates how species are distributed within trait space by measuring the deviation of individual species from the average distance to the centroid of the convex hull. Values range from 0 to 1 (Villéger et al., 2010; Laliberté et al., 2010).

$$\text{FDiv} = \frac{\sum_{i=1}^{S} a_i \left| x_i - \bar{x} \right|}{\sum_{i=1}^{S} a_i \left| x_i \right|}$$

where $a_i$ is the abundance of species $i$, $x_i$ is the distance of species $i$ from the centroid of the functional space, and $\bar{x}$ is the mean distance of all species to the centroid.

Rao's Quadratic Entropy (RaoQ) is a generalised form of the Simpson index that quantifies the amount of trait dissimilarity between two random individuals in the community (Villéger et al., 2010; Laliberté et al., 2010, Botta-Dukát, 2005).

$$\text{RaoQ} = \sum_{i=1}^{S} \sum_{j=1}^{S} p_i p_j d_{ij}$$

where $d_{ij}$ is the dissimilarity between species $i$ and $j$, and $p_i$ and $p_j$ are the relative abundances of species $i$ and $j$.

A scatter plot was finally made to represent the relation between functional richness and species richness.

# 3 Results

In 2023 metabarcoding, from 2,764 ASVs, 242 OTUs were identified. In 2018, 288 OTUs were extracted, and after data filtering, 196 OTUs remained. After merging the resulting OTU matrix, 367 species were identified at the species level across all sites, years, and months. These species belong to 34 orders and 235 genera. The number of reads is not relevant for this study as only presence/absence data is being considered.

## 3.1 Ecological attribute overview

Most present species in the data in terms of occurrence across months, year and locations are Asterales, Lamiales, Rosales, Ericales, Sapindales and Fabales (Table 11).

| Order | Species | Order | Species | Order | Species | Order | Species |
|-------|---------|-------|---------|-------|---------|-------|---------|
| Asterales | 44 | Aquifoliales | 6 | Apiales | 12 | Malvales | 3 |
| Lamiales | 30 | Dipsacales | 6 | Caryophyllales | 12 | Oxalidales | 3 |
| Rosales | 31 | Saxifragales | 6 | Solanales | 12 | Vitales | 3 |
| Ericales | 25 | Gentianales | 5 | Malpighiales | 12 | Arecales | 2 |
| Sapindales | 25 | Liliales | 5 | Ranunculales | 11 | Commelinales | 2 |
| Fabales | 21 | Magnoliales | 5 | Brassicales | 10 | Piperales | 2 |
| Poales | 16 | Boraginales | 4 | Cornales | 10 | Proteales | 1 |
| Fagales | 14 | Celastrales | 3 | Asparagales | 7 | | |
| Myrtales | 13 | Cucurbitales | 3 | Laurales | 3 | | |

Table 11: Species distribution across all recorded orders in monthly aggregate samples

The diagram in Figure 4 is visually structured to show the phylogenetic relationships among the species, with branches indicating evolutionary lineages. The occurence of each species in the samples is indicated by the dots The coloured orders show an evenly distribution of orders, whit no predominant orders.
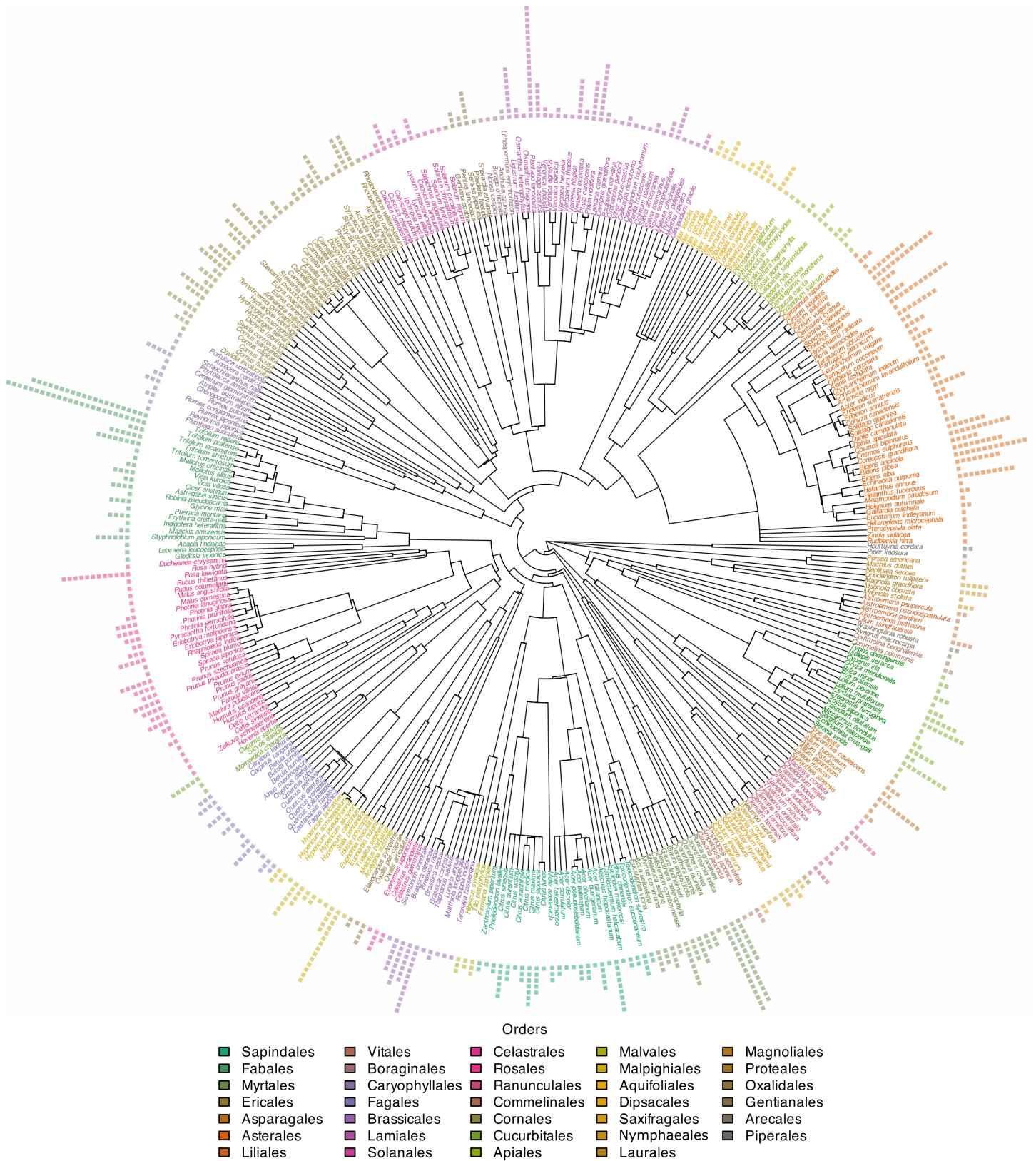
Figure 4: Phylogenetic diagram of all species-level identification in all samples, each dot after the species name represents the number of times the species was encountered in monthly aggregate samples.

Table 12 provides valuable insights into the taxonomic composition of pollen data across location an year. At the species level, *Trifolium pratense* and *Bidens pilosa* were among the most frequently recorded species, with other commonly observed species. At the genus level, *Trifolium* and *Bidens* showed high frequencies of occurrence. At the order level, Asterales was the most frequently occurring order with a particularly high occurrence in Nishichiba, while other orders like Fabales and Lamiales also appeared frequently but varied by location and year.

| | Species month occurrence | | | | Genus month occurence | | | | Order month occurence | | | |
| | 2018 | | 2023 | | 2018 | | 2023 | | 2018 | | 2023 | |
| | Species name | Month occurrence (n=5) | Species name | Month occurrence (n=5) | Genus name | Month occurrence (n=5) | Genus name | Month occurrence (n=5) | Order name | Month occurrence (n=5) | Order name | Month occurrence (n=5) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Kashiwanoha** | *Trifolium pratense* | 5 | *Oenothera rosea* | 5 | *Trifolium* | 14 | *Oenothera* | 10 | Asterales | 35 | Asterales | 17 |
| | *Centaurea cyanus* | 4 | *Plantago lanceolata* | 4 | *Brassica* | 6 | *Brassica* | 9 | Fabales | 23 | Myrtales | 17 |
| | *Mallotus barbartus* | 4 | *Trifolium pratense* | 4 | *Acer* | 5 | *Trifolium* | 8 | Lamiales | 18 | Lamiales | 15 |
| | *Trifolium repens* | 4 | *Trifolium repens* | 4 | *Oenothera* | 5 | *Prunus* | 6 | Ericales | 15 | Rosales | 14 |
| | *Aesculus hippocastanum* | 3 | *Brassica carinata* | 3 | *Phyla* | 5 | *Citrus* | 5 | Myrtales | 14 | Sapindales | 14 |
| | *Allium fistulosum* | 3 | *Brassica rapa* | 3 | *Allium* | 4 | *Cornus* | 5 | Sapindales | 14 | Ericales | 13 |
| | *Coreopsis grandiflora* | 3 | *Hypochaeris radicata* | 3 | *Centaurea* | 4 | *Ilex* | 4 | Poales | 13 | Fabales | 12 |
| | *Dichroa febrifuga* | 3 | *Ipomoea recta* | 3 | *Citrus* | 4 | *Plantago* | 4 | Fagales | 11 | Cornales | 12 |
| | *Erigeron annuus* | 3 | *Lolium multiflorum* | 3 | *Erigeron* | 4 | *Quercus* | 4 | Brassicales | 10 | Malpighiales | 9 |
| | *Lagerstroemia indica* | 3 | *Adinandra millettii* | 3 | *Mallotus* | 4 | *Acer* | 3 | Rosales | 9 | Solanales | 9 |
| | **Species name** | **Month occurrence (n=6)** | **Species name** | **Month occurrence (n=9)** | **Genus name** | **Month occurrence (n=6)** | **Genus name** | **Month occurrence (n=9)** | **Order name** | **Month occurrence (n=6)** | **Order name** | **Month occurrence (n=9)** |
| **Nishichiba** | *Trifolium pratense* | 6 | *Bidens alba* | 5 | *Trifolium* | 22 | *Bidens* | 14 | Fabales | 37 | Asterales | 60 |
| | *Trifolium repens* | 5 | *Bidens pilosa* | 4 | *Oenothera* | 8 | *Trifolium* | 10 | Asterales | 35 | Lamiales | 27 |
| | *Trifolium tomentosum* | 5 | *Cosmos sulphureus* | 4 | *Brassica* | 7 | *Brassica* | 9 | Lamiales | 23 | Ericales | 23 |
| | *Centaurea cyanus* | 4 | *Plantago lanceolata* | 4 | *Phyla* | 6 | *Citrus* | 9 | Myrtales | 21 | Rosales | 23 |
| | *Lagerstroemia indica* | 4 | *Trifolium pratense* | 3 | *Plantago* | 6 | *Camellia* | 8 | Rosales | 17 | Sapindales | 20 |
| | *Oenothera rosea* | 4 | *Bidens andicola* | 3 | *Carpinus* | 5 | *Cornus* | 8 | Ericales | 14 | Myrtales | 19 |
| | *Phyla canescens* | 4 | *Brassica carinata* | 3 | *Prunus* | 5 | *Cosmos* | 8 | Poales | 13 | Fabales | 17 |
| | *Plantago lanceolata* | 4 | *Brassica rapa* | 3 | *Allium* | 4 | *Oenothera* | 8 | Fagales | 11 | Fagales | 12 |
| | *Trifolium strictum* | 4 | *Hypochaeris radicata* | 3 | *Centaurea* | 4 | *Plantago* | 7 | Brassicales | 10 | Brassicales | 10 |
| | *Brassica napus* | 3 | *Oenothera rosea* | 3 | *Cosmos* | 4 | *Photinia* | 6 | Malpighiales | 8 | Malpighiales | 9 |
| | | | **Species name** | **Month occurrence (n=9)** | | | **Genus name** | **Month occurrence (n=9)** | | | **Order name** | **Month occurrence (n=9)** |
| **Sumida** | | | *Bidens pilosa* | 5 | | | *Bidens* | 11 | | | Asterales | 26 |
| | | | *Trifolium pratense* | 5 | | | *Citrus* | 9 | | | Rosales | 19 |
| | | | *Bidens andicola* | 4 | | | *Trifolium* | 9 | | | Lamiales | 15 |
| | | | *Trifolium repens* | 4 | | | *Photinia* | 7 | | | Sapindales | 15 |
| | | | *Ampelopsis aconitifolia* | 3 | | | *Camellia* | 6 | | | Ericales | 13 |
| | | | *Cirsium vulgare* | 3 | | | *Hypericum* | 5 | | | Fabales | 12 |
| | | | *Gazania splendens* | 3 | | | *Cirsium* | 4 | | | Brassicales | 10 |
| | | | *Lagerstroemia indica* | 3 | | | *Papaver* | 4 | | | Poales | 6 |
| | | | *Lolium multiflorum* | 3 | | | *Plantago* | 4 | | | Apiales | 5 |
| | | | *Papaver rhoeas* | 3 | | | *Verbena* | 4 | | | Caryophyllales | 5 |

Table 12: List of the top species, genus and order by month presence frequency for each site and year. The month occurrence is the number of times a species was encountered regarding each month, location and time combination. N is the number of months recorded for each modality.

Regarding ever the species encountered were native or introduced, of all species with the recorded plant origin, 67,1% were considered as introduced while 32.9% were considered native.

## 3.2   Taxonomic richness and diversity

### 3.2.1   Community composition

The NMDS plot (Figure 5) shows variability in species composition across all samples and months. The plot reveals distinct clusters corresponding to different months, indicating variations in species composition throughout the year. Winter months tend to group together, while spring months, such as April and May, form separate clusters. The monthly grouped plant communities are arranged along a cyclical gradient throughout the year, with consecutive months exhibiting similar community compositions to their neighboring months.
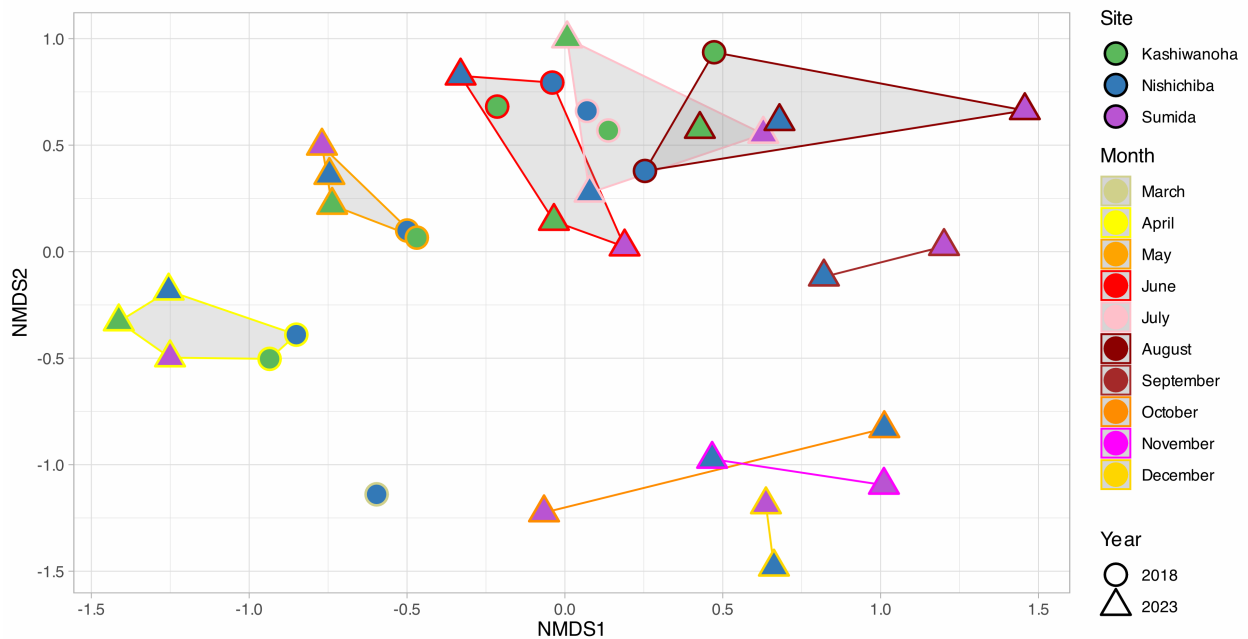


Figure 5: Non-metric multidimensional scaling (NMDS) of plant communities based on monthly grouped pollen incidence data. Each fill colour represents a site, each outer colour and lines corresponds to a month group. Each dot shape corresponds to a year.

The permutational multivariate analysis of variance (PERMANOVA) revealed significant effects of month, site, and year on the species composition of plant communities.

The month had the strongest effect on species composition ($R^2 = 0.540$, F = 3.83, p = 0.001). This indicates a pronounced seasonal pattern in the plant communities, with different species dominating in different months.

There was also a significant effect of site on species composition ($R^2 = 0.0561$, F = 1.79, p = 0.014). This suggests that the geographical location influences the species present, likely due to local environmental conditions and habitat characteristics.

The year showed a significant effect as well ($R^2 = 0.076$, F = 4.84, p = 0.001), indicating

interannual variability in species composition.

To have a more visual approach regarding the year disparity across samples, the year-oriented comparison between Kashiwanoha and sumida between 2018 and 2023 was done (Figure 6). Indeed, a clear separation can be seen between years, the top dots of the diagram are from 2023 while the bottom dots are from 2018.
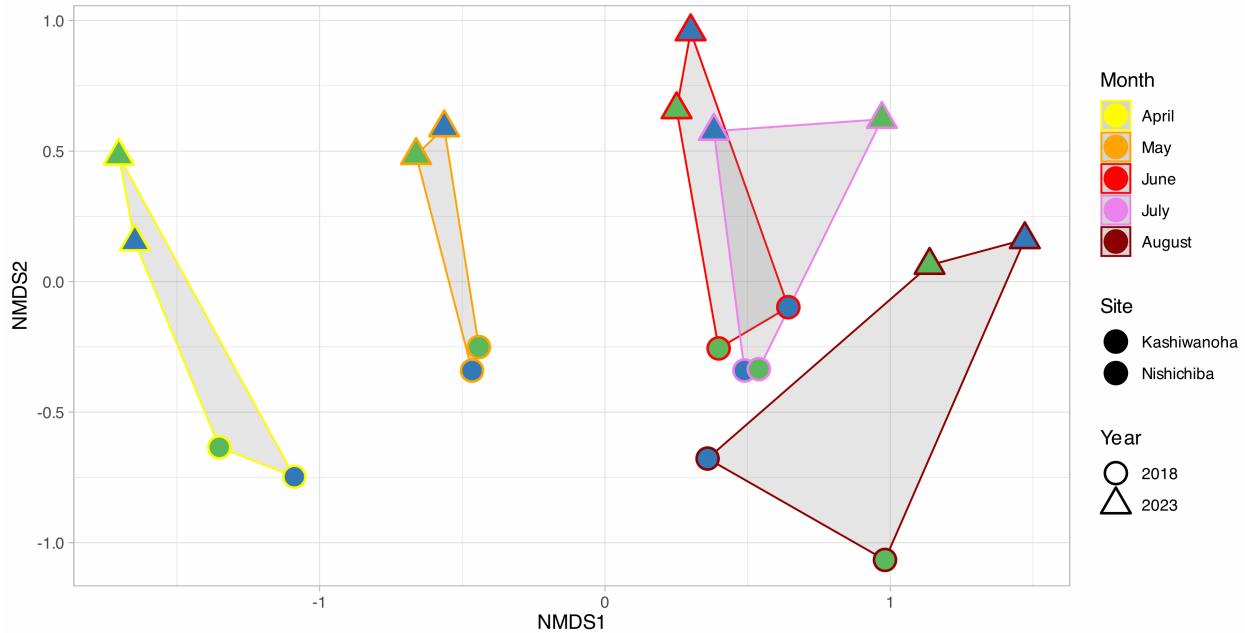


Figure 6: Non-metric multidimensional scaling (NMDS) of plant communities based on monthly grouped pollen incidence data (Year oriented comparison). Each fill colour represents a site, each outer colour and lines corresponds to a month group. Each dot shape corresponds to a year.

### 3.2.2 Taxonomic diversity indices comparison

Figure 7 presents a Venn diagram illustrating the overlap of plant species between Nishichiba and Kashiwanoha for the months of April to August in the years 2018 and 2023. All four year-site combinations share 30 species in common. Additionally, each year-site combination has approximately 40 species that are unique to that specific year and site.
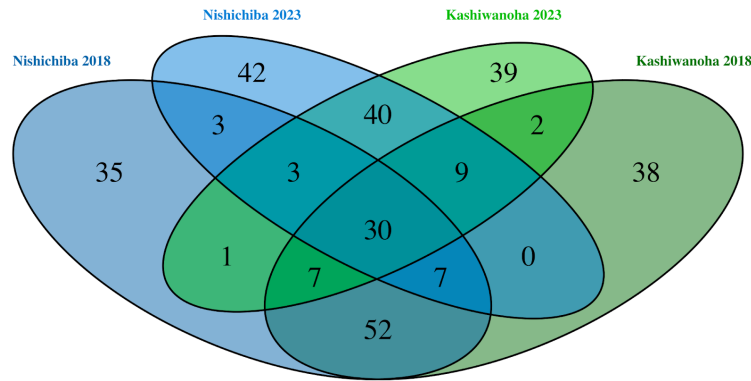
Figure 7: Venn Diagram illustrating the overlap of plant species within Nishichiba and Kashiwanoha in 2023 over April to August (Year Oriented Comparison).

Figure 8 presents a Venn diagram illustrating the overlap of plant species between Nishichiba and Sumida for the months of April to December in the year 2023. Both sites share 79 species in common. However, only 37 species are specific to Sumida, compared to 103 species specific to Nishichiba, indicating an important difference in species richness between the two sites, with Nishichiba being notably richer in plant species.
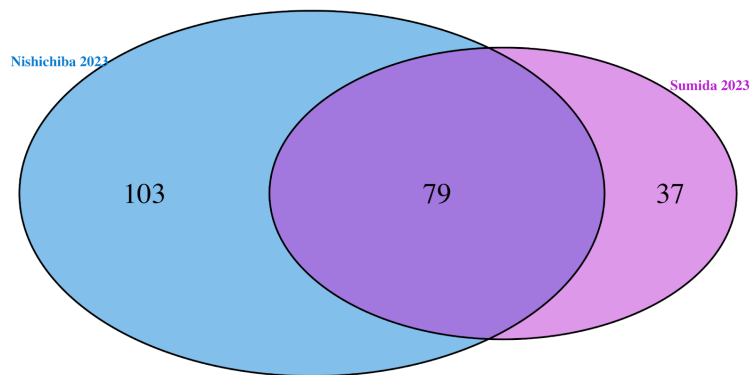


Figure 8: Venn Diagram illustrating the overlap of plant species within different Year (2018 and 2023) and location over April to December (Month Oriented Comparison).

The species richness GLM was performed using a Poisson distribution to model the count data. The results indicate that Month, Site, and Year are all significant predictors of species richness. The analysis details are as follows:

Month had the largest effect, showing a highly significant impact on species richness ($R^2 = 0.623$, $p < 2.20e\text{-}16$). Site also had a substantial effect, demonstrating significant influence on

species richness ($R^2$ = 0.200, p < 7.64e-13). Year, while having a smaller effect compared to Month and Site, still significantly influenced species richness ($R^2$ = 0.0260, p = 0.007). Detailed GLM results show that species richness in 2023 was significantly lower compared to 2018 (Estimate = -0.185).

For all indexes comparing Kashiwanoha and Nishichiba (Figure 9), the data shows that in 2018, the indexes peaked in May. In contrast, the 2023 indexes are more evenly distributed across the five months from April to August. In both cases, the indexes tend to be at their lowest in August.
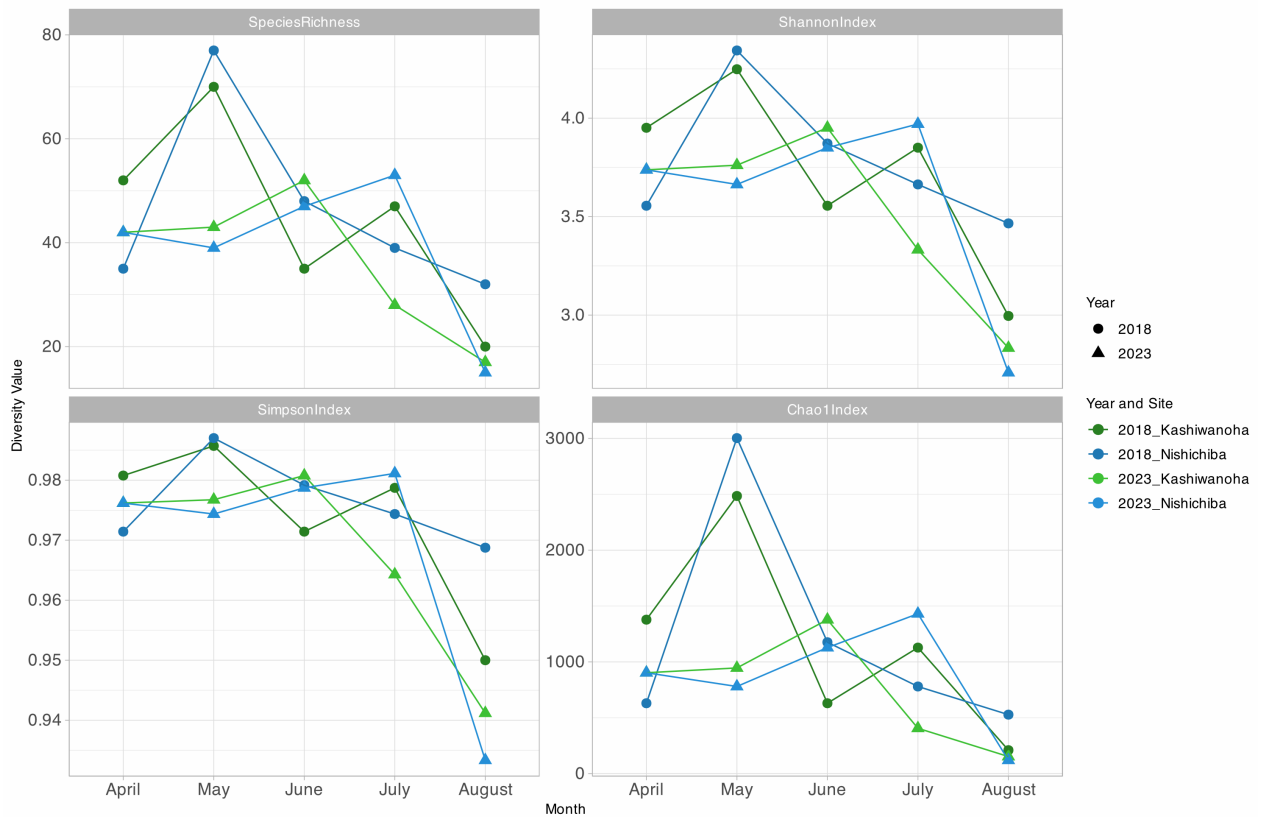


Figure 9: Richness diversity metrics (Year oriented comparison). Each fill colour represents a site. Each dot shape corresponds to a year. Lines connect points along months.

Diversity indexes comparing Nishichiba and Sumida across all months consistently show that Sumida has lower indexes than Nishichiba (Figure 10). While Nishichiba's indexes exhibit clear seasonal peaks, Sumida does not display a distinct positive peak in its indexes throughout the year. However, Sumida does experience a noticeable negative peak in October.

Figure 10: Richness diversity metrics (Year oriented comparison). Each fill colour represents a site. Lines connect points along months.

## 3.3   Functional traits analysis

In total, out of the 8 functional traits selected for the 367 species, 623 records in the functional trait matrix were NA, and 2,928 trait observations were recorded.

### 3.3.1   Functional traits measures comparison

Colours traits, being difficult traits to quantify and analyse, are displayed to have better insights in figure 11.
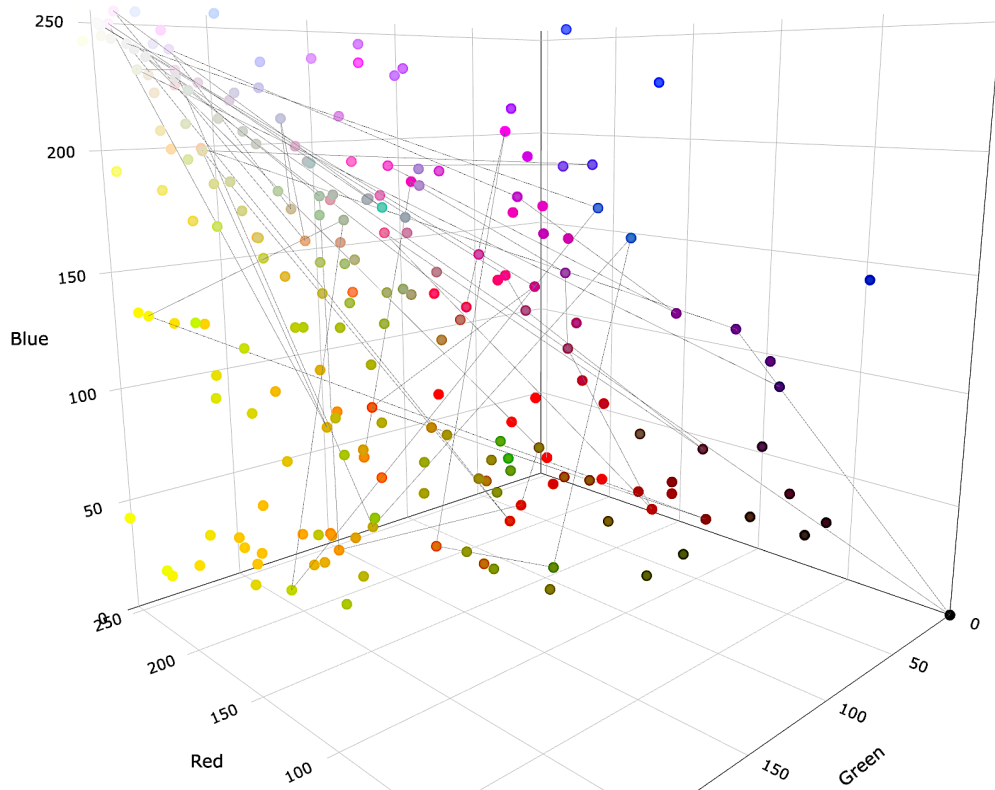


Figure 11: RGB representation of all inflorescence colours in a RGB 3D space in all used sample. Dot representing the inflorescence colour of the plant, links between dots indicate flowers that have a colour gradient between 2 colours.

The colours of inflorescences were analysed in the 3D space of Figure 12 by dividing it into 50 x 50 x 50 volumes. The centroid of each RGB volume was then calculated, and each centroid's RGB values were associated with a corresponding colour. These colours were subsequently displayed in a colour barplot to visualize their distribution.

Three different colours were above the mean plus standard deviation line: light gray, Peru (orange), and light goldenrod yellow. These colours are considered to be clear ones as they are on the right side of the figure.
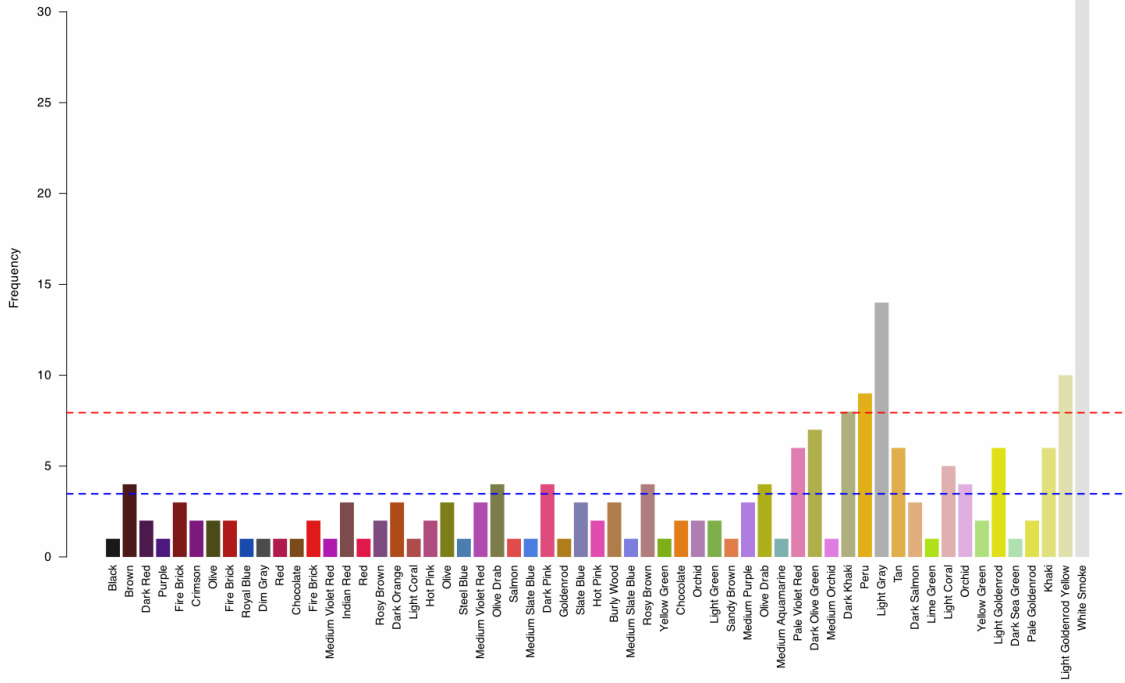
Figure 12: RGB representation of all inflorescence colour in a barplot along a luminosity gradient (From dark to clear), the blue line represents the mean, the red line represents the mean plus standard deviation

Table 13: Summary table of the selected functional traits analysed by Generalized Linear Model (GLM)

| Model | Response | Factor | Df | Deviance | Resid. Df | Resid. Dev | Pr(>Chi) | Significance |
|---|---|---|---|---|---|---|---|---|
| Gamma, log link | Plant height | Month | 9 | 155,00 | 940 | 1.58e+03 | 1.79e-14 | *** |
| | | Year | 1 | 22.6 | 939 | 1.56e+03 | 0.000443 | *** |
| | | Location | 2 | 0.344 | 937 | 1.56e+03 | 0.91 | |
| Gamma, log link | Inflorescence size | Month | 9 | 98.3 | 873 | 1.41e+03 | 1.18e-08 | *** |
| | | Year | 1 | 7.12 | 872 | 1.41e+03 | 0.0458 | * |
| | | Location | 2 | 6.31 | 870 | 1.4e+03 | 0.171 | |
| Gaussian, identity link | Luminosity | Month | 9 | 2.91e+04 | 939 | 3.22e+06 | 0.488 | |
| | | Year | 1 | 848 | 938 | 3.22e+06 | 0.619 | |
| | | Location | 2 | 3.28e+03 | 936 | 3.22e+06 | 0.621 | |
| Binomial, logit link | Flower symmetry | Month | 9 | 28.5 | 668 | 620 | 0.000794 | *** |
| | | Year | 1 | 1.64 | 667 | 618 | 0.201 | |
| | | Location | 2 | 1 | 665 | 617 | 0.605 | |
| Binomial, logit link | Flower conspicuous | Month | 9 | 18.8 | 972 | 955 | 0.0272 | * |
| | | Year | 1 | 0.121 | 971 | 954 | 0.728 | |
| | | Location | 2 | 1 | 969 | 953 | 0.605 | |
| Poisson, log link | Flower reproductive time | Month | 9 | 83.9 | 765 | 427 | 2.72e-14 | *** |
| | | Year | 1 | 0.996 | 764 | 426 | 0.318 | |
| | | Location | 2 | 0.086 | 762 | 425 | 0.958 | |
| Multinomial, logit link | Life form majority | Month | 54 | 150 | | | 6.17e-11 | *** |
| | | Year | 6 | 37.1 | | | 1.65e-06 | *** |
| | | Location | 12 | 6.71 | | | 0.876 | |
| Multinomial, logit link | Inflorescence type | Month | 18 | 44.1 | | | 0.000561 | *** |
| | | Year | 2 | 12.3 | | | 0.00209 | ** |
| | | Location | 4 | 1.01 | | | 0.908 | |

Multinomial models did not compute the different residuals. Additionally, their degrees of freedom are different since they are computed as (number of levels of Response-1) x (umber of levels of the predictor-1).

### 3.3.2   Functional diversity indices comparison

Table 14: Summary table of the functional diversity metrics analysed by Generalized Linear Model (GLM)

| Model | Response | Predictor | Df | Deviance | Residual Df | Residual Deviance | Pr(>Chi) | Significance |
|---|---|---|---|---|---|---|---|---|
| Gamma, log link | FRic | Month | 9 | 16.2 | 24 | 10.9 | 2.85e-14 | *** |
| | | Year | 1 | 0.234 | 21 | 6.19 | 0.271 | |
| | | Location | 2 | 4.52 | 22 | 6.42 | 8.27e-06 | *** |
| Gamma, log link | FEve | Month | 9 | 0.0385 | 24 | 0.0861 | 0.041 | * |
| | | Year | 1 | 0.000104 | 21 | 0.0455 | 0.828 | |
| | | Location | 2 | 0.0405 | 22 | 0.0456 | 9.79e-05 | *** |
| Gaussian, identity link | FDiv | Month | 9 | 0.0141 | 24 | 0.0215 | 0.0697 | . |
| | | Year | 1 | 0.00274 | 21 | 0.0186 | 0.0788 | . |
| | | Location | 2 | 0.000163 | 22 | 0.0213 | 0.912 | |
| Gaussiann, identity link | RaoQ | Month | 9 | 50.5 | 24 | 42.7 | 2.15e-07 | *** |
| | | Year | 1 | 1.89 | 21 | 21.9 | 0.178 | |
| | | Location | 2 | 18.9 | 22 | 23.8 | 0.000118 | *** |
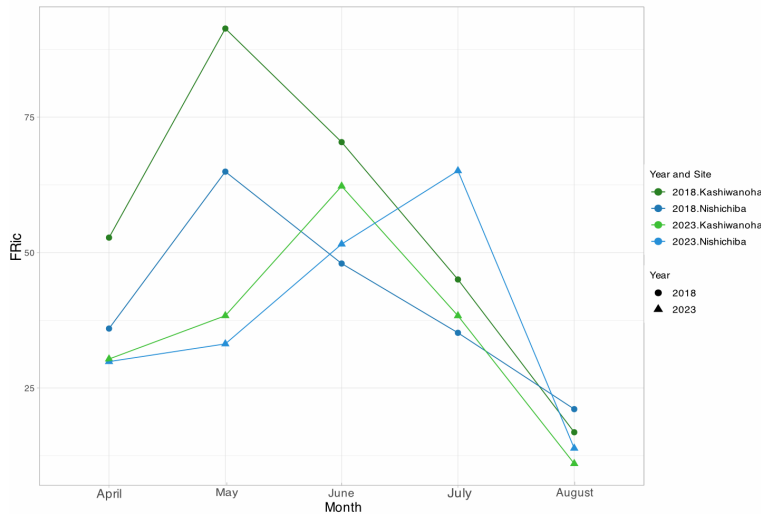


Figure 13: Functional richness (FRic) distribution accross sites and year combination throughout months (Year oriented comparison). Each fill colour represents a site. Lines connect points along months.

The linear model investigating the relationship between Functional Richness (FRic) and Species Richness yielded significant results (F-statistic = 117.6, p-value = 3.01e-12).
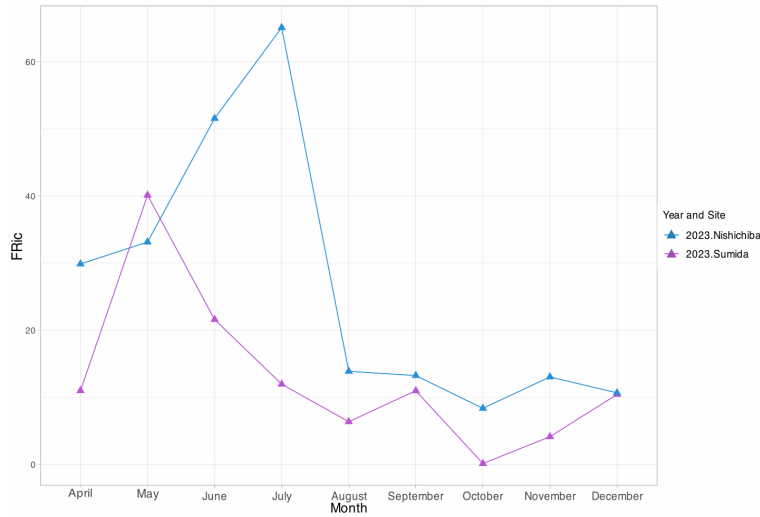
Figure 14: Functional richness (FRic) distribution accross sites and year combination throughout months (Month oriented comparison). Each fill colour represents a site. Lines connect points along months.
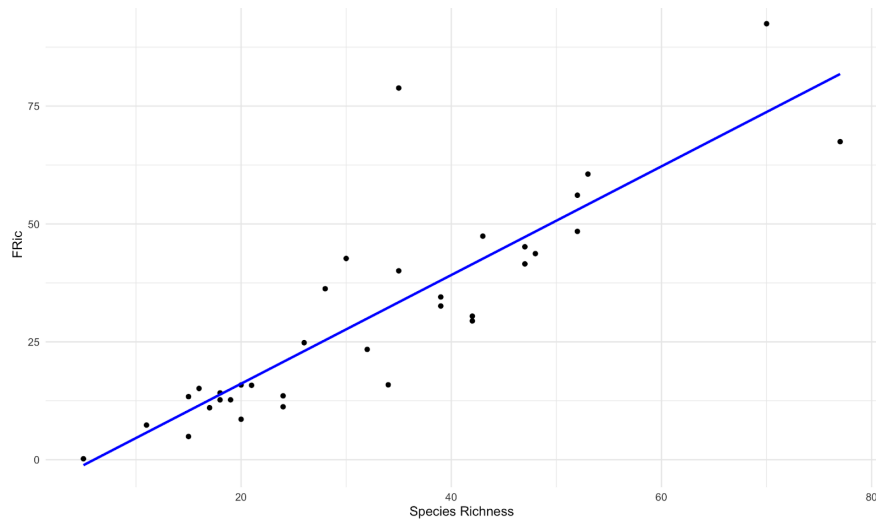


Figure 15: Scatter plot drawing the relation between species richness and functional richness of foraged plants by *A. mellifera*

The model equation is:

$$\text{FRic} = -8.89 + 1.2 \times \text{Species Richness}$$

The slope (1.20) is highly significant (t-value = 10.8, p-value = 3.01e-12), indicating a strong significant positive relationship. The model explains approximately 78% of the variance in FRic (R-squared = 0.786, adjusted R-squared = 0.779).

# 4 Discussion

This research aimed to gain a better understanding of *A. mellifera* ecology in urban spaces by examining the taxonomic range and functional diversity of foraged plants over different months, years, and locations thanks to their pollen analysis. Additionally, the study sought to improve our understanding of the relationship between the species richness and the functional richness of plants foraged by *A. mellifera*.

Pollen was collected from 3 different sites between 2018 and 2023, over a span of 10 months from March to December. Thanks to DNA metabarcoding, the pollen composition of the samples was identified, leading to the identification of 367 species. The generated data did not account for metabarcoding reads number and only considered species presence/absence (Bell et al., 2023).

## 4.1 Taxonomic richness and diversity discussion

**Taxonomy**

Regarding the taxonomy of the different foraged plants identified, it is common for certain species to be prevalent across multiple months. Indeed, species like *Trifolium pratense* and *Trifolium repens* are known to be preferred by pollinators (Ghosh et al., 2020), especially in urban areas (Kanduth et al., 2021), which could explain their frequent occurrence. Regarding, *Bidens pilosa* is identified as a species that bees often forage on, however, its nectar can be toxic and potentially harmful to bees in high doses due to its secondary metabolites (Tang et al., 2023; Palmer-Young et al., 2019). Interestingly, bees can develop a resilient gut microbiome that helps them survive despite the presence of toxic food sources such as *Bidens pilosa* (Tang et al., 2023; Danner et al., 2017). This suggests an important balance to consider between having a high abundance of certain plants that bees forage on and the potential toxicity of these plants. Bees might often forage on these plants, despite the risk of toxicity, due to their ability to develop a resilient gut microbiome (Tang et al., 2023) and possibly due to a lack of resources in their environment. In the majority of cases, the top plants identified are classified as cosmopolitan ("Plants of the World Online | Kew Science," July-17-2024), with most being non-native to Japan. This can be explained by the fact that *A. mellifera* is also a cosmopolitan species and not native to Japan (Purdy, 2024; Sarot, 2024).

The dominance of genera like *Trifolium* and *Brassica* is typical (Hawkins et al., 2015). The *Trifolium* genus provides the most protein for bees (Ghosh et al., 2020), so it is normal that its occurrence is high in pollen balls, which are the main protein source entering the hive. Genera like *Bidens* and *Oenothera* are also common in disturbed habitats (Centre national de données et d'informations sur la flore de Suisse, August 4, 2024), reflecting the environmental conditions at these sites.

The prominence of the order Asterales Fabales and Lamiales is usual, as it includes a large number of species known for their wide ecological amplitude and ability to colonise diverse

habitats explaining their high occurrence (Olivieri et al., 2012; Takhtajan, 2009).

This prevalence of some taxa can be explained by floral abundance, honest signaling and fidelity. Indeed, the bee might start to forage with an abundant flower as *Trifolium Pratense*, then if the flower provides a high enough reward to the bee (honest signaling), the bee will start to focus on this flower only and becomes faithful to this flower (floral fidelity) (Amaya-Márquez, 2009; Knauer et al., 2015).

### Community composition

The NMDS plot and PERMANOVA results reveal significant seasonal variations in species composition, with distinct clusters for different months, winter months grouping together, and notable shifts in spring, influenced by month, site, and year. These findings suggest that temporal factors, particularly the time of year, play a crucial role in shaping plant community composition. Comparing these results with similar studies, such as Noël et al. (2023), a seasonal gradient is also evident. Additionally, in studies analysing hobeybee's products like honey and beebread, this seasonal shift is observed as well (Leponiemi et al., 2023).

### Species richness

Species richness showed notable variations across the months for all sites and years. Those variations proved to be significant through the GLM applied on species richness. Kashiwanoha and Nishichiba have a pretty similar species richness; their evolution through the year seems to be really year-dependent, indices following a specific year pattern. While Nishichiba and Kashiwanoha tend to have significantly higher species richness overall than Sumida. This suggests that Nishichiba and Kashiwanoha have a more diverse array of flowering plants available throughout the year compared to Sumida (more urban). Another study found that pollen species richness of bees and wasps increases with distance from city centres. Additionally, insect richness also increases as urbanisation decreases (Dürrbaum et al., 2023).

Plus, it has been proven that in some cities , species richness would be higher in cities because of the fact that ornamental plants placed by humans are artificially increasing species richness (Wania et al., 2006; Marquardt et al., 2021). This particularity was not shown here, maybe due to the fact that honeybees foraged a lot of non-ornamental species (Table 12).

### Other diversity metrics

The Shannon Index followed similar patterns to species richness, showing a sharp increase in May 2018 followed by a decline, and higher values in Nishichiba in 2023, indicating more even species distribution. Sumida had lower values with a mid-year drop, indicating lower diversity. The Simpson Index remained relatively high, indicating no single species was overwhelmingly dominant, with slight fluctuations in 2018 and stable values in Nishichiba in 2023, but a significant drop in Sumida in October. The Chao1 Index showed significant peaks in May 2018 and higher values in Nishichiba throughout 2023, reflecting the presence of many rare species, while Sumida remained low, indicating less overall diversity. In terms of the Shannon index, a previous study found that it was not location-dependent but varied significantly over

months (Danner et al., 2017). The present study, however, indicates that location also plays a significant role, maybe due to the fact that Sumida is very urbanised.

## 4.2    Functional traits discussion

The analysis then focused on the functional trait compositions across site, year, and location. It examined various plant traits, including height, inflorescence size, inflorescence luminosity, flower symmetry, flower conspicuousness, flower reproductive time, life form majority, and inflorescence type. Such an approach is rather uncommon in studies analysing plants foraged by bees, which stops most of the time after assessing taxonomic diversity and some ecological attributes (Leponiemi et al., 2023; Noël et al., 2023).

### Inflorescence colour

Connections in the RGB 3D space (Figure 11) suggest many plants exhibit colour gradients, often trending towards lighter hues, while Figure 12 shows bees may prefer high-luminosity flowers, indicated by the prevalence of white and clear inflorescences. This preference can be explained by the need for inflorescences to contrast with their background, usually dark green leaves, to be visible to bees (Rohde et al., 2013; Bukovac et al., 2017). Therefore, it is logical that flowers need to be lighter to create this necessary contrast. This statement is also valuable for all pollinators that use visualisation to forage flowers, however, pollinators that rely more on floral bouquet are less dependent on flower colour (Milet-Pinheiro et al., 2021; Rachersberger et al., 2019).

### Functional traits

The results of the different models analysing various floral traits reveal significant patterns and insights.

Except for luminosity, all the functional traits showed significant changes throughout the different months of the year. This suggests that seasonality greatly influences functional trait measurements and should be considered in studies involving these traits (Römermann et al., 2016).

Height, inflorescence size, life form majority, and inflorescence type also varied significantly across years, indicating that the functional diversity of species is influenced by the year context in itself.

Regarding location, no significant differences were identified for any traits independently. This suggests that, at the functional trait level, the functional traits foraged by bees remain consistent across sites. It could indicate that bees tend to search for the same specific range of functional traits in plants, whatever the year, the time of the year and the location.

By examining functional traits independently, it was observed that plant height (and, to some extent, the predominant life form) was generally greater during the early months (Figure 18, Appendix part). This suggests that bees tend to forage on taller plants, such as trees,

more frequently in early spring. This trend likely occurs because trees typically bloom earlier than other plant species. Indeed, Bertrand et al. (2019) observed a significant shift in pollen sources for bees, especially honeybees, from predominantly trees in the spring to primarily herbaceous plants in the summer (see figure in the appendix). This pattern is likely influenced by plant phenology, as many dominant flowering trees and shrubs bloom relatively early, providing abundant floral resources early in the season (Requier et al., 2015).

Conversely, inflorescence size tends to peak at the end of summer (Figure 19, Appendix part). Although no specific bibliography was found describing its evolution across seasons, Brunet et al. (2021) emphasise that bees prefer flowers with larger display sizes when given a choice.

There is a more balanced proportion of flower symmetry during April, May, and June, which explains the significance of these months (Figure 23, Appendix part). Nevertheless, radial inflorescences are more common than bilateral ones.

Flower conspicuousness, except in Sumida, tends to be less conspicuous, especially in July and September (Figure 22, Appendix part). This may suggest that due to lower species richness and diversity, honeybees in Sumida have to search more extensively for inflorescences and will forage on less visible flowers as these are their only available food sources. This hypothesis aligns with Danner et al. (2017), who suggest that bees in areas with lower plant diversity will increase their pollen foraging range.

Reproductive time activity peaked in April, May, and June (Figure 21, Appendix part). This distribution aligns with the fact that the majority of plants flower in spring and early summer (Deng et al., 2019).

Regarding inflorescence type, it can be observed that towards the end of the year, inflorescences tend to be more colourful compared to those in early spring (Figure 24, Appendix part). This aligns with research indicating that flower colours are influenced by climatic factors and seasonal changes (Erickson et al., 2022).

**Functional diversity**

The results of the models analysing various functional diversity metrics (FRic, FEve, FDiv, and RaoQ) across months, sites, and years provide several significant insights. Plus, all indices exhibited significant variation across months, and all except FDiv showed significant differences across locations. Interestingly, functional diversity was almost significant regarding months and year.

Functional Richness (FRic) varies throughout the year and across different locations, indicating significant environmental and plant community differences, but remains consistent across years. Functional Evenness (FEve) changes seasonally and locally, reflecting shifts in dominant species and their traits. Functional Divergence (FDiv) shows slight temporal variation, with the spread of traits relative to the center of the trait space remaining stable. Rao's Quadratic Entropy (RaoQ) reveals that overall functional diversity changes throughout the year and differs across locations.

The results in Table 11 and 12 show that individual functional traits can exhibit significant yearly variation due to their sensitivity to specific environmental factors, while diversity indices, being more aggregated measures, might not show significant annual variation unless there are substantial shifts in community structure (Fu et al., 2023).

Conversely, the results indicate that functional diversity indices reveal significant variations across locations, as they integrate and measure complex interactions between functional traits and species. In contrast, analyses of individual traits may miss this complexity and appear more homogeneous across sites.

**Relation between functional richness and species richness**

The significant positive slope between functional and species richness indicates that as species richness increases, functional richness (FRic) also increases. Agreeing with **H5**, this suggests that areas with higher species diversity tend to have higher functional diversity. The model explains a substantial proportion of the variance in FRic, indicating that species richness is a strong predictor of functional richness.

This relation is not only for plant traits relating to flowers and pollination, but also for seed-related traits (Pallavicini et al., 2020). Furthermore, this kind of positive relationship between both indexes is also valuable in other ecology studies (Van De Perre et al., 2020). This suggests that for the majority of plant traits, there is a clear relation with species richness. The results highlight the importance of species diversity in maintaining functional diversity within ecological communities.

All of this highlights the reliance of bees on the availability of local food sources. Plus, this underscores the importance of species diversity in maintaining functional diversity within ecological communities. In other words, preserving or enhancing species richness in an area is likely to lead to a corresponding increase in the variety of functional traits present, which can enhance ecosystem stability and resilience. Furthermore, more traits can also be interesting for other pollinators and insects in general.

## 4.3   Predictors discussion

Months, years, and location were significant predictors of community composition, most of the selected functional traits, and most of the functional diversity metrics. Several factors can explain these differences.

Before discussing each predictor individually, it is important to consider some overarching factors that could account for the observed variations across months, years, and locations.

First, factors such as pests, diseases, pesticides, and competition with other pollinators can limit honeybee populations (Abudulai et al., 2022; Michez, 2019; Monceau et al., 2014). These factors can negatively impact colony health by reducing the number of bees, which in turn

may restrict the colony's foraging range and efficiency.

Additionally, honeybee colonies may adjust their feeding behavior depending on factors such as the beekeeper's decision to harvest more or less honey and the meteorological conditions of the year (Overturf et al., 2022; Steinhauer et al., 2021).

Since the bee colony operates as a superorganism, its foraging behavior can change over time and location as a unique entity (Page, 2012). In this organism, the presence of elite foragers, which select and return to certain flowers, may influence the overall foraging behavior of the colony (Amaya-Márquez, 2009; Klein et al., 2019). However, this elite-forager hypothesis is more relevant in areas with higher overall richness and diversity, as foraging availability is limited in urban environments.

## Month differences

Months were nearly all the time significant predictors for all the different tests. This significance can be explained by the seasonal shift in local plant communities. For example, regarding community composition, Danner et al. (2017) and Noël et al. (2023) also identified a special seasonal shift regarding foraged plant diversity.

This difference in months also impacted species richness, with significantly fewer species present in the winter months. As expected, the lower species richness during these months negatively affected functional richness, as the two are linearly correlated.

It also can be explained by the fact that the hive reaches its peak activity in spring and early summer, leading to a higher number of bees recruited for foraging (Brunet et al., 2024). Consequently, the monthly species richness may depend on the number of bees. However, one of the most probable hypotheses is that the main bee activity seasons coincide with the peak blooming periods of plants. Bees and plants may synchronise their activities to ensure pollination for plants and food availability for bees (mutualistic effect) (Pyke, 2016). Nevertheless, those results agree with **H1** because they indicate that taxonomic and functional diversity evolved positively during *A. mellifera* main activity time (spring and early summer).

## Year differences

Year predictors significantly influenced community composition, species richness, and certain individual functional traits. Several factors can explain these differences. For instance, meteorological conditions are known to affect plant phenology and blooming periods. Additionally, changes in land use over the years, with a trend towards urbanisation, have a significant impact on plant communities (see Location differences).

The fact that functional diversity indices do not change with years might indicate that bees forage for approximately the same amount and range of functional traits every year. This consistency is important because it suggests that, regardless of location, honeybees will still forage within the same number and range of functional traits. **H2** is only partially validated because, while the diversity indices did not change over the years, some individual functional traits and species diversity measures showed significant changes over time.

**Location differences**

Location predictors were significant for community composition, species richness, and most functional diversity metrics. Sumida was found to be less rich and diverse compared to Nishichiba and Kashiwanoha. The primary difference between these sites is the urbanisation gradient, with Sumida being highly urbanised. These results corroborate other studies which showed that more urbanised environments tend to have lower species richness and diversity (Udy et al., 2020). Consequently, these reductions in plant diversity also negatively affect the diversity of functional traits (Figure 15).

All these diversity results support **H3**, as the diversity and richness of foraged species decreases with urbanisation, with significantly less diversity and richness observed in Sumida, which is more urbanised than Nishichiba and Kashiwanoha (Figure 1).

**Recommendations**

Considering all the results and interpretations, several recommendations can be made to improve the situation of honeybees and other pollinators in urban environments:

Promoting plant species that are abundant and redundant in samples can help bees by providing a consistent food source throughout the year. It is preferable that these species are native, as native pollinators benefit more from native plants (Seitz et al., 2020). While many species found in Tokyo are cosmopolitan, prioritising native species will better support local pollinator populations. Indeed, ornamental species, being less redundant in the results, suggest that diversification should be achieved with a wide range of native plants, including trees and bushes, that flower throughout the year.

Regarding functional traits, it has been observed that bees forage on trees early in the season. Therefore, urban environments need trees to provide essential food sources for bees in the early part of the year (Hausmann et al., 2016). As the season progresses, bees need to forage on other plant species. Thus, urban greening should also include small herbaceous plants, preferably native ones, to ensure a continuous food supply for bees.

Since functional richness and diversity are positively correlated with species richness, it is crucial to increase plant diversity in all urban locations, especially the most urbanised ones. Higher species richness and diversity will create a richer environment for pollinators like *A. mellifera* and establish more functional ecological niches for other pollinators.

Honeybees, being cosmopolitan foragers, adapt well to their environment and local plant diversity to meet their needs (Purdy, 2024). However, this may not be the case for other pollinators. Therefore, plant diversification within cities is even more crucial for supporting a broader range of pollinators. Additionally, studies with a control (such as *A. mellifera*) on other pollinators' foraging behaviour using metabarcoding need to be conducted to better understand and support these diverse pollinator populations.

## 4.4   Limitations

This study has several limitations. Firstly, it compares and analyses metabarcoding data from two different years, with a five-year gap and different primer sets. To mitigate this heterogeneity, the same data cleaning methods were used for both years, and read counts were not considered. The goal was to minimise potential year biases that could falsely indicate significant differences when none exist.

However, not accounting for read abundance is also a limitation, as some studies suggest that read counts can provide a semi-quantitative measure of plant abundance in bee-foraged plants (Bell et al., 2023; Lamb et al., 2019).

Regarding spatial diversity, the study only compared two semi-urban locations with one urban location. It would have been beneficial to include more locations, especially those of different types such as rural intensive, rural extensive, and protected areas (Udy et al., 2020; Wania et al., 2006). This would provide a more comprehensive understanding of how different environments impact plant and bee communities (Udy et al., 2020; Wania et al., 2006).

In this study, urbanisation level was assessed arbitrarily using a satellite map of locations. Methods like NDVI (Normalised Difference Vegetation Index) could have been used to address the urbanisation level more accurately (Noël et al., 2023; Sarot, 2024). This ecological attribute could have helped quantify the relationship between species richness and urbanisation level, providing more precise insights.

This study did not take into account other relevant factors that impact plant development and, by extension, bee foraging, such as land use and meteorological data. These factors can significantly influence the availability and types of plants, thereby affecting bee foraging behaviour (Potts et al., 2003; Karbassioon et al., 2023).

The functional trait measurements were sometimes influenced by arbitrary choices. For example, quantitative traits were based on the general maximum height of plants, which can be biassed and depends heavily on the descriptions provided in online flora. Additionally, the general maximum height does not necessarily reflect the actual size of the plant in reality, introducing another layer of bias since direct measurements were not possible. Furthermore, traits like inflorescence size varied between male and female plants, and in such cases, the measurement was taken from the larger inflorescence, which may not accurately represent the species as a whole (Bauer et al., 2017). The luminosity trait was calculated using a function based on RGB values, which are suited for human vision. However, bees perceive colours differently than humans, including the ability to see ultraviolet (UV) light. Therefore, the conversion functions used for human vision may not fully capture the colour range seen by bees. An adapted equation that accounts for bees' unique vision, including UV sensitivity, could be more accurate.

Furthermore, more traits can also be interesting for other pollinators and insects in general. In fact, a broad range of functional traits, specifically floral bouquet-related traits, were not accounted for (Parachnowitsch et al., 2012; Milet-Pinheiro et al., 2021). These traits are

crucial in bee-plant interactions but were omitted due to a lack of coherent data across different plant species. This is a limitation of using online databases. Currently, these measurements can only be accurately obtained by directly measuring flower volatile compounds and scents from the flowers themselves.

Finally, it is challenging to determine whether the observed patterns in flower traits are due to bee preferences or if they are common traits among plants. That's why **H4** is difficult to prove. Additionally, the interaction between bees and plants might be a result of coevolution, leading to an equilibrium where both have adapted to each other's presence and characteristics.

Indeed, it would have been preferable to have another honeybee species such as *A.cerana* as a control to compare its foraging plant range in the same locations and time (Theisen-Jones et al., 2016).

# 5 Conclusion

This research provided a novel understanding of *Apis mellifera* L. ecology in urban spaces by examining the taxonomic range and functional diversity of foraged plants over different months, years, and locations through pollen analysis. The study identified 367 plant species using DNA metabarcoding, revealing significant patterns in species presence and diversity across the study period.

Predictors such as month, year, and location significantly influenced various aspects of taxonomic diversity and richness, as well as functional measures, diversity, and richness. This proves that honeybee populations are shaped by their environment at specific times and locations.

The findings highlight the importance of plant diversity in supporting honeybee populations. The study noted the prevalence of non-native, cosmopolitan plant species, which aligns with the cosmopolitan nature of *A. mellifera*. Indeed, European honeybees will generally find the necessary resources for survival in many situations. However, other pollinators might have more specialised ecological niches and therefore face difficulties in finding food before *A. mellifera* is impacted. It is crucial to also focus on supporting these other pollinators to ensure their survival and ecological health.

In conclusion, this research underscores the critical role of plant taxonomic and functional diversity. Recommendations include promoting native plant species, incorporating a mix of trees and herbaceous plants in urban greening efforts, and increasing plant diversity in highly urbanised areas. Future studies should focus on the foraging behaviour of other pollinators using similar methodologies to provide a more comprehensive understanding of urban pollinator ecology.

# 6  Personal contribution

This study marks the culmination of several years of collaboration between Liège University and Chiba University, focusing on the analysis of bees' diets through DNA metabarcoding.

This master study gave me the opportunity to work in Japan. Sampling pollen from the hives at Nishichiba, learning good beekeeping practices. Furthermore, for the first time, the initial part of the metabarcoding was conducted by the student.

The study went deeper into details by assessing the functional traits and functional diversity of the plants foraged by bees in the urban and semi urban part of the Kanto region in Japan.

I selected the majority of the relevant traits regarding bee-plant interactions that were sufficiently abundant in online plant databases. Plus, I assessed traits like inflorescence colours in a more detailed and quantitative way compared to previous studies. The generated data was then analysed through multiple plots and statistical analysis. I finally gave recommendations on what urban greening should focus on to promote pollinators development.

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