




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Contrasting patterns of foraging behavior in neotropical stingless bees using pollen and honey metabarcoding

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Stingless bees are major flower visitors in the tropics, but their foraging preferences and behavior are still poorly understood. Studying stingless bee interactions with angiosperms is methodologically challenging due to the high tropical plant diversity and inaccessibility of upper canopy flowers in forested habitats. Pollen DNA metabarcoding offers an opportunity of assessing floral visitation efficiently and was applied here to understand stingless bee floral resources spectra and foraging behavior. We analyzed pollen and honey from nests of three distantly related stingless bee species, with different body size and social behavior: *Melipona rufiventris*, *Scaptotrigona postica* and *Tetragonisca angustula*. Simultaneously, we evaluate the local floristic components through seventeen rapid botanical surveys conducted at different distances from the nests. We discovered a broad set of explored floral sources, with 46.3 plant species per bee species in honey samples and 53.67 in pollen samples. Plant families Myrtaceae, Asteraceae, Euphorbiaceae, Melastomataceae and Malpighiaceae dominated the records, indicating stingless bee preferences for abundant resources that flowers of these families provide in the region. Results also reinforce the preference of stingless bees for forest trees, even if only available at long distances. Our high-resolution results encourage future bee-plant studies using pollen and honey metabarcoding in hyper-diverse tropical environments.

Plant-pollinator interactions mediate most flowering plant reproduction, maintaining terrestrial ecosystems and crops¹. The current decline in pollinator abundance and diversity worldwide threatens pollination services, with direct consequences to nature conservation and food security². Therefore, understanding interactions between pollinators and flowering plants became crucial, since it can provide an information framework to subsidize conservation policies and decisions in a changing world. The most important group of animal pollinators—the bees—are completely dependent on floral resources to complete their life cycles³, while 87.5% of the animal-pollinated flowering plants depend on bees to reproduce⁴. Interaction between bees and angiosperms has been a major research focus in the last two decades, due to the potential threats of massive insect and bee declines⁵ on ecosystem services and food security^{6,7}. In this context, pollen, and honey DNA metabarcoding emerged as an efficient technique to identify plant taxa visited by pollinators based on samples extracted from bees' bodies or nests^{8–11}. DNA metabarcoding of pollen and honey has been largely applied to temperate systems, and recently to (sub) tropical species of stingless bees^{12,13}.

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The stingless bees (Apidae, tribe Meliponini) comprise c. 500 species of eusocial bees, most of which occur throughout tropical America (more than 400 species), but also in Africa, Asia and Australia^{3,14}. The role of stingless bees as pollinators of the neotropical flora could become even more relevant under climate change scenarios, if the predicted expansion of warmer temperatures pushes the distribution of predominantly temperate bees, such as *Apis* and *Bombus*, into cooler regions^{6,15}. Although domestication is still restricted to a few species, stingless bees are also explored commercially for honey production¹⁶, which can reduce the use of introduced honeybees and their impact on native species in these regions¹².

As all eusocial bees, stingless bees show a predominantly generalist pattern of floral exploitation, i.e. they visit a large number of species in several plant families, supposedly disregarding specific floral traits¹⁷. Particularities exist though, since stingless bees exhibit a huge diversity of body size (1.8 to 13.5 mm)³, flight distance (0.3 to 3 km)¹⁸, and foraging behavior¹⁹. However, the breadth of stingless bees diet—that is, how many different species of flowering plant they can forage on—and the extent of their role as pollinators of tropical plants are still largely open questions. Most studies aiming to answer these questions face methodological difficulties due to inaccessibility of visited flowers, which mostly occupy upper canopy strata especially in rainforests²⁰, and hyperdiversity of tropical plants, thus hampering easy identification through bee-pollen morphology^{21,22}.

^{21,22}In this study, we explored the diet breadth of three distantly related species of stingless bees of different body sizes and flight ranges in a hyper-diverse tropical ecosystem, the Cerrado savannas of central South America. The Cerrado is the most species-rich savanna in the world and a hotspot of biodiversity²³. The flora encompasses > 13,000 native plant species²⁴ in a highly patchy vegetation with several different physiognomies, ranging from grasslands, marshlands, and typical savanna to closed canopy riverine forests along waterways²⁵. The proportion of pollinator-dependent species in the Cerrado flora is still unknown, although this number is likely to be similar to that of tropical forests⁴, with some authors estimating c. 60% of angiosperm species being bee dependent^{26,27}.

We analyzed pollen and honey from the pots of the nests (henceforward pot-pollen and pot-honey respectively) from three commonly managed stingless bee species (*Melipona rufiventris* Lepeletier, 1836, *Scaptotrigona postica* (Latreille, 1807) and *Tetragonisca angustula* (Latreille, 1811)) native to the Cerrado to investigate: (i) How broad is the diet breadth of stingless bees in a hyper-diverse flora? (ii) Which plant species and families are the most important sources of pollen and/or nectar for stingless bees in the area? (iii) What can pollen and honey metabarcoding reveal, when combined with floristic surveys of the area, about stingless bees foraging behavior, particularly foraging distances, and floral preferences? We also discuss how efficiently pollen and honey metabarcoding identified plants visited by bees in the area, considering the low DNA sequence coverage of neotropical plant species in public databases²⁸, and the potential role of this technique in improving ecological understanding of bee-plant interactions in the tropics.

Material and methods

Study site. Our study was conducted in the Ecological Reserve of the Brazilian Institute of Geography and Statistics (IBGE) (15°56'41" S and 47°53'07" W) that, together with the contiguous Brasília Botanic Garden and the University of Brasília Experimental Field Station, preserves an area of c. 10,000 ha of native Cerrado in the Distrito Federal, Brazil. The IBGE reserve occupies a central position within the Cerrado domain and was chosen as our study site for being one of the most well-studied areas of Cerrado, with good prospects of building a relatively robust plant DNA reference library, a requirement for our analyses (see below). The climate in the area is typical tropical savanna climate (Aw Köppen classification system) with dry winters and rainy summers with an average annual precipitation of 1453 mm and altitude ranging from 1048 to 1160 m. The IBGE reserve contains the main vegetation types typical of the Cerrado domain: savannas (*cerrado sensu stricto*), palm swamps (*veredas*), grasslands (*campo limpo* and *campo sujo*) and riverine forests (*mata de galeria*), surrounded by natural and agricultural areas (Fig. 1). This habitat heterogeneity results in high plant biodiversity. The last published floristic survey in the area recorded 1798 species of angiosperms, of which 1457 are native, distributed in 138 families and 724 genera²⁹.

Stingless bee species and nest material sampling. Three native stingless bee species were chosen for our study based on differences in body size, differences in foraging behavior, and phylogenetic relationships. *Melipona rufiventris* is the largest with a body length of c. 9.5 mm, *Scaptotrigona postica* has an intermediate body size varying from 5.7 to 6 mm, and *Tetragonisca angustula* is amongst the smallest stingless bees with a total body length of c. 4 mm. Phylogenetically, the three genera are not closely related, i.e. they do not belong to sister groups³⁰. *Melipona rufiventris* is typically found in the more open vegetation types of eastern and central Brazil, *S. postica* occurs in a broader region in Central, Northeast and southeast Brazil, also associated with open vegetation, while *T. angustula* is widespread in the Neotropics (Mexico to South America)¹⁴.

The three species are commonly managed by local beekeepers and were chosen also for their relatively easy management in artificial colonies (Figure S1). The decision to use artificial colonies for sampling in our study relied on four main points: 1. to preserve the natural bee community in the area, by not destroying any nests for sampling; 2. to facilitate sampling, as pollen and honey are stored in accessible compartments in the wooden box; 3. to facilitate the access to the colonies, which in natural conditions would be randomly distributed, depending on availability of cavities, and 4. to make sure nests would have a strong population and enough pot-pollen and pot-honey for sampling. Eighteen pre-established nests were installed: three of *M. rufiventris*, eight of *S. postica* and seven of *T. angustula*. The nests were installed at a distance of about 5 m from each other and c. 150 cm above ground level, in typical savanna or *cerrado sensu stricto*²⁹ where most plant species are shrubs, shrubs or small trees.

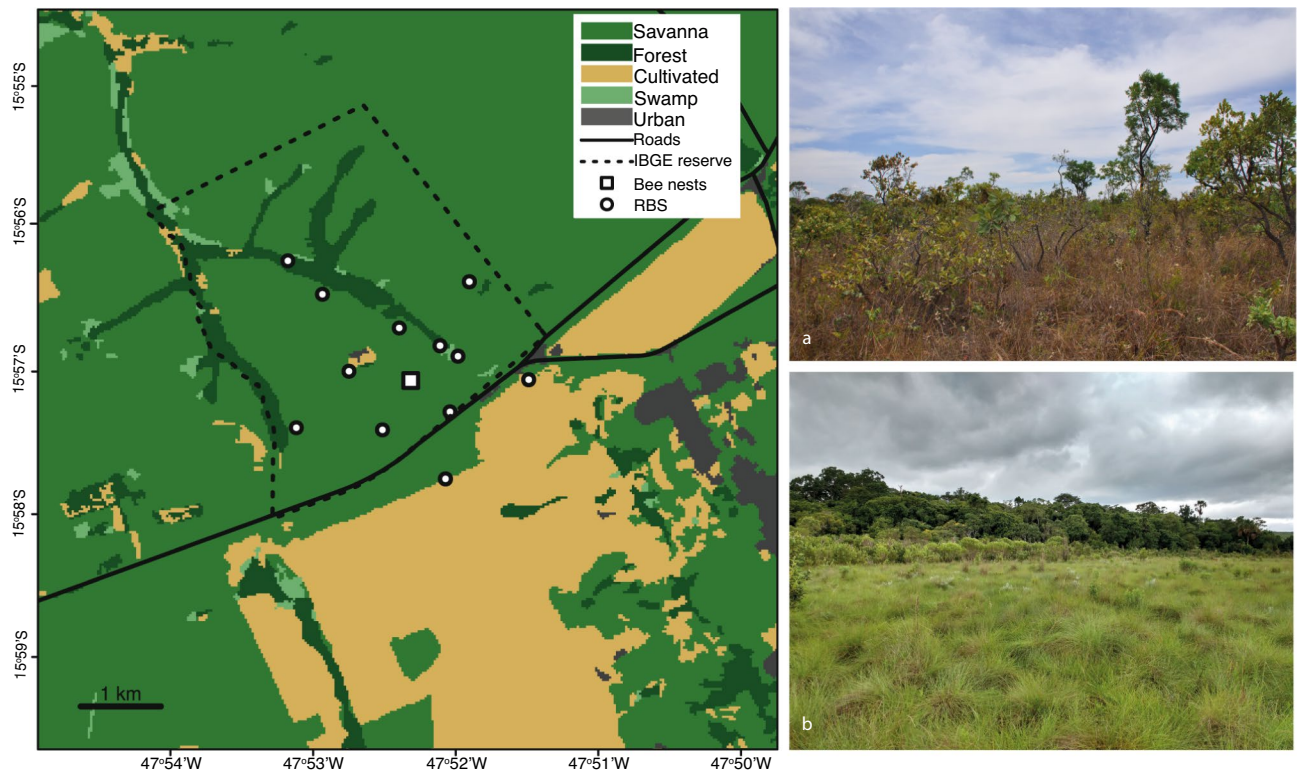


Figure 1. Map of the IBGE reserve and surroundings showing the location where bee nests were installed and the locations of Rapid Botanical Surveys. The image also shows main vegetational types, i.e. cerrado savanna, riverine forests, swamps, cultivated and urban areas. Photographs depict a. cerrado savanna vegetation type (Photo author: ACM) and b. area of transition between grassland and riverine forest (Photo author: AJCA). Vegetation cover: MapBiomias (www.mapbiomas.org). Reserve delimitation: IBGE.

Nests were moved to the study area eight weeks prior to the first sampling to allow bees time to accumulate pollen and honey from local species in the artificial nests. Pot-pollen and pot-honey samples were collected from the nests (Figure S2) once every 15 days for five months (July 2019–November 2019). This period started at the height of the dry season, moved through the transition between dry and wet seasons and ended at the beginning of the wet season. Samples were always collected from new pots—that is, those built in between two subsequent sampling events. Micropipettes (1000 μ L) were used to collect honey from the pots, while pollen was collected with plastic straws, which perforates the pollen mass while collecting it at the same time. Samples were subsequently stored in falcon tubes and stored in a -20°C freezer until extraction. In total, 191 samples (115 of pollen and 75 of honey) were collected from the three species: 29 of *M. rufiventris*, 81 of *S. postica* and 74 of *T. angustula*.

Metabarcoding protocol. DNA extractions of pollen samples from pot-pollen and pot-honey followed different methodologies, due to the different natures of the samples. For pot-honey, we extracted DNA using the Machery-Nagel (Düren, Germany) NucleoSpin Food Kit; for pot-pollen we used the Machery-Nagel (Düren, Germany) NucleoSpin Plant II.

Pot-pollen DNA extraction. To extract pollen genomic DNA, we added to the pooled samples (weight ranging from 0.1 g to 2 g) 4 mL of deionized and autoclaved water and homogenized it using a vortex. We then placed 200 μ L of this emulsion in a 1.5 mL microcentrifuge tube and centrifuged it for 15 min at 8000 rpm. We discarded the supernatant material, froze the pellet obtained in liquid nitrogen, and then used mortar and pestle to break the pollen exine, and the NucleoSpin Plant II Kit to promote cell lysis and to isolate the DNA according to the manufacturer's instructions.

Pot-honey DNA extraction. To extract pollen genomic DNA from honey, we added deionized and autoclaved water to the samples until the volume of each sample tube reached 1.5 mL. We incubated the tubes at 65°C for 30 min and, over that period, inverted the tubes slowly to homogenize the material. We then pooled the honey samples collected from the same nest and day by pouring them into falcon tubes, to which deionized and autoclaved water was added until completing 10 mL. Afterwards, we centrifuged these pooled samples for 15 min at 5000 rpm and discarded the supernatant material. Each precipitated pooled honey sample was resuspended in 200 μ L deionized and autoclaved water and placed in a 1.5-mL microcentrifuge tube. This procedure was done twice. Finally, we centrifuged the samples for 15 min at 5000 rpm, discarded the supernatant material, dried the

pellet in a drying cabinet at 35 °C, and then ground the samples inside the microcentrifuge tube using micropestles and liquid nitrogen. We then used the NucleoSpin Food Kit to promote cell lysis and to isolate the DNA according to the manufacturer's instructions.

The protocol of amplification utilizes a dual-indexing strategy⁹ to amplify the ITS2 region, using the primers ITS-S2F and ITS4R. Primer sequences, references and other amplification methodological details can be found in Sickel (2015)⁹ and Campos et al. (2021)³¹. The triplicate PCR reactions were combined per samples, well mixed and checked on 1% agarose gel using 5 µL of the combined products for quality. PCR products of each sample were then normalized to ensure library concentrations were approximately equivalent across samples using the SequalPrep Normalisation kit (Invitrogen, CA, USA) according to the manufacturer's protocol. After pooling the multiplex-index samples, we quantitated the pools with a dsDNA High Sensitivity Assay on a Qubit Fluorometer and assessed fragment lengths with a Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies, CA, USA). For library dilution, we followed the Illumina Sample Preparation Guide for a 2 nM library where 5% consisted of PhiX control library. In addition, the reagent cassette of the sequencing kit was spiked with the Read1, Read 2 and index primers according to Sickel et al. (2015). Sequencing was then performed on the Illumina MiSeq system at the University of Würzburg. Sequence data are available at NCBI (Bioproject 976708).

Bioinformatic data analyses. We used VSEARCH v2.14.2³² to join paired ends of forward and reverse reads and to remove reads shorter than 150 bp, quality filtering ($EE < 1$)³³, *de-novo* chimera filtering (following UCHIME3)³⁴, and determination of amplicon sequence variants (ASVs)³⁴, as previously done for pollen metabarcoding networks¹². Reads were first directly mapped iteratively with global alignments using VSEARCH against five flowering plant ITS2 reference databases (see below for construction details) for the study region and an identity cut-off threshold of at least 97% (higher percentages are prioritized). These reference databases were created with BCdatabaser, then automatically curated³⁵ from GenBank entries with default parameters (length between 200 and 2000 bp, maximum nine sequences per species), from the following species lists: 1) all plant species recorded from IBGE. This database was then manually curated to remove voucher-less entries for greater trustworthiness. Remaining unclassified sequences were then tracked by iterative searches against geographically broadening public sequence reference data, i.e., 2) species lists of the flora of the Distrito Federal, then 3) the large, neighboring state of Goiás, and 4) the entire Cerrado domain flora to increase completeness of reads. These reference databases were created with the BCdatabaser³⁶ from GenBank entries given above mentioned species lists and default parameters (length between 200 and 2000 bp, maximum nine sequences per species). For still unclassified reads, we used SINTAX³⁷ to assign taxonomic levels as deep as possible. Finally, we used 5) global reference database³⁶. After classification, we performed plausibility checks according to geolocation and phenology with the results to verify validity. Thirteen species were automatically matched to genus level only, but were manually attributed to species based on these being the only species of the genus to occur in the Distrito Federal.

Floristic surveys and vegetation characterization. To improve our knowledge of the flora surrounding the nests, we conducted Rapid Botanical Surveys (RBS) in small plots that were demarcated *in loco* as homogeneous to vegetation type. These plots were exhaustively surveyed for all flowering plant species of all life forms, fertile or not, by a team of 3–5 researchers, where one was the booker, i.e. the most experienced person in the group, who identified the plants in the field and discarded duplicated species; other team member collected and pressed the vouchers (for additional methodological details see³⁸).

Eleven RBS plots had been initially chosen to correspond to one plot near the nests (henceforward nest plot) and ten other plots established at the vertices of two pentagons; the inner pentagon was established with its vertices at 700 m from the nests and the outer pentagon with vertices at 1500 m from the nests. These distances were chosen based on the literature of the flight capabilities of other stingless bees¹⁸. These eleven RBS plots mostly fell in areas of well-preserved savanna within the IBGE Reserve, ranging from the more open, grass and herb-rich areas with few shrubs and trees (*campo sujo*), to dense savanna woodland (*cerradão*); one outer pentagon plot fell in disturbed *cerrado* and another in heavily degraded secondary vegetation out of the IBGE. Because none of the plots fell in riverine gallery forest, we included six additional RBS plots in this vegetation type: three in the riverine gallery forest nearest to the nests (Nascente do Roncador, c. 630 m from the nests), and three in a more distant gallery forest (Ponte do Corujão, c. 2070 m from the nests), measured as the crow flies, totaling 17 RBS plots. Lastly, we also surveyed the plants and weeds growing in the ornamental gardens associated with the Main Building and Seat of the Reserva Ecológica do IBGE, which is located c. 650 m from the nests. All specimens collected in RBS inventories were identified by the author CEBP and JEQF and were deposited in the UB Herbarium (University of Brasilia) and the records are available online in the Species Link Network (<https://specieslink.net/search/>) by searching on the collector name "Projeto Barcode Cerrado". From all species collected, three are categorized as endangered at some level: *Virola urbaniana* Warb. and *Cedrela fissilis* Vell. (vulnerable) and *Anemopaegma arvense* (Vell.) Stellfeld ex de Souza (endangered). The vast majority of herbarium collections (all collections of the two vulnerable and one endangered species) did not require uprooting.

Data integration. The 30 most abundant plant species in the pollen and honey samples were classified by ubiquity (i.e., presence in pollen or honey samples of two or all bee species). We then crossed this information with data from the RBS floristic surveys: distance from the nests, i.e., if they were sampled at nest plot, inner pentagon plots, outer pentagon plots, nearest or furthest gallery forest plots, or the gardens. These 30 species were also characterized from the literature in terms of type of resources (e.g. pollen, nectar, oil, resin), their habitat (savanna, forest or cultivated/weedy) and habit (trees, shrubs, subshrubs, hemiparasites) (Table 1).

Higher taxon	Species	Habitat	Habit	Honey	Pollen	Floral reward
Asteraceae	<i>Baccharis dracunculifolia</i>	Savanna (0)	Shrub	SP, TA	ALL	PN
Myrtaceae	<i>Blepharocalyx salicifolius</i>	Savanna (7) Forest (1)	Tree, shrub	ALL	ALL	P
Malpighiaceae	<i>Byrsonima basiloba</i>	Savanna (1)	Shrub	MR, TA	TA	PO
Malpighiaceae	<i>Byrsonima pachyphylla</i>	Savanna (8)	Tree, shrub	SP, TA	ALL	PO
Clusiaceae	<i>Clusia criuva</i>	Forest (1)	Tree, shrub	SP, TA	SP, TA	PR
Euphorbiaceae	<i>Croton conduplicatus</i>	Savanna (0)	Shrub, subshrub	ALL	TA	PN
Myrtaceae	<i>Eucalyptus</i> sp.	Cultivated	Tree, shrub	ALL	ALL	PN
Nyctaginaceae	<i>Guapira graciliflora</i>	Savanna (6)	Tree, shrub	ALL	ALL	PN
Chloranthaceae	<i>Hedyosmum brasiliense</i>	Forest (4)	Tree, shrub	SP, TA	ALL	P
Melastomataceae	<i>Leandra polystachya</i>	Savanna (1)	Shrub, subshrub	MR, SP	MR, SP	P
Euphorbiaceae	<i>Mabea fistulifera</i>	Savanna (1)	Tree, shrub	ALL	SP, TA	PN
Euphorbiaceae	<i>Maprounea guianensis</i>	Savanna (9)	Tree	SP, TA	ALL	P
Sapindaceae	<i>Matayba guianensis</i>	Savanna (3) Forest (1)	Tree, shrub	ALL	ALL	PN
Melastomataceae	<i>Miconia hirtella</i>	Forest (2)	Tree, shrub	MR	MR, SP	PN
Melastomataceae	<i>Miconia leucocarpa</i>	Savanna (2)	Tree, shrub	ALL	MR, SP	P
Melastomataceae	<i>Miconia stenostachya</i>	Savanna (2)	Shrub	ALL	ALL	P
Myrtaceae	<i>Myrcia guianensis</i>	Savanna (9)	Tree, shrub, subshrub	MR, SP	ALL	P
Myrtaceae	<i>Myrcia linearifolia</i>	Savanna (9)	Shrub, subshrub	ALL	ALL	P
Myrtaceae	<i>Myrcia pinifolia</i>	Savanna (0)	Shrub	ALL	ALL	P
Myrtaceae	<i>Myrcia tomentosa</i>	Savanna (2) Forest (2)	Tree, shrub	SP	SP, TA	P
Primulaceae	<i>Myrsine</i> sp.	?	?	ALL	SP, TA	P
Myrtaceae	<i>Myrtaceae</i> sp.	?	?	ALL	ALL	?
Piperaceae	<i>Piper aduncum</i>	Forest (1)	Tree, shrub	ALL	ALL	P
Phyllanthaceae	<i>Richeria grandis</i>	Forest (5)	Tree, shrub	ALL	ALL	PN
Rosaceae	<i>Rubus urticifolius</i>	Forest (4)	Climber, shrub, subshrub	ALL	ALL	PN
Loranthaceae	<i>Struthanthus/Psittacanthus</i> sp.	?	Hemiparasite	ALL	ALL	PN
Fabaceae	<i>Stryphnodendron</i> sp.	Savanna (8)	?	MR, SP	ALL	PN
Myrtaceae	<i>Syzygium cumini</i>	Forest (2 cultivated)	Tree	ALL	ALL	PN
Anacardiaceae	<i>Tapirira guianensis</i>	Forest (1) Savanna (3)	Tree	ALL	ALL	PN
Anacardiaceae	<i>Toxicodendron succedaneum</i>	Cultivated (0)	Tree	SP, TA	SP, TA	PN

Table 1. Thirty most frequent taxa in ASVs, their habitats and habits (tree, shrub, subshrub, climber, hemiparasite), presence in pollen or honey and floral resource offered (P: pollen; N: nectar; O: oil; R: resin). Habitat data from floristic inventory in this study; numbers in parenthesis represent a record in each RBS plot (forest plots surveyed: 6; savanna plots surveyed: 11). Habit data from Flora & Funga do Brasil (2023).

Statistical analysis of pollen and honey samples. Data was processed for analyses using R 4.2.2³⁹ and the packages phyloseq⁴⁰ vegan⁴¹, bipartite⁴², circlize⁴³ and viridis⁴⁴ (all code required to replicate the analyses is available at github.com/tncvasconcelos/barcode_cerrado). In R, non-plant sequences were removed from the dataset, as well as the data transformed to relative read abundances (RRAs) per sample. ASVs that were classified as the same plant species were accumulated at the species level. Low abundance taxa that contributed less than 1% to a sample were removed from those samples. The Shannon diversity index was calculated for each sample (pollen and honey) for each bee species. The diversity was tested for significant differences between stingless bee species using the Kruskal-Wallis test, separately for pollen and honey samples. We also performed an NMDS ordination to visualize clustering of samples of pollen and nectar using Bray-Curtis beta-diversity dissimilarities. The ordination represented by proximity of points shows how similar two samples are in terms of composition and abundance of taxa. We tested for differences between species by using a PERMANOVA, separately for honey and pollen samples. We further calculated network indices of the three stingless bee species to account for their overlap and complementarity in the visited plant resources, i.e. the d' for each bee species and $H2'$ for the entire network.

Results

Pollen and honey metabarcoding yielded a total of 5,079,123 quality filtered reads, with mean throughput per sample of 27,307.11 reads \pm 1756.635 (SE). Significant reads (more than 1% of reads in any sampling) accounted for 110 ASVs, in 86 genera and 40 plant families; c. 36% of these reads were only matched to generic level or above. In total, 95 out of the 110 ASVs recovered from the samples had been previously recorded in the IBGE Reserve flora²⁹; 12 of the 15 absent taxa were exotic cultivated or weedy species. A detailed list of all significant

plant species present in pot-pollen and pot-honey samples is available in Table S2. Reads below the threshold value (190 ASVs) still showed a high number of matches to species known to occur in IBGE (86 species, c. 45%) of which 41 were also recorded by us in the RBS floristic inventories.

How broad is the floral resource exploitation by stingless bees in Cerrado Savanna? Overall, the interaction network was highly generalized ($H2' = 0.2895575$), and consequently also that of the three species within the network (*M. rufiventris* $d' = 0.22$, *S. postica* $d' = 0.04$, and *T. angustula* $d' = 0.22$) (Fig. 2). More than a half of plant species appeared in the samples of at least two of the bee species. In terms of relative plant species abundances as evaluated by combined honey plus pollen samples, bees showed an opportunistic foraging pattern, with most plant species with low abundance and a few highly abundant.

Differences among pattern of floral sources exploitation of bee species. The comparison between alpha diversity among samples of different bee species showed that the plant species richness in the pot-honey was higher than in the pot-pollen for all species, but the difference was only significant for *M. rufiventris*

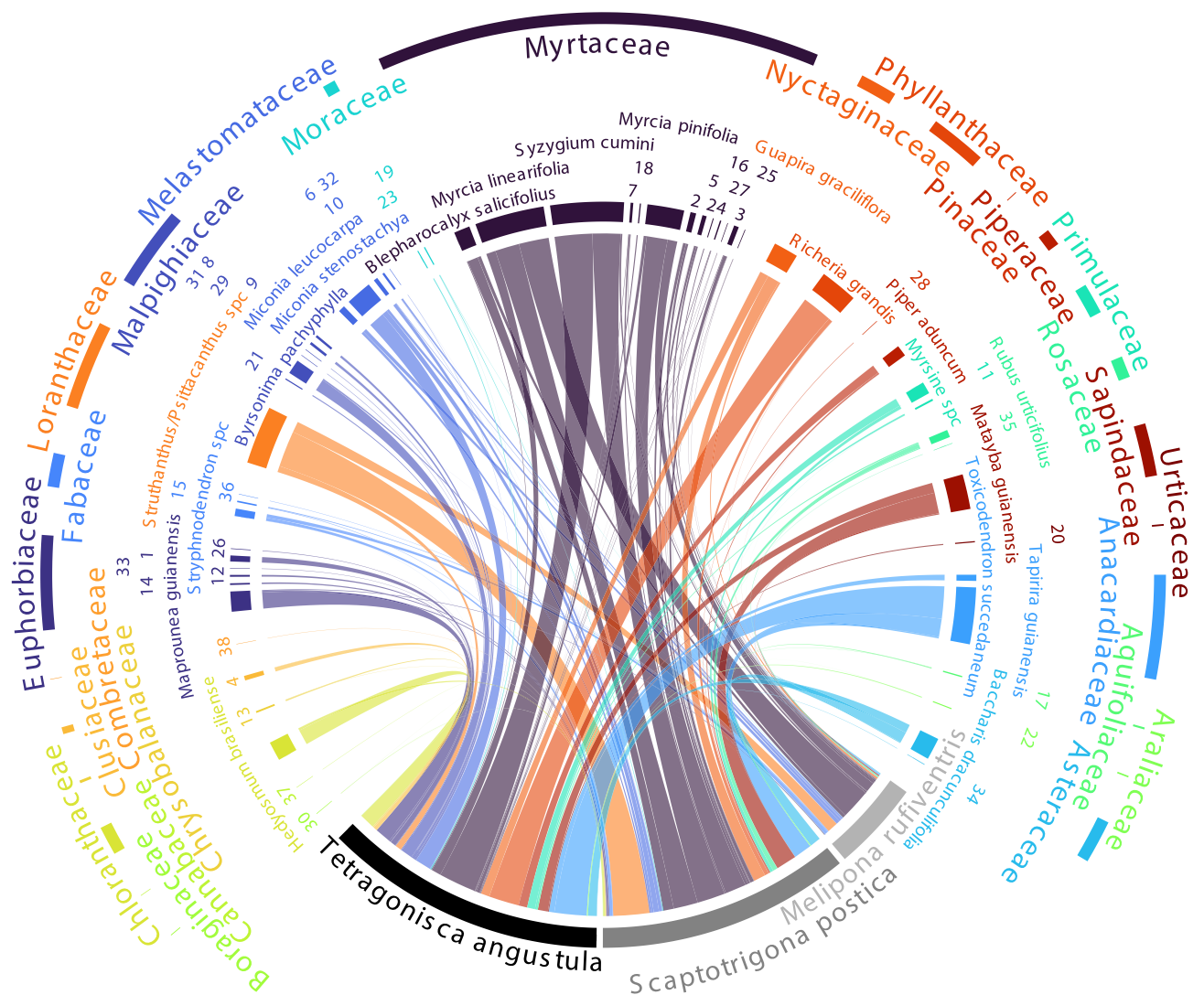


Figure 2. Interaction network of three stingless bee species and the 30 most frequent species in honey and pollen samples (Table S3). Bars connecting bee species and plant species indicate reported interaction (i.e. that plant species was present in the sequencing reads of pollen and/or honey metabarcoding in significant numbers). Some plant species are represented by numbers: 1. *Croton conduplicatus*; 2. *Eucalyptus*; 3. Myrtaceae; 4. *Clusia criuva*; 5. *Myrcia guianensis*; 6. *Miconia hirtella*; 7. *Myrcia splendens*; 8. *Byrsonima basiloba*; 9. *Byrsonima laxiflora*; 10. *Leandra polystachya*; 11. *Myrsine umbellata*; 12. *Acalypha*; 13. *Couepia*; 14. *Mabea fistulifera*; 15. Fabaceae; 16. *Myrcia tomentosa*; 17. *Ilex affinis*; 18. *Eugenia involucrata*; 19. Moraceae; 20. *Cecropia pachystachya*; 21. *Byrsonima crassifolia*; 22. *Schefflera macrocarpa*; 23. *Artocarpus heterophyllus*; 24. *Campomanesia pubescens*; 25. *Myrcia pubescens*; 26. *Stillingia*; 27. *Syzygium*; 28. *Pinus*; 29. *Banisteriopsis*; 30. *Borago officinalis*; 31. *Byrsonima viminifolia*; 32. Melastomataceae; 33. *Euphorbia potentilloides*; 34. Asteraceae; 35. *Rosa chinensis*; 36. *Copaifera*; 37. *Trema micranthum*; 38. *Terminalia*.

(Fig. 3). In a comparison among the three bee species, Shannon diversity of plant species in pollen samples was not significantly different between bee species (Kruskal–Wallis rank sum test, chi-squared = 4.5138, $df=2$, p -value > 0.05), neither was plant species richness (Kruskal–Wallis rank sum test, chi-squared = 1.4733, $df=2$, p -value > 0.05). The same applied for honey samples with Shannon diversity (Kruskal–Wallis rank sum test, chi-squared = 2.6469, $df=2$, p -value > 0.05) and species richness (Kruskal–Wallis rank sum test, chi-squared = 4.9389, $df=2$, p -value > 0.05).

Although the most frequent plant species are shared among the three stingless bee species, samples from different bee species have several compositional particularities, as shown by the NMDS (Fig. 4). The NMDS showed the composition of plants collected differed strongly between bee species, both for pollen (PERMANOVA, $df=2$, $R^2=0.12516$, $F=7.2246$, $p<0.001^{***}$) and honey (PERMANOVA, $df=2$, $R^2=0.10751$, $F=3.8548$, $p<0.001^{***}$). The NMDS also points to different plant species composition between samples of three species, but in the honey samples little ordination is observed (Fig. 4A). Among pollen samples, on the other hand, we can observe different patterns among the three species, with more overlap between *M. rufiventris* and *S. postica* (Fig. 4B).

Most frequent plant species and families recovered from pot-pollen and pot-honey samples. The 30 ubiquitously found plant species in pot-honey and pot-pollen samples belong to the following families: Myrtaceae, Loranthaceae, Anacardiaceae, Phyllanthaceae, Sapindaceae, Melastomataceae, Euphorbiaceae, Primulaceae, Nyctaginaceae, Rosaceae, Asteraceae, Malpighiaceae, Cloranthaceae, Piperaceae, Fabaceae, and Clusiaceae (Fig. 5, Table S3). Out of 110 ASVs, some plant taxa stand out as most frequent in samples of all the three bee species: Myrtaceae: *Syzygium cumini* (L.) Skeels, *Myrcia linearifolia* Cambess. and *Myrcia pinifolia* Cambess.; Loranthaceae: *Struthanthus/Psittacanthus*, Anacardiaceae: *Tapirira guianensis* Aubl., Phyllanthaceae: *Richeria grandis* Vahl, Sapindaceae: *Matayba guianensis* Aubl., and Melastomataceae: *Miconia stenostachya* DC. Most of them offer pollen and nectar, except the pollen-only *Miconia* and the two *Myrcia* species. Thirteen of these ubiquitous species were nectar or oil flowers (i.e., they provide additional resources beyond pollen). Five highly abundant reads were incompletely matched, i.e. could not be identified to species level (*Eucalyptus* sp., Myrtaceae sp., *Myrsine* sp., *Croton* sp, *Struthanthus/Psittacanthus*) but *Croton*, *Eucalyptus*, *Psittacanthus* and *Struthanthus* are known to produce floral nectar. Pollen-only flowers were found in honey samples of all three species: *Myrsine* sp., *Blepharocalyx salicifolius* (Kunth) O.Berg, *Piper aduncum* L., *Miconia leucocarpa* DC. and several *Myrcia* species, thus indicating some kind of mixing nectar and pollen trips, manipulation or spill-over inside the nests. Pollen records include similar diversity numbers of pollen-only flowers and flowers offering

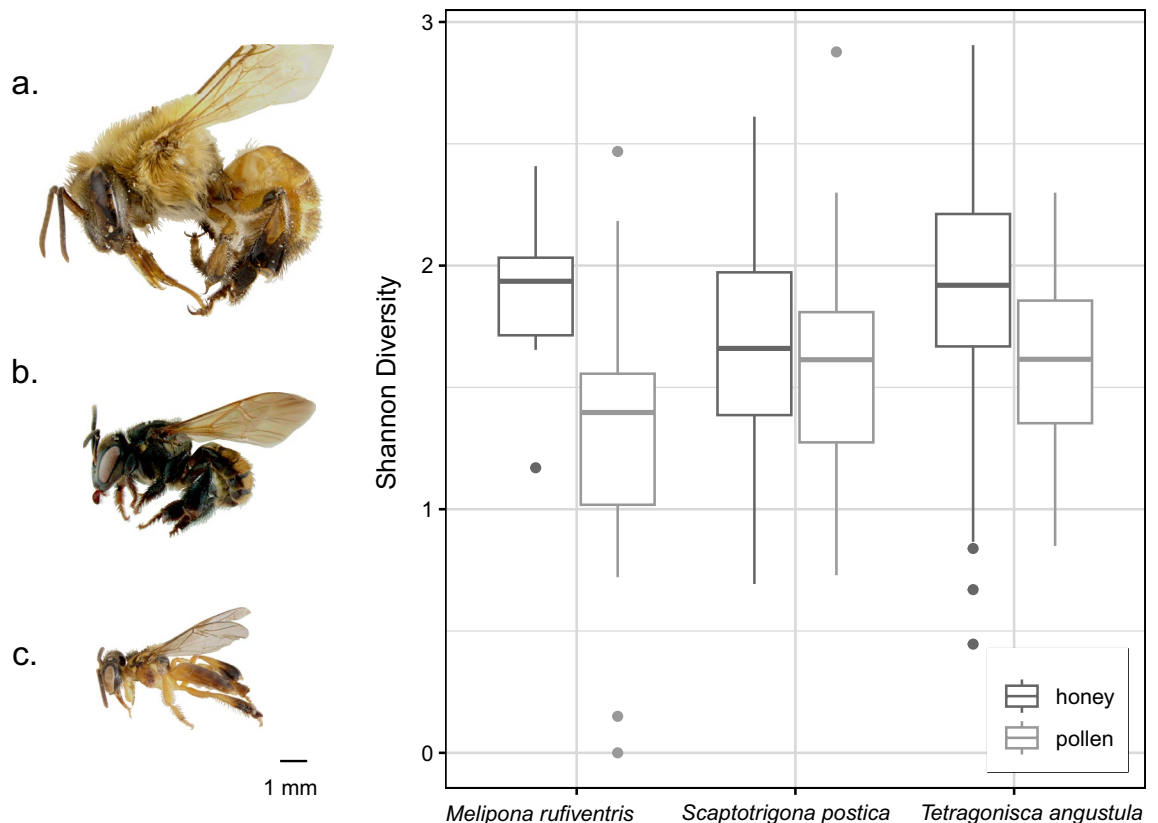


Figure 3. Boxplot of Shannon diversity indexes of plant species found in the honey (dark grey) and pollen (light grey) pots. Boxplots display the median (thick horizontal middle bars), lower (0.25) and upper (0.75) quartile (box limiting thin horizontal bars), minimum and maximum values (vertical lines). Solid dots represent an individual outlier sample. On the left, the three studied bee species in lateral view and in scale to show body size: (a) *Melipona rufiventris*, (b) *Scaptotrigona postica*, (c) *Tetragonisca angustula*.

nectar and pollen. Only four out of the 110 ASVs were not recorded in our RBSS: *Baccharis dracunculifolia* DC. and *M. pinifolia* Cambess., both native Cerrado species that occur in IBGE, and exotic *Eucalyptus* sp. and *Toxicodendron succedaneum* (L.) Kuntze.

These 30 most abundant plant species had the following characteristics: all were woody perennials, and most were trees or large shrubs (except one climber and one hemiparasite). They could be grouped into two dominant groups according to a combination of the habitat and floral resources. *Group 1* is composed of riverine forest species that offer pollen and nectar, recorded as very common in the Forest RBS surveys: *Syzigium cumini*, *Tapirira guianensis*, *Richeria grandis*, *Matayba guianensis*, *Rubus urticifolius* Poir. *Group 2* includes Cerrado shrubs or trees offering only pollen and recorded as common around the nests, in the Cerrado RBS surveys: *Myrcia linearifolia*, *Blepharocalyx salicifolius* and *Maprounea guianensis* Aubl. (Table 1).

Discussion

Pollen and honey metabarcoding of three stingless bee species in the genus *Melipona*, *Scaptotrigona* and *Tetragonisca* revealed a broad generalized set of used floral sources regarding number of species and plant families explored. We recovered 110 plant species in pot-honey and pot-pollen retrieved from nests of the three stingless bee species. This reveals a broader spectrum of food sources than found by previous surveys on neotropical stingless bees that relied on non-DNA based methods such as field observations, field collections, and palynological studies. For instance, non-DNA based studies in another hyper diverse area in the Neotropics, the Amazon, revealed from 80 to 122 pollen types in nests and pollen loads of 10–15 species of stingless bees⁴⁵. Other similar studies in species-rich areas of the Neotropics show comparatively lower numbers²². While these studies recorded a maximum of five to eight plant species per bee species, we found a mean of 46.3 plant species per bee species in honey samples and 53.67 in pollen samples. The interaction network and high number of species found in honey and pollen of the three analyzed stingless bee species point to a generalist foraging behavior, known to be common in eusocial bees and in stingless bees in particular^{17,20}. It also points to probable scouting investigative trips, followed by heavy recruitment and opportunistic behavior when a high-quality resource is located, with most plant species with low abundance and a few highly abundant. Note that our results may still be an underestimation, since samples were collected during only 6 months, i.e., did not include all seasons.

The power of pollen DNA metabarcoding in revealing broad food sources for stingless bees had only been demonstrated before in Southeast Asia and Australia. In Sumatra, a study of *Tetragonula laeviceps* (Smith, 1857) using pollen metabarcoding coupled with light microscopy revealed 99 plant species⁴⁶. Similarly, a study with *Tetragonula carbonaria* (Smith, 1854) in Queensland retrieved 302 plant species in pollen samples across seven sites at different seasons of the year over a two-year period¹³. These are promising results, especially when considering expanding this technique to tropical and subtropical forests in the Neotropics. Studies of pollination and floral biology in these habitats is often very difficult because flowers are often in the upper canopy and difficult to reach. Therefore, direct observations of bees on flowers in tropical and subtropical forests are rare²⁰, and records of stingless bee—flower interaction in these environments became almost restricted to palynological analyses of pollen loads or pot pollen analyses²². Although their utility is undeniable⁴⁷, morphological identification of pollen may become obsolete for pollination biology studies when compared with the efficiency of DNA metabarcoding to identify different plant species in extremely rich floras.

Pollen analyses via DNA metabarcoding also have the advantage of revealing unexpected food sources used by bees that would perhaps be unnoticed in studies using other methodologies. For instance, our analyses revealed that DNA from 13 wind-pollinated plant species were found among the 50 most abundant species in the sample of the three species, including monocots (Poaceae, Cyperaceae), eudicots (Euphorbiaceae: *Acalypha*, Amaranthaceae: *Amaranthus*, Urticaceae: *Cecropia*, Cannabaceae: *Trema*), and a conifer genus, the introduced *Pinus* (Table S2). The presence of non-melitophyllous angiosperms and gymnosperms (e.g. Cyperaceae, Poaceae, Taxaceae and Pinaceae) is relatively common in melisso-palynological studies^{12,22,46}. Despite previous studies demonstrating that pollen from anemophilous species might be a contamination in melisso-palynological samples⁴⁸, bees are regularly reported visiting such taxa^{49,50}. Our results confirm active collection of pollen from anemophilous species, since their abundance in our analyzed samples is relatively high. One of the most abundant plant species in the pollen analysis was *Hedyosmum brasiliense* Mart. ex Miq (Chloranthaceae), widely cited in the literature as wind-pollinated⁵¹. This species was not only recorded in the pollen samples of all three species of bees, but was amongst the 10 most abundant records for *T. angustula* in our results. These results reinforce the theory that anemophilous plants, which account for 10% of angiosperms and most gymnosperms, produce enough pollen⁵² to be attractive to social bees, under certain conditions of colony size and food demands. However, the role of bees and other insects as true pollinators of anemophilous plants remains unresolved, in spite of the importance of wind-pollinated crops⁵³ and of the several records showing that anemophilous plant pollen is important for several bee species (see references above).

A surprising and novel observation is the significant amount of Marchanthiophyte DNA from the liverwort *Dumortiera hirsuta* (Sw.) Nees found in pot pollen from the three studied stingless bee species (Table S2). Future research would need to seek evidence if the DNA results from the collection of spores or perhaps some chemical compounds from liverworts by stingless bees. Bees collecting spores from fungi and plants is not a novelty, as there is evidence of active collecting⁵⁴ as well as records of spores in samples of pollen and honey⁵⁵. In lieu of pollen, spores supposedly have nutritional benefits⁵⁶. Stingless bees might also visit liverworts to collect lipidic compounds, e.g. terpenoids used in communication among individuals⁵⁷ commonly occurring in liverworts⁵⁸.

The high degree of overlap between plant profiles found in the honey of the three bee species suggests that bees may be competing for the same nectar resources. Pollen plant profiles on the other hand showed far less overlap between species, corroborating evidence that pollen exploitation and digestion require a higher degree of specialization⁵⁹, even in generalist bees⁶⁰, which is often facilitated by each species' gut microbiome⁶¹. Although

some plant species appeared in the samples of all three bee species, *S. postica* and *M. rufiventris* shared more species while *T. angustula* differed from both. Considering body size vs. flower matching, the smallest species, *T. angustula*, visits the highest number of species of the three, potentially due to solitary foraging behavior, in which females forage alone without recruiting other workers.

Melipona species present a unique foraging pattern among stingless bees, not only because they are amongst the largest stingless bees (up to 15 mm)³, but because they show clear preferences towards some groups of plants^{22,62}. *Melipona* are also the only stingless bees capable of buzzing to harvest pollen⁶³, but pollen-flowers that require buzz-pollination for pollen harvesting were not abundant in the samples, even though species with poricidal anthers were observed flowering around the nests during the months of collection (e.g., *Miconia ferruginata* DC, *Pleroma stenocarpum* (Schrank & Mart. ex DC.) Triana, *Solanum falciforme* Farruggia).

Our botanical surveys also reinforced the patterns of floral exploitation among the three species, such as the apparent preference for trees with mass flowering by stingless bees, even though their exploitation demands a long flight range. Some stingless bees' sophisticated communication abilities allow a massive recruitment of foragers when mass blooming plants are available¹⁹. In the case of *T. angustula*, which is considered a solitary forager, the range of pollen sources is wider and seems less biased towards mass blooming plants. In the Atlantic rainforest, another hyper diverse neotropical ecosystem, stingless bees have a preference for upper canopy stratum with small hermaphroditic or monoecious whitish flowers and abundant resources (pollen and/or nectar)²⁹. Importantly, most of their preferred trees flower in mass, i.e. produce a large number of flowers over a short period of time²⁰. In the Cerrado savannas, where the nests were, we observed the typical high frequency of shrubs and herbaceous species in stingless bees pollen (ca. 38% of samples), which reflects the savanna physiognomy where herbs and shrubs are predominant⁶⁴. However, despite the high availability of flowers in the savanna surrounding their nests, they still flew up to riverine forests at least 630 m far from the nests to collect resources where mass-flowering species were more common.

Flight distance in bees is usually related to body size (larger bees tend to have wider flight ranges)⁶⁵ and social behavior (social bees have a larger foraging distance than solitary bees due to the potential communication and recruitment between individuals)⁶⁶. Given that the closest riverine forest is located at a distance of 630 m to the nests, and that species from this habitat were among the most abundant in the samples, this suggests that all three stingless bee species will forage and probably recruit at least 630 m from their nests, supporting the hypothesis of long-distance foraging when attractive rewards are available²⁰. This distance is well within the known flight range of *Melipona* whose typical flight distance is about 2 km, but can be extended up to 10 km¹⁸, but it is more surprising for *Scaptotrigona* and *Tetragonisca* whose reported maximum flight distances are 1.7 to 0.6 km, respectively¹⁸.

These estimates of minimum foraging distance of 630 m are considered trustworthy based on the high frequency of pollen from species occurring only in riverine forests, e.g. *Syzygium cumini*, an introduced species that only occurs in a small portion of the nearest riverine forest to the nests. Other highly abundant species in our samples are common in the Distrito Federal riverine forests (*Clusia cruiva* Cambess, *Hedyosmum brasiliense* Mart. ex Miq., *Miconia hirtella* Cogn., *Piper aduncum* L., *Richeria grandis*)^{67–69} and were only found in our surveys of the riverine forests (Table 1).

Some plant families stand out as the most important floral sources for the three stingless bee species, i.e. have one or more species amongst the 30 most frequent ASVs. Amongst them, Myrtaceae, Anacardiaceae, Sapindaceae, Melastomataceae, Euphorbiaceae, and Asteraceae are well-known as common resources for stingless bees globally¹⁷, while Loranthaceae and Malpighiaceae are frequent in other studies⁶². Phyllanthaceae, Primulaceae, Chloranthaceae and Piperaceae, however, have been only rarely reported²². Asteraceae, Myrtaceae, and Melastomataceae are amongst the most speciose plant families in the IBGE reserve, representing at least 300 species with different life forms (from herbs to trees) in the flora²⁹, but it is surprising that other diverse plant families in the IBGE area, i.e. Fabaceae, Lamiaceae and Orchidaceae, which also represent close to 300 species combined²⁹, are less conspicuous or totally absent from our most frequent 30 taxa. This means that, although the important floral sources for stingless bees partially overlap with the most common plants in the area, indicating that abundant sources are preferred, this is not always the case. This could simply mean that species within these families were not flowering at the time of sampling, but it is worth noting that Lamiaceae, papilionoid legumes and orchids share complex floral morphologies that are different from those of the families recorded as most abundant in our samples. These three families tend to present flowers with bilateral symmetry, specialized petals and androecia, and deep, hidden resources that often forces floral visitors to approach and handle the flowers in a specific way⁷⁰. Our results confirm the hypothesis that stingless bees may be specialized in exploiting small, open resource “bowl-type” flowers⁵², with exposed stamens and nectar, that are produced in large numbers²⁰. They may also favor plant species with a “big bang” flowering phenology i.e., that undergo mass blooming for short periods. Floral morphology, floral chemistry and phenology of plants exploited by stingless bees deserve further investigation. Investigations of plant resources exploited by stingless bees using metabarcoding over a longer time periods, in other types of vegetation, and of other bee species, would also be desirable to consolidate our knowledge of stingless bee ecology in the Neotropics.

In conclusion, our high-resolution results should encourage future studies on bee-plant interactions in hyper diverse tropical environments using pollen or honey DNA metabarcoding, even considering the currently low DNA sequence coverage of tropical plant species in public databases²⁸. The method has been proved useful not only for pot-pollen, but also for pollen loads carried by bees in their scopae or corbiculae¹². Given the importance of social and solitary bees for pollination of tropical crops and natural ecosystems, this methodology has the power to answer various important ecological questions, regarding the use of floral resources by bees, the dependence of bees on specific floral sources, their role as pollinators in crops or how well they compete with different species.

Data availability

The data that support the findings of this study is available at public repositories: metabarcoding sequence data is available at NCBI (<https://www.ncbi.nlm.nih.gov/bioproject/976708>); all plant species vouchers were deposited in the Herbarium of the University of Brasilia (UB) and all related information, including deposit number, is available in the Species Link network by searching under the collector name “Projeto Barcode Cerrado” (<https://specieslink.net/search/>). Analytical R codes used in our analysis are deposited at https://github.com/tncvasconcelos/barcode_cerrado.git and the bioinformatics analysis pipeline used for data analysis is available at https://github.com/chiras/metabarcoding_pipeline.

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Competing interests

The authors declare no competing interests.

Additional information

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