Received: 15 October 2020 | Accepted: 10 November 2021

DOI: 10.1002/2688-8319.12120

Ecological Solutions and Evidence | BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

Monitoring of honey bee floral resources with pollen DNA metabarcoding as a complementary tool to vegetation surveys

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Funding information
CSIRO Environomics Future Science Platform

Handling Editor: Scott MacIvor

Abstract

1. Monitoring biodiversity is a growing and pressing challenge, particularly as climate change threatens species with extinction and leads to widespread shifts in plant distribution and phenology. Tracking changes via ground vegetation surveys is costly and time-consuming, hence monitoring of complex and heterogenous communities remains an ongoing challenge.
2. Molecular DNA methods are rapidly being developed to provide fast and reproducible results for environmental monitoring, including diet and ecosystem assessments. Here, we used DNA metabarcoding of pollen foraged by European honey bees (*Apis mellifera*) to investigate their floral resource use in an urban reserve. We collected three different pollen samples from hives: individual bees, raw honey and pollen traps, and identified plants using two metabarcoding markers (ITS2 and trnL). We then compared the results to a ground vegetation survey of surrounding flowering taxa.
3. Pollen DNA metabarcoding detected 74 taxa (48.6% identified to species) across all pollen sources, compared to 44 taxa recorded by the survey (93% identified to species). Within the metabarcoding results, we identified 25% of the genera and 9% of the species found during the survey, with three of the top 10 flowering genera represented. While honey was the most taxon-rich pollen source (mean = 8.5, *SD* = 3.5), followed by honey bees (mean = 5.8, *SD* = 6.1) and pollen traps (mean = 4.2, *SD* = 1.7), combining the results of six individual bees could detect similar taxa numbers to honey, while 20 bees were required to detect as many taxa as the survey.
4. We demonstrate how DNA metabarcoding of the pollen foraged by honey bees can detect more flowering taxa than traditional survey methods, and how different pollen sources and genetic markers affect the level of detection of plant taxa. The foraging choices of honey bees matched few species detected by the vegetation survey, therefore pollen metabarcoding is recommended as a complementary approach to ground surveys. Rigorous validation and stringent filtering of

metabarcoding results were also required to exclude potential false positives. Altogether, this molecular approach can be used to augment vegetation surveys, while tracking the floral resources used by bees.

KEYWORDS
Apis mellifera, biomonitoring, honey, ITS2, metabarcoding, pollen, trnL

1 | INTRODUCTION

The global effects of climate change on plant species diversity and distribution have been well documented (Kelly & Goulden, 2008; Pearson et al., 2013; Walther et al., 2005). The detection of shifts in vegetation patterns has been largely enabled by long-term ground surveys (Crimmins et al., 2011; Elmendorf et al., 2012; Gonzalez et al., 2010) providing vital information for monitoring, management and conservation (Chytrý et al., 2011). Traditionally, ground surveys are conducted by researchers systematically identifying, measuring and recording plant, soil and climatic traits along transects or in plots (Austin & Heyligers, 1989), resulting in very fine-scale resolution of vegetation. This approach is both labour and cost intensive and requires significant botanical expertise. Remote sensing methods can supplement ground surveys by inspecting vast areas and collecting large amounts of data using high (below 1 m) and ultra-high (below 20 cm) resolution satellite imagery (Antwi et al., 2008; Felinks et al., 1998; Fernández-Guisuraga et al., 2018). However, remote sensing methods are limited to mapping broad vegetation types or detecting a few target species due to a trade-off between resolution and coverage (Larson et al., 2020). Rapid and scalable surveys that can detect multiple individual species, particularly smaller plants, within complex and heterogenous landscapes would be a significant advantage to monitoring programs.

In recent years, high-throughput molecular analyses, especially those dealing with traces of DNA in the environment (known as eDNA), have been successfully employed to monitor the presence of species in wide-ranging habitats, such as fish and mammals in oceans (Bessey et al., 2020; Foote et al., 2012), invertebrates in soil (Bienert et al., 2012) and aquatic plants in wetlands (Shackleton et al., 2019). Another common application of eDNA analysis is diet assessment of animals (De Barba et al., 2014; Kartzinel et al., 2015; Pompanon et al., 2012). Few eDNA studies to date have measured vegetation diversity in terrestrial environments. Analyses of soil eDNA have successfully described above and below ground plant diversity, but detection can be obscured by the abundance of non-target organisms, such as fungi (Fahner et al., 2016), or inhibited by environmental conditions (van der Heyde et al., 2020). Animal scats have also been proposed as a source of plant eDNA for biomonitoring (van der Heyde et al., 2020), but few results are yet available. An alternative approach to monitoring the presence of angiosperms is via the analysis of plant DNA found in pollen, a technique that has the potential to discriminate species more efficiently than conventional microscopy (Keller et al., 2015; Milla et al., 2021; Smart et al., 2017; Valentini et al., 2010).

Several studies have used pollen DNA metabarcoding to examine the diet and foraging preferences of European honey bees (*Apis mellifera* L.) (Cornman et al., 2015; de Vere et al., 2017; Galimberti et al., 2014; Nürnberger et al., 2019; Richardson, Lin, Quijia, et al., 2015). Honey bees are one of the most common insect pollinators worldwide, extensively used in agricultural and urban settings for their generalist pollination ability and honey production (Aizen et al., 2009; Deelstra & Girardet, 2000; Klein et al., 2007). Large global declines in colony numbers (Dainat et al., 2012; Ellis et al., 2010; Seitz et al., 2015; vanEngelsdorp et al., 2010) have led to growing interest in improving hive management practices. Pollen diversity in honey bee diet can contribute to longer lifespans and improved immunocompetency (Alaux et al., 2010; Pasquale et al., 2013), thus ensuring bees have access to a variety of floral resources can help maintain hive health (Decourtye et al., 2010). A single colony can visit thousands of flowers each day (Requier et al., 2015), with the average foraging distance ranging from a few hundred metres to a few kilometres depending on resource availability, landscape and season (Beekman & Ratnieks, 2000; Couvillon et al., 2015; Danner et al., 2017; Sponsler et al., 2017; Steffan-Dewenter & Kuhn, 2003). Pollen DNA analyses have shown that pollen collected by honey bees can be used to detect a botanical geographic signature (Milla et al., 2021; Utzeri et al., 2018), changes in plant phenology (Cornman et al., 2015) and the presence of rare, toxic or invasive species (Bruni et al., 2015; Galimberti et al., 2014; Tremblay et al., 2019; Utzeri et al., 2018). For these reasons, identifying the pollen collected by honey bees has been suggested as a potential vegetation monitoring tool (Cornman et al., 2015; Richardson, Lin, Sponsler, et al., 2015; Tremblay et al., 2019). However, it is unclear how well different sources of collected pollen reflect the surrounding plant diversity, with previous findings suggesting bees visit only small fraction of the surrounding flowering vegetation (de Vere et al., 2017).

We compare here for the first time three different honey bee pollen sources (individual bees, pollen collected with traps and pollen extracted from honey) and compare the plants foraged by hives placed within an urban reserve against flowering plants recorded by a ground vegetation survey of the reserve and to those historically recorded within a 5-km radius. For the metabarcoding part of the study, we selected two widely used plant markers, ITS2 and trnL, which are relatively short and suitable for degraded DNA (Chen et al., 2010; Hollingsworth et al., 2011; Taberl et al., 2006). In this study, we address two main questions. First, we assess how different pollen sources and metabarcoding genetic markers compare in terms of the number of plant taxa detected and identified. Second, we compare metabarcoding

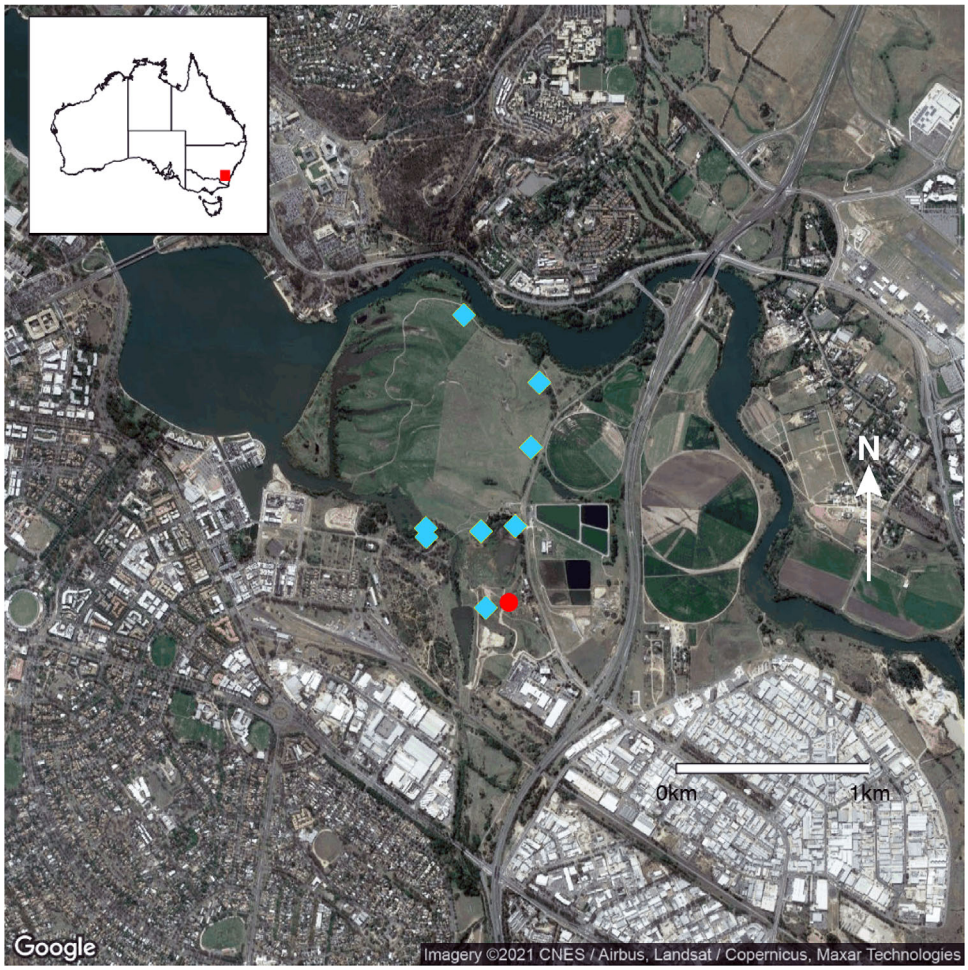

FIGURE 1 Map of Jerrabomberra wetlands (centre) and the surrounding urban area in ACT, Australia. Location of vegetation survey plots (blue squares) and location of beehives (red circle) are shown

of honey bee-collected pollen to the taxa recorded during the ground vegetation survey.

2 | MATERIALS AND METHODS

2.1 | Vegetation survey and pollen collection

The study site was located at Jerrabomberra wetlands, an urban reserve in Canberra, Australia. Vegetation surveys of angiosperms were carried out on 31 October 2019 and 2 November 2019, using eight randomly selected 50 m × 50 m plots at locations shown in Figure 1. Counts of individual plants for every flowering species found within a plot were recorded, the total number of flowers (if any) estimated, and plant vouchers were identified by botanists at the Australian National Herbarium (Figure S1). On 31 October 2019, pollen was sampled from three honey bee hives that had been located at the site for several months. Ten bees from each hive were collected in sterile 50-ml tubes as they returned to the hive, representing pollen collected by individual bees during foraging. Between 2.4 and 9.7 g of honey was collected from each hive by scraping honeycomb directly into sterile 50-ml tubes, representing pollen and nectar collected over several weeks. Two pollen traps (Bee Equipment Australia) were placed at the entrance of two hives and left for a period of 24 h. Pollen pellets collected by worker bees were caught in a tray below. The traps were removed on 1 November 2019, and the pellets collected were stored with all other samples at −20°C until processing.

2.2 | Pollen DNA amplification and sequencing

We followed suitable pollen isolation and DNA extraction protocols for each of the pollen sources, as described in Supplementary Materials S1. Metabarcoding works by amplifying short genetic markers that can discriminate species through interspecific sequence differences, or "barcode gaps" (Hebert et al., 2003). The internal transcribed spacer region 2 (ITS2) is a variable region between 100 and 700 bp in plants (Yao et al., 2010), and it has been shown to provide good discriminatory power in pollen metabarcoding studies, particularly at the genus level (Keller et al., 2015; Richardson et al., 2019; Richardson, Lin,

Sponsler, et al., 2015). The P6 loop of the chloroplast transfer RNA gene for Leucine (trnL P6) is a very short region between 10 and 143 bases with high amplification success, and it has been used in various metabarcoding studies dealing with degraded DNA, including honey pollen (Pornon et al., 2016; Valentini et al., 2010). Both markers were amplified via a two-step PCR process, as well as four DNA extraction controls, and three PCRs each of blank controls and positive controls. Full protocol details are in Supplementary Materials S1.

2.3 | ZOTU prediction and taxonomy assignment

An overview of the data processing workflow is illustrated in Figure S2. Briefly, the demultiplexed paired FASTQ reads were merged with USEARCH v11.0.667_i86linux32 (Edgar, 2010). Gene-specific primer sequences were removed from the merged reads using cutadapt 2.7 (Martin, 2011). We used the fastqfilter function of USEARCH to filter trimmed reads to Q20. Sequences were denoised and zero-radius operational taxonomic units (ZOTUs, or sequences representing individual taxa) were chosen using the USEARCH unoise3 function using a minimum cluster size of 16 reads.

To create custom reference databases, we downloaded Viridiplantae sequences from the ITS2DB (Ankenbrand et al., 2015; Koetschan et al., 2010, 2012; Merget et al., 2012; Selig et al., 2008) website at http://its2.bioapps.biozentrum.uni-wuerzburg.de and the trnL UAA intron region file (trnL_GH.fasta) from the PlantAligDB (Santos et al., 2019) website at http://plantaligdb.portugene.com on 20 August 2020. We used mothur (Schloss et al., 2009) to remove duplicates and retain sequences between 100 and 800 bp for trnL and between 50 and 1000 bp for ITS2. To filter sequences to species recorded in the honey bee foraging area, we drew a 5-km radius from the approximate location of the hives (−35.32, 149.161) using the Atlas of Living Australia (ALA) website (www.ala.org.au) and exported all plant records, filtered to those species that had at least three spatially validated entries since 1960. Using a custom python script, we filtered the downloaded ITS2DB and trnL_GH files to species names from the ALA plant list and generated searchable databases with the USEARCH makeudb program. To assign taxonomy to predicted ZOTUs, we used the USEARCH sintax function with an 95% bootstrap cut-off against the corresponding custom databases. The sintax command uses the kmer-based SINTAX algorithm (Edgar, 2016) to make taxonomic predictions. Secondary identifications were assigned using blastn against the full NCBI nt database, returning the top 10 matches. The top BLAST results were parsed using the python script taxonomy_assignment_BLAST_V1.py (github: Joseph7e/Assign-Taxonomy-with-BLAST), which assigns the consensus lowest taxonomic level possible from all BLAST hits that match the provided identity percent cut-offs. We set a minimum e-value of $1e^{-10}$, minimum identity cut-offs of 95%, 90% and 80% for species, family and phylum, respectively, a minimum query cover of 90% and a maximum of 0.5% divergence from the best hit to be included in the consensus.

2.4 | Data analysis

Further analyses were performed using the R statistical software (R Core Team, 2019) and are shown in Supplementary Materials S2. To deal with potentially erroneous taxonomy assignments, we checked any mismatches at family and genus level between the USEARCH assignment and the BLAST results where the BLAST identity score was at least 99%. We excluded ZOTUs where there was a family mismatch between the USEARCH and BLAST results and checked each mismatch at the genus level. We assigned the BLAST results to ZOTUs where no identification to at least family level was assigned by USEARCH, and then removed all remaining ZOTUs without a taxonomic assignment. To address potential contamination, we used the R package metabaR (Zinger et al., 2020). First, we identified contaminant ZOTUs based on their presence in negative controls using the contaslayer function. Secondly, we determined an adequate abundance filtering threshold to reduce the number of false positives. Using the function tagjumpslayer, we tested a range of filtering thresholds to reduce the number of ZOTUs present in the negative controls. Adequate sampling was checked with rarefaction curves using the rarefy function of the R package vegan 2.5.6 (Oksanen et al., 2007).

After removing contaminants and filtering reads to lower the rate of false positives, the remaining ZOTUs were merged by their taxonomic identification using the tax_glom function of the phyloseq package (McMurdie & Holmes, 2013). We subsequently use the terms taxon or taxa to refer to ZOTUs identified and merged to the lowest level of identification (either family, genus or species). We further excluded any taxa representing less than 1% of the reads of a sample. We fitted a Gamma probability distribution to both datasets based on the best fitting distribution calculated during data exploration. We compared the results using generalized linear models, with species richness as the response variable, and pollen source and hive as factors, and compared richness between groups using Kruskal–Wallis tests. To compare beta diversity, we converted counts to relative abundances. We calculated distance indices and tested differences using Wilcoxon rank sum tests. Differences between pollen substrates and hives for each marker were visualized with Principal Coordinates Analysis (PCoA) ordination and tested using PerMANOVA with the adonis function in vegan. We also tested the differences in dispersion (variance) for each pollen source with the betadisper command in vegan. We repeated the PerMANOVA analyses using presence/absence data by converting abundances greater than 0 to 1.

To compare the metabarcoding results with those of the vegetation survey, we first examined the overlap in families, genera and species detected by each method, with the metabarcoding results from the three sample types (bees, honey and pollen traps) and markers combined. Because resolution of metabarcoding results to species level was low, we then compared all genera and the top 10 taxa (based on highest number of samples detected) detected by metabarcoding in each sample type to the genera and top 10 taxa (based on highest number of plots) detected by the survey. Finally, we calculated the

proportion of taxa detected by each method compared to the taxa recorded in the area in the ALA at all identification levels.

3 | RESULTS

3.1 | Database creation and amplicon sequencing

The ALA search within 5 km of the hives returned a total of 601 plant species in the area. We found reference sequences for 412 (68%) species in ITS2DB and 248 (41.3%) species in trnL_GH. From the sequencing results, we obtained a total of 4,629,533 reads for ITS2 after trimming and filtering. We predicted 823 ITS2 ZOTUs, of which 291 (with 56% of reads) were identified to at least family level by the USEARCH algorithm. We removed 23 fungal ZOTUs with 18,200 ITS2 reads from the analysis, as well as 304,934 reads that did not match any plant sequences. There were no family mismatches and one genus mismatch (*Lolium*, USEARCH, 97% support compared to *Bromus hordeaceus*, BLAST, 100% identity) between the custom ITS2 database and the BLAST hits. The mismatch was removed. We assigned taxonomy from BLAST results to an additional 336 ITS2 ZOTUs not identified by USEARCH. We removed 10 ZOTUs identified as potential contaminants and set a threshold of 0.3% minimum ZOTU abundance per sample to reduce false positives (Figure S3). One bee sample was removed as rarefaction analysis indicated it had been under-sampled.

For trnL, we obtained a total of 6,208,784 reads after trimming and filtering. We predicted 105 trnL ZOTUs, of which 61 (93.7% of reads) were identified to at least family level by the USEARCH algorithm against the filtered PlantAligDB. We removed 490 trnL reads that could not be classified as plants. We found one family mismatch (Betulaceae, USEARCH, 96% support and Casuarinaceae, BLAST, 100% identity) that was removed. We assigned taxonomy from BLAST results to an additional 28 ZOTUs not identified by USEARCH. We identified and removed seven ZOTUs as potential contaminants in the trnL dataset and set a threshold of 0.3% minimum ZOTU abundance per sample to reduce false positives. We also removed one bee sample as rarefaction analysis indicated it had been under-sampled.

After filtering reads to remove false positives and potential contamination, we merged the remaining ZOTUs by taxonomic identification, resulting in 19 unique taxa from trnL and 116 unique taxa from ITS2. Removing low abundance taxa (below 1%), resulted in 73 taxa from ITS2 and 10 taxa from trnL, with 9 taxa detected by both markers (Figure 4a).

3.2 | Comparison of metabarcoding markers and pollen sources

With ITS2, we detected an average of 10.3 plant taxa per bee, 5.5 taxa per pollen trap sample and 11.2 taxa per honey sample. With trnL, the mean detected taxa were 1.2 per bee, 3 per pollen trap sample and 5.8 per honey sample (Figure 2a). The numbers of detected taxa by each pollen source were not significantly different using ITS2, but they were using trnL (Kruskal–Wallis, ITS2: $\chi^2 = 2.74$, $p = 0.25$; trnL: $\chi^2 = 26.05$, $p < 0.001$). We found significant differences between the relative abundances of plant taxa detected by each marker in pollen sources combined (Wilcoxon rank sum, $p < 0.001$) and individually (ITS2: $F = 5.27$, $df = 2$, $p = 0.001$; trnL: $F = 27.18$, $df = 2$, $p = 0.001$; also Figure S5a,c). These differences in pollen source were also significant when the data were converted to presence/absence of taxa (ITS2: $F = 4.99$, $df = 2$, $p = 0.001$; trnL: $F = 8.57$, $df = 2$, $p = 0.001$; also Figure S5b,d). Using the species accumulation curve for bees, we calculated that we could detect as many taxa as the average number (+2 *SD*) found in a pollen trap sample by combining the results of four bees (Figure 2b). We also estimated that combining the results from six bees would detect as many taxa as the average honey sample (+2 *SD*) (Figure 2b).

When comparing the results of metabarcoding markers from combined pollen sources, we found that 90% of trnL taxa was also detected by ITS2 (Figure 4a). After combining markers, we found pooled individual bees detected around half of the taxa (41 or 55.4%), with only a small overlap between the three pollen sources (Figure 4b). We found greater overlaps in taxa detected by the individual hives ranging between 21.4% (pollen traps, two hives) and 34.3% (bees, three hives) (Figure S6). With both metabarcoding markers combined, 74 distinct taxa were detected. ITS2 provided much higher taxonomic resolution than trnL, with 47% of 73 detected taxa identified to species level, compared to 20% of 10 taxa detected by trnL. By combining both markers, we identified a total of 36 species, 45 genera and 25 families (Figure 4c), with 85% of unique taxa identified to at least genus level.

The taxa with the highest mean read percentages per sample detected by each marker were Rosaceae (mean: 75% of reads), Rhamnaceae (57%) and Salicaceae (48%) for trnL, and Rosaceae (43%), *Malva* (33%) and *Salix* (30%) for ITS2. Both markers had high mean proportions of reads (>10%) mapping to *Quercus robur* and *Acer*. Salicaceae species were detected in high levels within honey samples (mean: 45%), while Malvaceae species were in the most abundant taxa for bees and pollen traps, but not for honey.

3.3 | Comparison of honey bee-foraged plants to vegetation survey

The vegetation survey detected a total of 44 taxa (with 41 or 93% identified to species level, Figure S1) belonging to 32 plant genera and 20 families in the eight 50-m plots (Figure 4c). Within each plot, we recorded between four and 28 species. Overall, we recorded open flowers on 39 species (88.6%) from 29 genera (90%) and 19 families (95%) (Figure S1). Using the species accumulation curve for all bees, we calculated that the combined results of 20 bees could detect at least as many taxa as the ground survey (Figure 2b).

The most frequently detected genera in the survey plots were three non-native herbaceous plants: *Plantago* (75% of plots), *Hypochaeris* (62%) and *Sonchus* (50%) (Figure 3 and Figure 5). In terms of highest proportion of flowers, the top three genera in the survey were *Eucalyptus*, a widespread genus endemic to Australia (26.7% of flowers recorded, at least five species), *Conium* (18.3%, one species, *C. macu-*

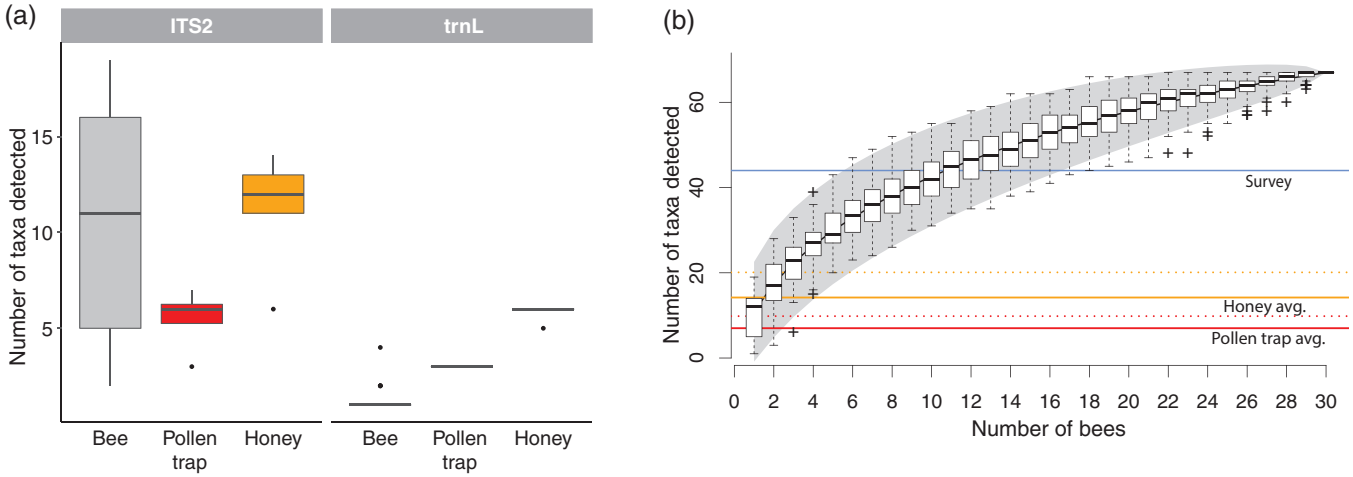

FIGURE 2 (a) Numbers of detected taxa at different taxonomic levels for each of the pollen source types (grey for bees, red for pollen traps and orange for honey) and both metabarcoding markers (ITS2 on left, trnL on right). (b) Species accumulation curve for taxa detected by sampling increasing numbers of individual bees. Grey area indicates region within ±2 *SD* of taxa detected by bees. Horizontal lines represent average species richness detected by pollen traps (red) and honey (yellow), and total species detected by survey (blue). Dotted lines represent +2 *SD* for pollen traps and honey species richness

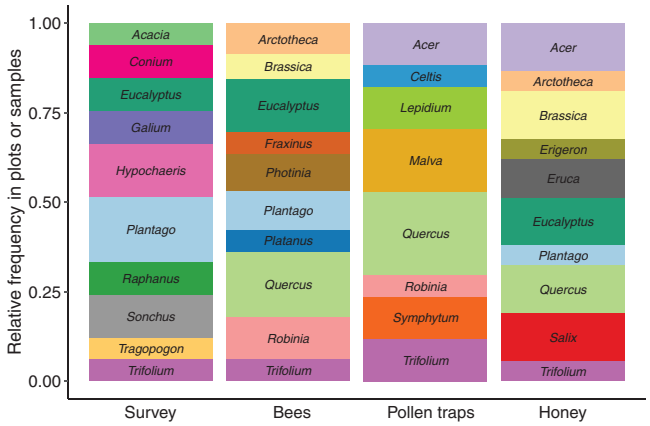

FIGURE 3 Relative frequency of the top 10 genera detected in survey plots or in pollen samples. Bar heights represent the relative frequency of each plant genus in the top 10 based on all survey plots or all samples of each pollen sample type. Colours are used to differentiate each plant genus. For pollen samples, results from ITS2 and trnL metabarcoding markers have been combined

latum) and *Raphanus* (10.8%, one species, *R. raphanistrum*) (Figure S1). The three genera and families most frequently detected by metabarcoding were *Quercus* (90% of bees, 100% of honey and pollen trap samples), undifferentiated Rosaceae species (87% of bees, 100% of honey and pollen trap samples) and *Eucalyptus* (73% of bees and 100% of honey samples) (Figure 3 and Figure 5). While we observed standing trees of *Quercus* in the vicinity of the hives, they were not present in our survey plots nor in ALA records. *Trifolium* (30% of bees, 50% of pollen trap and 40% of honey samples) and *Acer* (23% of bees, 50% of pollen trap and 100% of honey samples) were also commonly detected taxa among pollen sources. Three of the top 10 genera in the survey were also detected in the top 10 metabarcoding results: *Plantago*, *Eucalyptus* and *Trifolium* (Figure 3). Overall, we found 28.6% of families, 11.6% of genera and 5.3% of species in common between our survey and the metabarcoding results (Figure 4c).

When compared to the ALA records within a 5-km radius, we found that 22 (21%) families, 30 (8.6%) genera and 15 (2.4%) species were detected in the pollen foraged by bees (Figure 4d). Comparatively, the survey (which covered a smaller area of approximately 2 km^2) detected 20 (19.6%) families, 30 (8.9%) genera and 32 (5.2%) species recorded in the area (Figure 4d). Both methods detected taxa not found within the ALA records, with metabarcoding detecting an extra 21 species, 15 genera and three families, and the survey detecting an extra 12 species and two genera.

4 | DISCUSSION

Efficient and comprehensive detection and monitoring of biodiversity is critical, as global changes including land clearing (Bradshaw, 2012; Tilman et al., 2001), invasive species (Mooney & Cleland, 2001) and climate change (Godfree et al., 2019; Hutyra et al., 2005; Liu et al., 2010) continue to threaten ecosystems. One way to assess impacts on vegetation is through regular plant community ground surveys, but these are resource intensive and require extensive botanical expertise. Although relatively new, pollen DNA metabarcoding has been demonstrated to be a powerful tool to investigate the foraging choices of honey bees (de Vere et al., 2017; Richardson et al., 2019). Its value in monitoring flowering plant diversity, however, has not been well established.

Our findings here suggest that DNA metabarcoding of pollen collected by honey bees can provide useful information on the surrounding flowering vegetation. We detected higher numbers of taxa (74) and genera (45) but identified fewer species (36) from 39 pollen samples

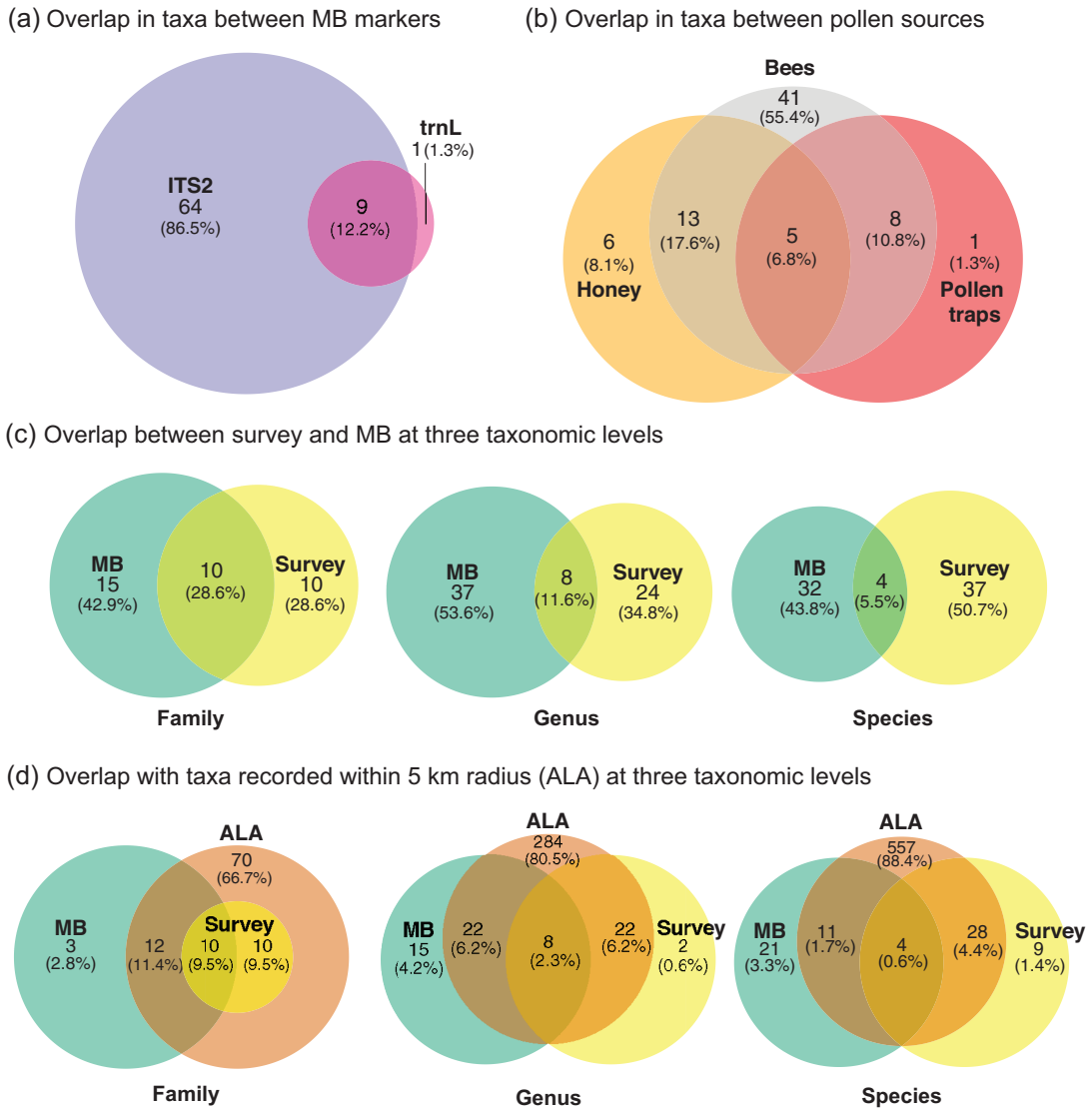

FIGURE 4 Overlap between taxa (lowest possible level of taxonomic identification) detected by different combinations: (a) in all pollen samples by each metabarcoding marker (purple for ITS2, pink for trnL), (b) in all pollen samples by pollen source type (grey for bees, red for pollen traps and orange for honey), (c) overlap between taxa at three different levels of taxonomic identification (family, genus or species) in all pollen samples by metabarcoding (green) and in all plots by survey (yellow), (d) overlap between taxa at three different levels of taxonomic identification (family, genus or species) by metabarcoding and survey compared to historical records from 5-km radius of hives (brown, *source*: Atlas of Living Australia [ALA]). For metabarcoding results in panels b–d, ITS2 and trnL results have been combined

compared to the ground survey of eight plots (44 taxa and 32 genera, with 41 taxa identified to species). The plants foraged by honey bees poorly matched the species identified by the survey, with only a quarter of the genera and less than 10% of the species detected in the survey found in the pollen samples. Although honey bees are supergeneralist foragers (Crane, 1990; Huryn, 1997), their foraging preferences can vary over time and according to flowering resources (Coffey & Breen, 1997), thus they are unlikely to visit the same plants detected by a systematic survey. Pollen sampling at different time intervals could help account for changes in bees' foraging preferences and detect a wider range of flowering taxa. Pollen metabarcoding is highly scalable and offers better taxonomic resolution than alternative pollen identification methods such as microscopy (Smart et al., 2017). While metabarcoding costs are not trivial (between $3000 and $4500 USD plus labour for 90 samples), Bell et al. (2017) concluded the cost of similar work using microscopy could be much higher as palynological expertise is very specialized and varies among palynologists. Additionally, the costs of sequencing have decreased over time and more labs are becoming proficient in PCR techniques. Overall, the reduction in time spent in the field and the scalability of the method make pollen metabarcoding a promising complementary tool for monitoring of flowering taxa, with further research required into optimizing the number of hives, their placement and sampling frequency in order to incorporate this approach into monitoring programs.

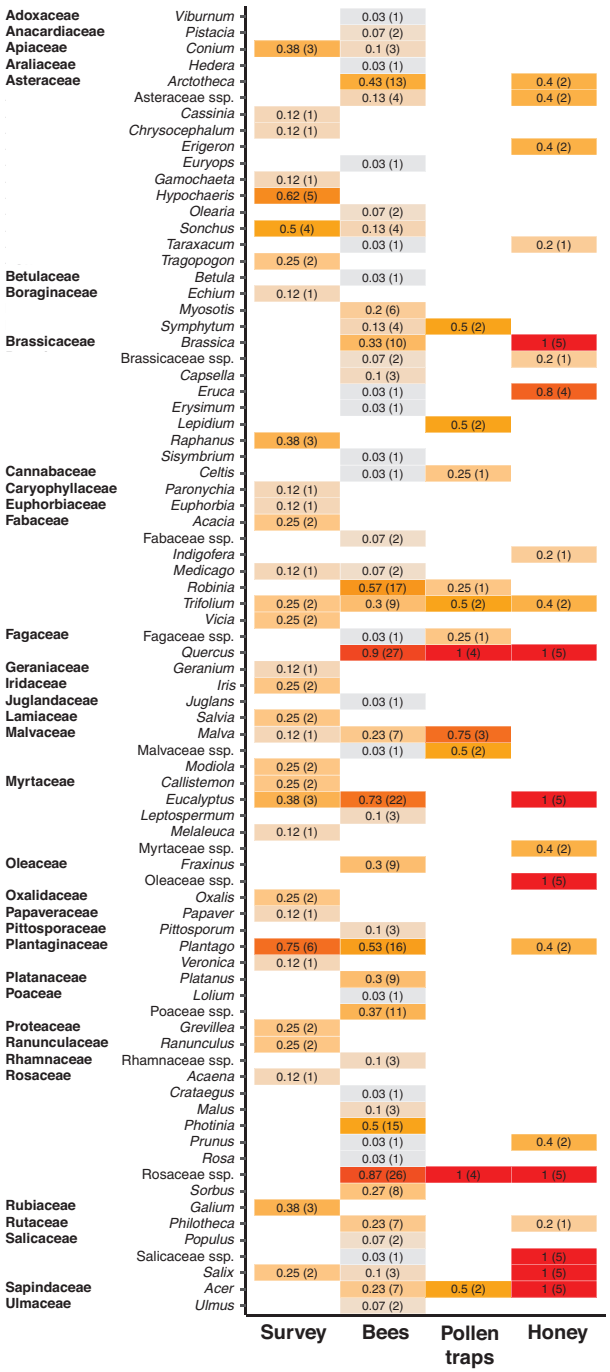

FIGURE 5 Families and genera detected by vegetation survey and by metabarcoding of bee, pollen trap and honey pollen samples. For pollen samples, ITS2 and trnL results have been combined. Numbers in cells indicate proportion of total plots (survey) or total samples (metabarcoding) where a given genus was detected, with the counts of plots (survey) and samples (metabarcoding) where the genus was detected in brackets. Cell colours indicate the size of the proportion, with the spectrum from grey (low) to orange (mid) to red (high) signifying increasing proportion

4.1 | Selection of metabarcoding markers for floral resource monitoring

A key consideration for DNA-based monitoring programs is the combination of appropriate genetic markers to deliver the desired detection sensitivity and taxonomic resolution. The relatively small overlap between ITS2 and trnL in taxa detected (nine out of 74 overall) emphasized the need to use multiple markers (Prosser & Hebert, 2017; Richardson et al., 2019; Richardson, Lin, Quijia, et al., 2015). We found that incorporating a range of quality control checks was critical for dealing with potential false positives and increasing robustness of the results: namely, using positive and negative controls to identify likely contaminants, comparing results against a global database to confirm taxonomic identifications and stringent filtering of the results to remove low abundance taxa. Accurate quantification of pollen from metabarcoding is still problematic (Baksay et al., 2020; Bell et al., 2019), with common issues such as PCR primer bias (Deiner et al., 2017) and the interaction between plant species (Pornon et al., 2016) potentially affecting results. While longer fragments of trnL have been found to quantify airborne pollen species relatively well (Kraaijeveld et al., 2015), it is unclear from our results whether the much shorter P6 loop shares the same characteristics.

Identification of taxa to species level through metabarcoding was low (48.6%), while identification to at least genus level was high (85.1%). The taxonomic resolution of a marker is highly dependent on a reference database match. Missing species can produce a false negative result, and closely related species without a "barcode gap" cannot be discriminated (Bell et al., 2019; Wilkinson et al., 2017). In our custom databases, there were also fewer representative sequences for trnL (340) than for ITS2 (703). While searching against a large public database, such as NCBI's GenBank, can generate more identifications, errors can be introduced due to incorrect annotations. To improve detection of a particular species of interest (for example, an invasive weed), specific markers could be developed to differentiate target sequences from closely related species. Furthermore, pollen metabarcoding has the potential to be extended to monitoring associated plant pathogens and other biota (Tremblay et al., 2019) by adding different markers without the need to repeat the sampling effort.

4.2 | Selection of pollen sources for metabarcoding

Most metabarcoding studies examining floral resource usage by honey bees have used pollen traps to collect pollen (Melin et al., 2020; Nürnberger et al., 2019; Richardson et al., 2019; Tremblay et al., 2019) or sourced it from honey (de Vere et al., 2017; Prosser & Hebert, 2017; Utzeri et al., 2018). It is commonly thought that individual honey bees exhibit high floral fidelity during foraging trips (Free, 1963; Westerkamp, 1991), thus bulk collecting samples of pellets or honey should detect a wider range of plants. Individual bees can carry large amounts of pollen, with estimates ranging from around 1400 (Horskins & Turner, 1999) to more than 11,000 grains per individual, excluding corbicula (Escaravage & Wagner, 2004). Microscopic analyses of pollen loads on

bees have identified dominant species (Escaravage & Wagner, 2004), but diverse pollen can be carried over from visiting several species (Horskins & Turner, 1999), or passively acquired through wind transport or contact with other bees. Our results suggest that some honey bees collect, or perhaps passively sample, several pollen species. Pollen DNA from a few bees (between four and seven per hive) produced as many taxa as the average pollen trap sample. Pollen traps greatly reduce the amount of pollen reaching the hive, and may impact the bees' health and foraging activity (Dubois et al., 2018; Webster et al., 1985). Honey is a natural product of honey bees; however, its production is contingent upon several conditions, such as colony size and available resources. While healthy colonies can produce up to 25 pounds of honey in a single day during spring (Cale et al., 1986), honey production is highly reduced or absent over winter. Because honey production is ongoing, it is difficult to infer when a particular pollen found in honey was collected. Therefore, repeated sampling using individual bees during target flowering periods is recommended. To detect longer term phenological changes such as relative shifts in flowering times over multiple years or large vegetation changes in the landscape due to agricultural intensification, honey could be a more suitable choice, as pollen in honey naturally accumulates over time. For example, Jones et al. (2021) used pollen DNA metabarcoding of honey samples to detect shifts in plants foraged by honey bees in the United Kingdom between 1952 and 2017, with intensification of agriculture, one of the factors leading to changes in floral resource availability. Using honey pollen could be used to sample longer intervals than recommended with pollen traps and avoid the need to collect many individual bees to cover the same period.

There were some differences in the plant composition of the pollen samples. *Salix* (Willow) was one of the major components of honey (mean abundance of 18% with ITS2) but found in much lower quantities in pollen trap and bee samples. *Salix* flower from September to October (Cremer, 2003) and were observed on the edges of the wetlands. As honey accumulates over longer periods of time, it could be used to detect plants with short flowering times, which may be missed by sampling individual bees or pollen traps at discrete intervals. The presence or absence of plants in pollen samples can be used to infer changes in phenology; however, sample replicates are essential. False positives may appear due to incorrect sequence identifications (tag jumps), which are unused index combinations that can spuriously appear in sequencing studies (Schnell et al., 2015). Residual pollen, such as pollen picked up by bees coming into contact with the previous season's resources (Richardson, Lin, Sponsler, et al., 2015), may inflate numbers of foraged taxa. False negatives are also possible due to low DNA abundance, taxon or PCR bias (Deagle et al., 2018; Pornon et al., 2016). Other potential sources of sample variation are the DNA extraction protocols. In this study, the pollen trap protocol specified drying of pollen at 60°C for 60 h and honey was incubated at 65°C for half an hour, whereas pollen from individual bees was not heated. It is not known whether the long drying period for pollen trap samples contributed to additional DNA degradation and may have affected the results, therefore consistent protocols and minimal pollen processing are recommended.

4.3 | Using honey bee-foraged pollen as a tool for angiosperm monitoring

One of the main advantages of using pollinating insects for monitoring flowering vegetation is their ability to visit plants in sensitive or difficult to survey areas, such as alpine regions (Mayr et al., 2021; Pornon et al., 2016). However, a major consideration of using managed honey bees for biomonitoring is their potential negative impact on wild pollinators (Cane & Tepedino, 2017; Henry & Rodet, 2018; Valido et al., 2019). Introducing hives is highly discouraged where honey bees are not native or common. Another major disadvantage of pollen metabarcoding compared to vegetation surveys is that accurate location information cannot be inferred from pollen DNA. In some cases, however, an approximate location within the foraging range of honey bees may be adequate, such as in rapid, continuous assessments of flowering vegetation or detecting the arrival of invasive species within a new area. As the amounts and types of pollen collected by hives can vary due to colony size, season and life stage, further research is needed on the optimal number of hives at each survey point. In our study, each additional hive led to a small increase in the number of species detected by bees (between 9% and 15% more taxa); therefore, more than three hives at each survey point may not be cost-effective. Alternatively, collecting multiple pollen samples from hives placed at the same location over an extended period can provide more opportunities to detect plants flowering at different times. As foraging distance will be influenced by landscape diversity and resource availability (Danner et al., 2017; Steffan-Dewenter & Kuhn, 2003), more research is also needed to determine the most effective placement of hives to survey increasingly complex habitats.

While there was little overlap between the taxa detected by honey bees and our vegetation survey, the plants foraged by bees provided useful additional information regarding the flowering vegetation of the study area. Several common genera recorded in the area such as *Quercus*, *Malus*, *Brassica* and *Prunus* were detected only via metabarcoding. *Malus*, *Brassica* and *Prunus* were present in a small, cultivated plot less than 100 m from the hives, which we were unable to survey as it was located within a private allotment. Similarly, there were several *Quercus* trees in the vicinity, a genus commonly found in the city of Canberra (Zhang & Brack, 2021), but they were outside the survey plots. Honey bees are known to exploit pollen resources from anemophilous plants (Giovanetti & Aronne, 2011; Keller et al., 2005), including *Quercus* (Aronne et al., 2012; Saunders, 2018). Another possibility is that pollen from wind-pollinated plants could adhere to bees during flight, as airborne particles have been detected on their bodies (Negri et al., 2015), although we have not tested this. Both the survey and metabarcoding were able to detect plants that were not present in the ALA records with more than half the species and a third of the genera detected by metabarcoding, and nearly a third of species recorded in the survey were not in the ALA list. However, the proportion of taxa identified to species level was much higher for the survey (93.2%) than for metabarcoding (48.6%). Identifying more taxa through metabarcoding could only be achieved here by expanding the sequence search to the larger NCBI nt database. Carefully curated

databases have been shown to achieve a high percentage of detection at species level (Galimberti et al., 2014; Richardson et al., 2020; Valentini et al., 2010). As more species are detected via direct surveys or via robust metabarcoding studies supported by local vegetation knowledge, custom plant databases can become more comprehensive and useful. However, species-level identification of plants from metabarcoding will remain a challenge for some groups, such as frequently hybridizing genera like *Eucalyptus* (Griffin et al., 1988), which show low genetic marker differentiation between species (Prosser & Hebert, 2017).

The foraging choices of honey bees mean that unattractive or morphologically unsuitable plants might not be visited (Hung et al., 2018), thus not all flowering plants may be suitable targets for monitoring using honey bees. Some flowering plants may also be underrepresented in pollen metabarcoding when pollen production is low. Non-entomophilous species are not necessarily excluded from detection, as pollens from wind-pollinated genera such as *Quercus* and *Acer* are often detected on honey bees (Bruni et al., 2015; de Vere et al., 2017; Hawkins et al., 2015; Tremblay et al., 2019; Utzeri et al., 2018). Using pollen metabarcoding on a diverse range of native pollinators could help address several challenges. Pollen metabarcoding can be applied to other pollinator groups, such as hoverflies and moths (Lucas et al., 2018; Macgregor et al., 2019; Pornon et al., 2016), extending monitoring to pollinators with diverse foraging preferences to fill in gaps in plant detection. Combining multiple pollinators for vegetation monitoring can not only target a wide range of flowering plants, but also provide valuable information on phenological changes and pollination services through the analysis of pollen transport or interaction networks (Gray et al., 2014; Pornon et al., 2016; Richardson et al., 2019). Native pollinators, particularly social bees (e.g. bumble bees), could be sampled non-destructively for pollen (Bänsch et al., 2020; Potter et al., 2019), potentially contributing towards species conservation efforts.

5 | CONCLUSIONS

Our results demonstrate that the use of pollinators and pollen DNA metabarcoding could be complementary to traditional methods surveying terrestrial ecosystems. Undoubtedly, ground vegetation surveys provide an important advantage over pollen-based molecular methods as they provide estimates of plant location, abundance and trait data (Austin & Heyligers, 1989), as well as being able to detect non-flowering taxa. In our study, expert taxonomic identification of plants was superior to the identification obtained by metabarcoding using the currently available data (i.e. reference sequence databases and occurrence data). However, the scalability and high-throughput nature of molecular methods, together with the capacity of pollinators to forage in relatively large and difficult-to-access areas, make the combination of pollen metabarcoding and insects a promising tool for tracking floral resources and complementing vegetation monitoring programs.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the contribution of Bioplatforms Australia in the generation of data used in this publication. Bioplatforms Australia is enabled by NCRIS. We also thank Alan Wade and the Canberra Region Beekeepers Association for their help providing their hives, their assistance collecting samples and many insightful discussions. We would also like to thank several reviewers and editors who provided detailed and thoughtful comments, and whose input has greatly improved this manuscript. This project was funded by the CSIRO Environomics Future Science Platform.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

FEV, ASL and LM conceived the ideas and designed methodology. FEV, ASL and LM collected the data. ASL identified plant vouchers. LM and JB conducted the lab work. LM and FEV analysed the data. LM and FEV led the writing of the manuscript. Our colleague and co-author Jessica Bovill sadly passed away before publication of this manuscript. All authors contributed critically to the drafts, and all except JB gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the CSIRO Data Access Portal: https://doi.org/10.25919/w5qg-c293 (Milla et al., 2020).

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How to cite this article: Milla, L., Schmidt-Lebuhn, A., Bovill, J., & Encinas-Viso, F. (2022). Monitoring of honey bee floral resources with pollen DNA metabarcoding as a complementary tool to vegetation surveys. *Ecological Solutions and Evidence*, *3*, e12120. https://doi.org/10.1002/2688-8319.12120