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Capabilities and limitations of using DNA metabarcoding to study plant–pollinator interactions

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Abstract

Many pollinator populations are experiencing declines, emphasizing the need for a better understanding of the complex relationship between bees and flowering plants. Using DNA metabarcoding to describe plant–pollinator interactions eliminates many challenges associated with traditional methods and has the potential to reveal a more comprehensive understanding of foraging behaviour and pollinator life history. Here we use DNA metabarcoding of ITS2 and *rbcL* gene regions to identify plant species present in pollen loads of 404 bees from three habitats in eastern Oregon. Our specific objectives were to (i) determine whether plant species identified using DNA metabarcoding are consistent with plant species identified using observations, (ii) compare characterizations of diet breadth derived from foraging observations to those based on plant species assignments obtained using DNA metabarcoding, and (iii) compare plant species assignments produced by DNA metabarcoding using a "regional" reference database to those produced using a "local" database. At the three locations, 31%–86% of foraging observations were consistent with DNA metabarcoding data, 8%–50% of diet breadth characterizations based on observations differed from those based on DNA metabarcoding data, and 22%–25% of plant species detected using the regional database were not known to occur in the study area in question. Plant–pollinator networks produced from DNA metabarcoding data had higher sampling completeness and significantly lower specialization than networks based on observations. Here, we examine some strengths and limitations of using DNA metabarcoding to identify plant species present in bee pollen loads, make ecological inferences about foraging behaviour and provide guidance for future research.

KEYWORDS
bees, DNA, metabarcoding, network, pollen, pollinator

1 | INTRODUCTION

Pollination is a crucial biological interaction that sustains ecosystem function and aids in human food production (Buchmann & Nabhan, 2012; Losey & Vaughan, 2006; Ollerton et al., 2011). This ecosystem service is performed by a diverse group of organisms including insects, birds, bats and some nonflying mammals. In recent years, many pollinator populations have experienced human-induced stressors including habitat loss, climate change and misuse of pesticides (Goulson et al., 2015; Vanbergen and Initiative, 2013;

Winfree et al., 2009) . Rufous hummingbird populations have experienced significant declines in the Pacific Northwest (Sauer et al., 2017), monarch butterfly populations continue to struggle in North America (Thogmartin et al., 2017) and bee populations are declining worldwide (Koh et al., 2016; Potts et al., 2010). Understanding the cause of these declines and how to reduce the resulting negative impacts requires a greater understanding of plant–pollinator relationships.

All pollinating species visit plants to search for food, often in the form of nectar and pollen (Ford et al., 1979; Gould, 1978; Ginsberg, 1983; Goulson, 1999; Mevi-Schütz and Erhardt, 2005; Nicolson, 2007; Reed Haisworth & Wolf, 1976). As individuals move from flower to flower searching for food, they often move pollen among flowers in the process, aiding in pollination. While many pollinating species forage primarily for nectar to meet their immediate energetic needs (e.g., hummingbirds, bats, butterflies), females of most bee species also collect protein-rich pollen to provision their nests in one of two general ways. For mass-provisioning bees (which include all nonparasitic, solitary bee species), females construct nests with individual brood cells for each offspring, and provision each cell with a mass of pollen, often mixed with some nectar, upon which the female will lay an egg. The cell is closed off until the larva develops into an adult and leaves the nest. Therefore, each female must provide sufficient resources to rear her larva to adulthood before closing the brood cell. Consequently, the fitness of mass-provisioning bees is highly dependent on the quantity and quality of pollen the adult female collects in a relatively brief period of time (Batra, 1984). For progressive-provisioning bees (which include social bees such as bumble bees and honey bees), females feed pollen and nectar to larvae in the colony as they develop. Thus, the productivity of the colony throughout the growing season, including the ultimate production of reproductive females and males, depends on the foraging behaviour of individual female workers throughout the growing season (Field, 2005). In both cases, a bee's foraging decisions have direct impacts on its fitness.

Because of its potential impact on fitness, much attention has been paid to the degree of specialization among bee species with regard to pollen foraging. Bee species can vary along a continuum of being pollen specialists (i.e., "oligolectic," bees that forage for pollen on one or a few plant species) to pollen generalists (i.e., "polylectic," bees that forage for pollen on many plant species) (Danforth et al., 2019; Robertson, 1925). When describing foraging behaviour in bees, it is important to distinguish between dietary specialization and floral constancy (i.e., when an individual forager utilizes the flowers of a single species during a foraging bout) (Grant, 1950). A species may exhibit polylectic behaviour as a whole, while simultaneously engaging in floral constancy during individual foraging trips.

Examples of specialist bees include species that have evolved specific relationships with their host plants, emerging from their nests as their host plant begins blooming (Minckley et al., 1994; Neff et al., 1982; Willis Chan & Kevan, 1995). There are several benefits to specialization, including provisioning brood cells quickly and foraging on plant species that produce pollen with high protein content and overall nutritional value (Danforth, 1990). Generalist bee species may have a more difficult time provisioning their nests because the nutritional content of pollen varies widely amongst different plant taxa (Danforth et al., 2019). However, generalist bee species may be less vulnerable to extinction or local extirpation because they can readily shift their diet to the resources that are currently available to them (McCann, 2000). Additionally, bee species with relatively long adult stages (e.g., *Bombus* spp.) are often characterized as polylectic, foraging on different plant taxa throughout the growing season (Miller-Struttmann et al., 2015; Russell et al., 2017).

A major puzzle in pollination ecology is how many bee species truly fall on the specialist end of the pollen specialist–generalist continuum. Although evidence suggests that numerous bee species are oligolectic (Fowler, 2016; Pekkarinen, 1997; Robertson, 1926), limitations in research methodologies cast uncertainty on these conclusions. Many studies of pollen foraging of bees are based on single observations of flower visits, and often do not differentiate between nectar feeding and pollen collecting (Daniels et al., 2020; Hass et al., 2018; Roof et al., 2018). Characterizing foraging behaviour using observations of bees visiting flowers presents several challenges and could produce biased results. Direct observation is time-consuming and is limited by the observer's field of vision. The difficulty of following a foraging bee from one flower to another often limits the observer to viewing one or two flower visits per bee, which can lead to overestimates of floral fidelity (Free, 1970; van der Niet et al., 2020). Solitary bees can spend anywhere from a few minutes to a few hours on a single foraging trip (Bohart & Youssef, 1976; Cameron & Ramírez, 2001; Gebhardt & Rohr, 1987). Therefore, extrapolating data from a single foraging observation to describe a bee's entire foraging trip may be misleading. Collecting data throughout entire foraging trips might decrease both estimates of flower fidelity for particular species and estimates of the proportion of "oligolectic" species comprising bee communities.

One way to examine entire foraging trips is to use microscopy to separate and identify the plant species in a bee's pollen load based on morphological characteristics of pollen grains (Erdtman, 1943). This method allows for the detection of all plant species from which a bee has collected pollen during the foraging trip in which it was sampled, but it also presents challenges and limitations. Plant species identification by microscopy of pollen is time-consuming, requires specialized expertise and often results in low taxonomic resolution (Cornman et al., 2015; Rahl, 2008). In addition, typically only a subsample of an insect's pollen load is analysed using this technique, which can result in a lack of detection of pollen from plant species present in low abundance (Sajwani et al., 2014; Von Der Ohe et al., 2004; Whittington et al., 2004).

A more recent technique, DNA metabarcoding, can be used to identify multiple species from a single environmental sample (García-Robledo et al., 2013; Kartzinel et al., 2015). Its use for pollen collected from foraging bees has been introduced as a promising tool for identifying a more complete representation of plant species that are present in pollen loads and investigating plant–pollinator networks (Bell et al., 2017; Cornman et al., 2015; Keller et al., 2015; Pornon et al., 2017; Richardson, Lin, Quijia, et al., 2015; Richardson,

Lin, Sponsler, et al., 2015; Smart et al., 2017). DNA metabarcoding combines species identification using DNA barcoding with next generation sequencing technology. In pollen DNA metabarcoding methods, DNA is extracted from pollen samples containing one or more plant species, and barcode regions with low intraspecific and high interspecific variation are amplified and sequenced using next generation sequencing. The species composition of a pollen sample is then determined based on the presence of DNA sequencing "barcodes" in the pollen samples and their similarity to sequences of known reference plant species (Hebert et al., 2003; Sickel et al., 2015).

Although traditional methods are still used more frequently to describe plant–pollinator networks than DNA metabarcoding (Morandin & Kremen, 2013; Wood et al., 2015), several studies have compared the taxonomic resolution of DNA metabarcoding with that of microscopy and found that DNA metabarcoding resulted in equal or higher taxon richness (Keller et al., 2015; Macgregor et al., 2019; Richardson, Lin, Quijia, et al., 2015; Richardson, Lin, Sponsler, et al., 2015; Smart et al., 2017). Yet very few studies have compared observations of bee foraging with DNA metabarcoding data, despite the fact that studies involving bee foraging observations are becoming more common because of the rising popularity of citizen science (Domroese & Johnson, 2017). The few studies that have compared these approaches either did not examine bees exclusively (Galliot et al., 2017; Pornon et al., 2017) or lacked taxonomic resolution of plants and pollinators (Potter et al., 2019). Examining the consistency, or lack of consistency, between plant–pollinator networks derived from bee foraging observations and DNA metabarcoding of bee pollen loads is important for determining whether observations alone can sufficiently identify major food sources used by bees, or whether they tend to oversimplify plant–pollinator networks. This understanding is necessary for characterizing the degree of pollen specialization of specific bee species and the basic ecology of bee communities, including functional trait composition.

There are challenges associated with pollen metabarcoding, including the issue of correctly identifying plant taxa to the species level (Keller et al., 2015; Kraaijeveld et al., 2015; Richardson, Lin, Sponsler, et al., 2015). While DNA metabarcoding increases the number of taxa assigned at the species level compared to traditional methods, species assignments obtained through DNA metabarcoding can be prone to sequencing and database errors, resulting in erroneous, or equivocal, species identifications (Cornman et al., 2015; Keller et al., 2015; Smart et al., 2017). The accuracy of plant species assignments is highly dependent on the reference database. Reference databases are constructed by acquiring DNA sequences of known plant species from curated databases or the National Center for Biotechnology Information (NCBI) nucleotide database (Sickel et al., 2015). Researchers often include all plant DNA sequences in a database for a specific barcode marker (Cornman et al., 2015; Galliot et al., 2017; Keller et al.; 2015; Sickel et al., 2015) or from a geographical location consistent with their area of study, which is often as large as an entire state or country (de Vere et al., 2017; Potter et al., 2019; Richardson, Lin, Quijia, et al., 2015). This ensures that all possible plant species used as forage resources are included in the database, but it can also increase the potential for false detections associated with equivocal identification. This equivocal identification may occur because species and genera of certain plant families (e.g., Asteraceae) share a significant portion of their DNA, making differentiation of these species difficult (Gao et al., 2010). Currently no studies have been conducted to document the potential benefits of using reference databases that are limited to plant species that occur in the location of interest (i.e., a "local" reference database) vs. a reference database that includes all plant species known to occur in a larger region (i.e., a "regional" reference database). One potential benefit of using a local reference database is reducing the potential for equivocal identification that can occur when using a larger reference database that includes plant species that are not known to occur in the study area but cannot be differentiated from closely related species that do occur in the study area. This benefit may outweigh the extra time and effort required to conduct plant surveys of an area before using plant species assignments derived from DNA metabarcoding of pollen to study the plant–pollinator interactions occurring there.

Here, we examine the accuracy of plant species assignments using DNA metabarcoding of pollen in the context of a study aimed at examining native bee–plant associations in eastern Oregon. The specific objectives of this study were to (i) determine whether plant–pollinator networks based on plant species identifications from DNA metabarcoding of pollen are consistent with plant–pollinator networks based on bee foraging observations, (ii) compare polylectic and oligolectic bee species diet breadth characterizations using foraging observations to those based on plant species assignments obtained using DNA metabarcoding, and (iii) compare plant species assignments obtained using a regional reference database to species assignments obtained using a local reference database.

2 | MATERIALS AND METHODS

2.1 | Study sites

The field component of this study was conducted in June, July and August of 2018 at three locations in eastern Oregon, USA: Threemile Canyon Farms (45.7513°N, 119.9376°W), the United States Forest Service (USFS) Starkey Experimental Forest and Range (45.2332°N, 118.5511°W) and The Nature Conservancy's (TNC's) Zumwalt Prairie Preserve (45.5559°N, 116.9587°W). These locations were chosen with the goal of examining plant–bee associations in different land use types (i.e., agroecosystem, riparian forest and grassland, respectively). The three study sites occur within 350 km of each other and are all located in the Blue Mountains Ecoregion.

Threemile Canyon Farms (Threemile) is a 37,636-ha industrial, pivot-irrigated farm located in Morrow County (elevation 100–300 m). Threemile has 9,308 ha in conservation area, 15,985 ha of irrigated conventionally managed cropland and 6,178 ha of irrigated organically managed cropland. The conservation area consists of arid grassland and shrub-steppe as well as field margins, which are dominated by non-native vegetation.

The USFS Starkey Experimental Forest and Range (Starkey) is located in Union County (elevation 1,130–1,500 m); this long-term research site established in 1940 is grazed by cattle under a standard USFS grazing permit, and also supports herds of deer (*Odocoileus* spp.) and elk (*Cervus canadensis*) (Rowland et al., 1997). Sampling at Starkey occurred along Meadow Creek, a major tributary of the upper Grande Ronde River that flows through Starkey. Dominant riparian shrubs include willow (*Salix* spp.), black hawthorn (*Crataegus douglasii* Lindl.), thinleaf alder (*Alnus incana* [L.] Moench ssp. *Tenuifolia* [Nutt.] Breitung), black cottonwood (*Populus balsamifera* L. ssp. *trichocarpa* [Torr. & A. Gray ex Hook.] Brayshaw) and common snowberry (*Symphoricarpos albus* [L.] S.F. Blake). Scattered ponderosa pine (*Pinus ponderosa* Lawson & C. Lawson), Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) and western larch (*Larix occidentalis* Nutt.) are also found in the riparian corridor (Averett et al., 2017). Herbaceous vegetation includes >200 species of forbs (Roof et al., 2018).

TNC's Zumwalt Prairie Preserve (Zumwalt), is a 13,269-ha remnant bunchgrass prairie in Wallowa County (elevation 1,100–1,700 m). The Zumwalt Prairie has been used as summer pasture for horse, sheep and cattle for over 100 years, but the majority of the area remains dominated by native plant species including Idaho fescue (*Festuca idahoensis* Elmer), prairie Junegrass (*Koeleria macrantha* [Ledeb.] Schult.) and bluebunch wheatgrass (*Pseudoroegneria spicata* [Pursh] Á. Löve) (Kennedy et al., 2009) and a rich forb community (>112 species of forbs) (Kimoto et al., 2012).

2.2 | Bee sampling

Bees were sampled during peak foraging hours (9 a.m. to 6 p.m.) once a month from each site during three bouts (June 13–28, July 3–25, August 6–31). Each bee was caught directly in a glass vial, if possible, or with an insect net and placed in an individual glass vial. All vials and nets were soaked in 10% bleach for at least 1 min and dried prior to sampling. Nets were replaced with clean, bleached nets after each use to prevent pollen contamination among samples. No killing agent was used in the vials, and bees were frozen at the end of each field day. Vials were labelled with the time, date, location and flower species that the bee was foraging on when it was caught. The flower species noted for each bee was considered the foraging observation for that specimen. Each bee was given a unique sample ID so that it could later be associated with its specific pollen load. Bees were pinned, labelled, sexed and identified to the lowest taxonomic level possible, usually species, using the methods described in Kuhlman and Burrows (2017).

2.3 | Pollen isolation

Bees were washed in the glass vial in which they were collected in the field. Sterilized water was added to the vial, and the vial was shaken vigorously until all visible pollen was removed from the bee. The pollen solution was pipetted from the vial and transferred to a 50-ml centrifuge tube. If pollen was still visible on the bee or the walls of the vial, it was rinsed with additional sterilized water, and the solution was pipetted from the vial and transferred to the same 50-ml centrifuge tube. The bees were then stored in a freezer until they could be pinned and identified. Each tube containing an individual bee's pollen load was centrifuged at 448 x *g* for 2 min, and the supernatant was discarded. The pollen pellet was resuspended in 1 ml of water, and the solution was transferred to a 1.5-ml screw cap microcentrifuge tube. The screw cap tubes were centrifuged at 14,403 x *g* for 30 s and the supernatant was discarded. The pollen pellet was washed in 1 ml of 100% ethanol, and the supernatant was discarded. The pollen pellet was then dried for 30 min in an Eppendorf vacufuge. Dried pollen pellets were stored at −20°C until further processing.

2.4 | DNA extraction and PCR

DNA extractions took place during October and November 2018 using the Nucleospin Food kit (Macherey-Nagel). We followed the "isolation of genomic DNA from honey or pollen" supplementary protocol. We added 1-mm glass beads to each 1.5-ml screw cap microcentrifuge tube and homogenized the samples in a Mini-BeadBeater-24 (Biospec Products). We included negative controls with each round of DNA extraction using sterilized water instead of pollen.

We used the dual-indexing pollen DNA metabarcoding strategy of Sickel et al. (2015). We used the second internal transcribed spacer (ITS2) primers ITS S2F and ITS4R used by Sickel et al. (2015) and existing universal ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) primers, *rbcL*2 (Palmieri et al., 2009) and *rbcL*aR (Kress & Erickson, 2007). Illumina overhang adapter sequences were added to each of the primers (Klindworth et al., 2013). Polymerase chain reaction (PCR) was conducted as a multiplex, targeting both sequence regions for amplification in one reaction. PCRs contained 10 μl of 5× Green GoTaq Reaction Buffer (Promega), 0.3 μl of 10 mM dNTPs, 1 μl of each primer, 0.2 μl of GoTaq DNA Polymerase (Promega), 33.5 μl of water and 2 μl of the template DNA for a total volume of 50 μl per reaction. PCR cycles included an initial period of heat activation for 3 min at 95°C. This was followed by 35 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C. There was a final extension of 10 min at 72°C and then the samples were held at 10°C until further processing. A negative control was included in each round of PCR, where 2 μl of water was used instead of template DNA. A 5-μl sample of each PCR product was electrophoresed in a 2% agarose gel, stained with GelRed (Biotium), and visualized under UV light to confirm the presence of the appropriately sized amplicons. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA was quantified for each sample using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific), and the samples were diluted with sterilized water in 96-well plates. The samples were indexed, pooled and sequenced at the

Center for Genome Research and Biocomputing at Oregon State University in a standard flow cell v3 300-bp paired-end full service run of the Illumina MiSeq instrument.

2.5 | Bioinformatics

2.5.1 | Read quality filtering and denoising

The dual indexed sequencing reads were first subjected to a custom python script to separate ITS2 and *rbcL* reads by matching the PCR primers. As a result, a total of 41,152,294 and 735,102 raw, paired-end reads were retrieved across 557 samples for ITS2 and *rbcL*, respectively. The open-source QIIME2 pipeline (version 2020.11.1) (Bolyen et al., 2019) was used to analyse the species composition of pollen loads (Figure S1). Initially, the QIIME2 DADA2 plugin (Callahan et al., 2016) was used to filter read quality and denoise reads. Sequence reads with a median sequencing quality score below 30 (ITS2: –p-trunc-len-f 298 –p-trunc-len-r 235; *rbcL* –p-trunc-len-f 300 –p-trunc-len-r 244) and noisy reads were filtered out. Contigs were created by joining paired-end reads, and duplicate sequences and sequences with chimeras were removed. The resulting feature table containing optimized sequences and amplicon sequence variants (ASVs) were used for additional analyses (e.g., taxonomic classification). After filtering and denoising, ~19,944,308 and 499,348 paired-end reads were retained for ITS2 and *rbcL*, respectively. Negative controls made up 0.03% of the total, retained paired-end reads, with 6,389 and 156 reads for ITS2 and *rbcL*, respectively.

2.6 | Reference database construction

Lists of all plant species known to occur at the three study sites have been developed over multiple years by botanists conducting field work at each site. Currently, the lists consist of 205, 601 and 602 plant species at Threemile, Starkey and Zumwalt sites, respectively. After removing grasslike species seldom visited by bees (i.e., sedges, rushes and grasses), the lists were comprised of 181, 485 and 466 forb and shrub species at Threemile, Starkey and Zumwalt sites, respectively. Some overlap occurred among the three plant lists (Figure S2). A regional plant list was developed that included 786 unique species for each study site. A customized python package (NCBI-COMPANION, https://github.com/lixiaopi1985/NCBI_Companion) was developed to search the NCBI nucleotide database with our plant species list and matching sequences were downloaded to a local Structured Query Language (SQL) database. For ITS2 sequences, we used the inquiry term "internal transcribed spacer 2"; for *rbcL* sequences, we used the inquiry term "rbcL." Those reference sequences were further processed to remove duplications and correct formatting. Additionally, we Sanger sequenced the ITS2 and *rbcL* markers of species on our regional plant list that were not available in the NCBI nucleotide database. The paired-end reads were joined using sangeranalyser (Chao et al., 2021), and the resulting contigs were appended to the local SQL database containing sequences from NCBI. The final "regional" ITS2 and *rbcL* databases were then split into three smaller, "local" databases for ITS2 and *rbcL* that contained only species known to occur at each location. From the master plant list with all unique plant species, the regional database contained 83% and 80% of the total species for ITS2 and *rbcL*, respectively. The local Threemile, Starkey and Zumwalt ITS2 databases contained 74%, 82% and 77% of the total species at each study site, respectively, and the *rbcL* databases contained 70%, 83% and 76% of the total species at each study site, respectively.

2.6.1 | Taxonomy classification

The database was trained using the Naïve Bayes algorithm of the QIIME2 feature classifier plugin (Bokulich et al., 2018) with default settings. The same plugin was used to assign taxonomy to each ASV. In addition to analysing the regional data, we separated the raw paired-end reads for ITS2 and *rbcL* by the study sites. For each site, the reads were subjected to the pipeline above (DADA2) to produce an ASV table and representative sequences with adjusted truncating parameters to maintain appropriate overlapping length for reads pairing (ITS: Zumwalt –p-trunc-len-f 299 –p-trunc-len-r 258; Starkey –p-trunc-len-f 300 –p-trunc-len-r 249; Threemile –p-trunc-len-f 300 –p-trunc-len-r 258. *rbcL* paired end: Zumwalt –p-trunc-len-f 300 –p-trunc-len-r 262; Starkey –p-trunc-len-f 300 –p-trunc-len-r 265; Threemile –p-trunc-len-f 300 –p-trunc-len-r 259). Corresponding local databases were used to assign taxonomy annotation to the features for each study site.

2.6.2 | Sequence count removal threshold

We found the average and the standard deviation of the number of sequencing reads found in the negative control samples. We added 1.645 standard deviations to the average and used this number as our sequence count removal threshold, accounting for 95% of possible "background noise" detected in the pollen samples (Armbruster & Pry, 2008). We set the sequence count to zero for any taxonomic assignment whose read count fell below the threshold.

2.7 | Network analyses

Bipartite plant–pollinator networks were created, and network statistics were calculated using the bipartite package in R (Dormann et al., 2008). Plant–pollinator networks were based on either bee foraging observations or plant species assignments derived from DNA metabarcoding data using reference libraries of regional (MB-RDB) and local (MB-LDB) plant species. For each location, data from all sampling periods were combined into one network for each detection method. We estimated sampling completeness for observation networks using an abundance-based estimator on aggregated data,

TABLE 1 Number of plant taxonomic units assigned by each detection method for all pollen loads collected at a location

		MB-RDB			MB-LDB		
	Observations	ITS2	*rbcL*	ITS2 + *rbcL*	ITS2	*rbcL*	ITS2 + *rbcL*
Threemile							
Families	4	6	5	6	5	5	6
Genera	8	12	5	13	10	7	14
Species	9	14	5	16	12	5	15
Starkey							
Families	10	17	13	23	15	14	21
Genera	22	41	21	49	35	21	44
Species	26	44	18	51	40	20	50
Zumwalt							
Families	14	16	11	18	16	11	19
Genera	21	28	11	32	28	13	32
Species	23	28	9	32	27	9	30

Note: MB-RDB, DNA metabarcoding using a regional reference database, MB-LDB, DNA metabarcoding using a local reference database.

and we used an incidence-based estimator on individual-level data for metabarcoding networks (Macgregor et al., 2017). Four quantitative metrics describing network complexity and specialization were calculated. Linkage density (the weighted diversity of interactions per species; Bersier et al., 2002) was calculated because increased detection of interactions could increase network complexity. We calculated H2' (a network level measure of specialization; Blüthgen et al., 2006) and bee generality (the weighted mean number of plant species per bee species, Bersier et al., 2002) because increased detection of interactions could also alter the perceived specialization of the bee species at each location. Lastly, we calculated robustness (area below the "secondary extinction curve"; Burgos et al., 2007) because increased complexity is believed to be correlated with greater resilience to species loss (Dunne et al., 2002; Gilbert, 2009; Thébault & Fontaine, 2010). Analysis of variance (ANOVA), blocked by location, was used to compare H2', linkage density and robustness of pollinator networks created using each detection method. A Kruskal–Wallace test was used to compare bee generality of pollinator networks because the assumption of homogeneity of variance was not met. Tukey's honest significant difference test (HSD) was used to compare means among detection methods.

2.8 | Diet breadth characterization

Bee species with a minimum sample of three females were included in comparisons of diet breadth characterization among techniques (Müller & Kuhlmann, 2008; Wood et al., 2016). Using foraging observations, a species was described as monolectic if all females were observed visiting the same plant species, oligolectic if at least 90% of females were observed visiting the same plant family or genus, and polylectic if less than 90% of females were observed visiting the same plant family or genus. Using metabarcoding data, a species was described as monolectic if all females carried pollen from a single plant species, oligolectic if at least 90% of females carried pure pollen loads from the same plant family or genus, and polylectic if less than 90% of females carried pure pollen loads from the same plant family or genus (Sipes & Tepedino, 2005). We documented floral constancy for polylectic species in which at least 90% of females collected pure pollen loads, but from multiple plant families or genera.

3 | RESULTS

3.1 | Bee species and pollen load composition

Each sampling location was characterized by a distinctive group of bees interacting with differing plant communities. At Threemile Canyon Farms, the 140 bees used in the network analyses included 14 genera and 22 species. *Anthophora curta* was the most abundant species, comprising 24% of bees sampled, and was observed visiting four plant species. Other common bee species included *Agapostemon femoratus*, *Melissodes bimatris* and *Melissodes pallidisignatus* (Appendix 1). Pollen loads of bees sampled from Threemile contained an average of 1.6 plant species when using both MB-RDB and MB-LDB and a maximum of four and five plant species when using MB-RDB and MB-LDB, respectively (Arstingstall et al., 2021).

At Starkey, the 168 bees used in the network analyses included 12 genera and 39 species. *Bombus bifarius* was the most commonly sampled species, making up 21% of bees sampled, and was observed visiting 12 plant species. Other common species included *Bombus flavifrons*, *Halictus ligatus* and *Bombus californicus* (Appendix 1). Pollen loads of bees sampled from Starkey contained an average of 2.4 and 2.6 plant species identified using MB-RDB and MB-LDB, respectively, and a maximum of nine plant species using either reference library (Arstingstall et al., 2021).

At Zumwalt, the 96 bees used in the network analyses included nine genera and 35 species. *Bombus californicus* and *Bombus centralis* were the most commonly sampled species, making up 17% and 13% of bees

sampled, respectively (Appendix 1). *B. californicus* was observed visiting eight plant species, and *B. centralis* was observed visiting six plant species. Pollen loads of bees sampled from Zumwalt contained a maximum of five plant species and average of 1.9 and 1.7 plant species identified using MB-RDB and MB-LDB, respectively (Arstingstall et al., 2021).

3.2 | Comparing observation networks with metabarcoding networks

When using taxonomic assignments from ITS2 and *rbcL* combined, MB-RDB and MB-LDB detected and assigned more plant taxonomic units than bee foraging observations at all taxonomic levels (Table 1). The estimated sampling completeness of interactions was higher for all metabarcoding networks than observation networks (Figure 1). Plant–pollinator networks created from MB-RDB and MB-LDB were more complex than those created from bee foraging observations (Figures 2–4). H2' was significantly higher for networks produced from bee foraging observations compared to those produced by MB-RDB and MB-LDB (Figure 5a; $F = 45.00$, $p < .01$). Bee generality and linkage density were greater for networks created using MB-RDB and MB-LDB compared to networks based on foraging observations, but the differences in means were not significant (Figure 5b; χ^2 (2, 3) =5.42, $p = .07$; Figure 5c; $F = 4.671$, $p = .09$). Robustness was not significantly different among detection methods (Figure 5d; $F = 0.615$, $p = .58$). The mean number of plant species detected per pollen load using MB-RDB and MB-LDB was significantly greater than the mean number of plant species identified per individual using foraging observations (Figure 6; $F = 141.5$, $p < .001$).

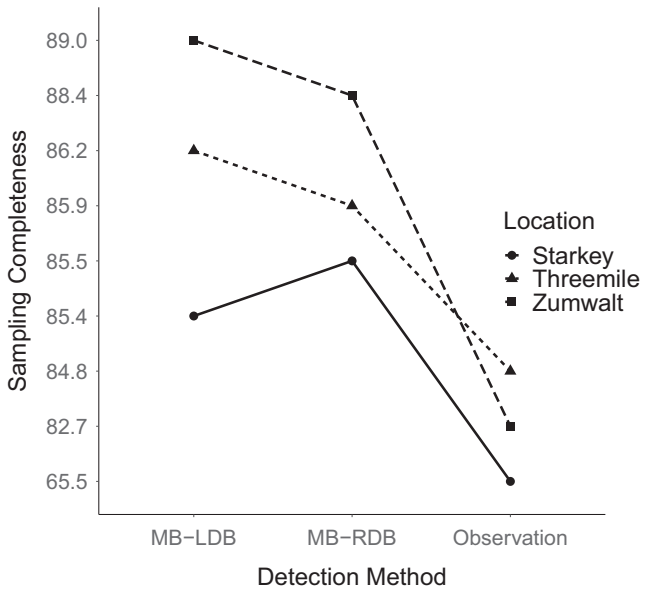

FIGURE 1 The estimated sampling completeness of interactions for plant–pollinator networks created from DNA metabarcoding data using a local reference database (MB-LDB), DNA metabarcoding data using a regional reference database (MB-RDB) and foraging observations

3.3 | Comparison of diet breadth characterization among detection methods

Twelve bee species sampled from Threemile were characterized for diet breadth. When examining plant families or genera visited, 42% and 50% had differing diet breadth characterizations depending on the detection method (Table 2). When using MB-RDB and MB-LDB, 51% of pollen loads from individual bees contained pollen from only one plant species, indicating floral constancy (Appendix 2; Arstingstall et al., 2021).

Twelve bee species sampled from Starkey were characterized with regard to diet breadth. When examining plant families or plant genera visited, 33% and 17% of bee species, respectively, had differing diet breadth characterizations depending on the detection method (Table 2). When using MB-RDB and MB-LDB, 40% and 28% of individual bees, respectively, had pollen from only one plant species in their loads, indicating floral constancy (Appendix 2; Arstingstall et al., 2021).

Ten bee species sampled from Zumwalt were characterized for diet breadth. When examining plant families visited, 8% of bee species had differing diet breadth characterizations depending on the detection method (Table 2). When using MB-RDB and MB-LDB, 49% and 47% of individual pollen loads, respectively, contained pollen from only one plant species, indicating floral constancy (Appendix 2; Arstingstall et al., 2021).

3.4 | Comparing plant species assignments obtained using MB-RDB and MB-LDB

Of the 10 plant species detected in the greatest number of pollen loads using each detection method (Table 3), MB-RDB and MB-LDB assigned 80%, 50% and 80% of the same species at Threemile, Starkey and Zumwalt, respectively. At all locations, we found that using the local database to assign taxonomy resulted in an equal or higher percentage of detections of the plant species that bees were visiting when sampled (Table 4). In this study, we estimated the potential for false detections associated with equivocal identification by quantifying regional mismatches, which we define as a plant species identified using MB-RDB but that is not known to occur in the local area of interest. We determined that 25%, 22% and 22% of the plant species identified using MB-RDB at Threemile, Starkey and Zumwalt, respectively, were regional mismatches (Table 5).

3.5 | Inconsistencies among behavioural observations and DNA metabarcoding data

At Threemile, three of the nine plant species that bees were observed visiting comprised 80% of observations: diffuse knapweed (*Centaurea diffusa* Lam.) (41%), hoary tansyaster (*Dieteria canescens* [Pursh] Nutt.) (24%), yellow star-thistle (*Centaurea solstitialis* L.) (16%) and tall tumblemustard (*Sisymbrium altissimum* L.) (5%) (Table 3).

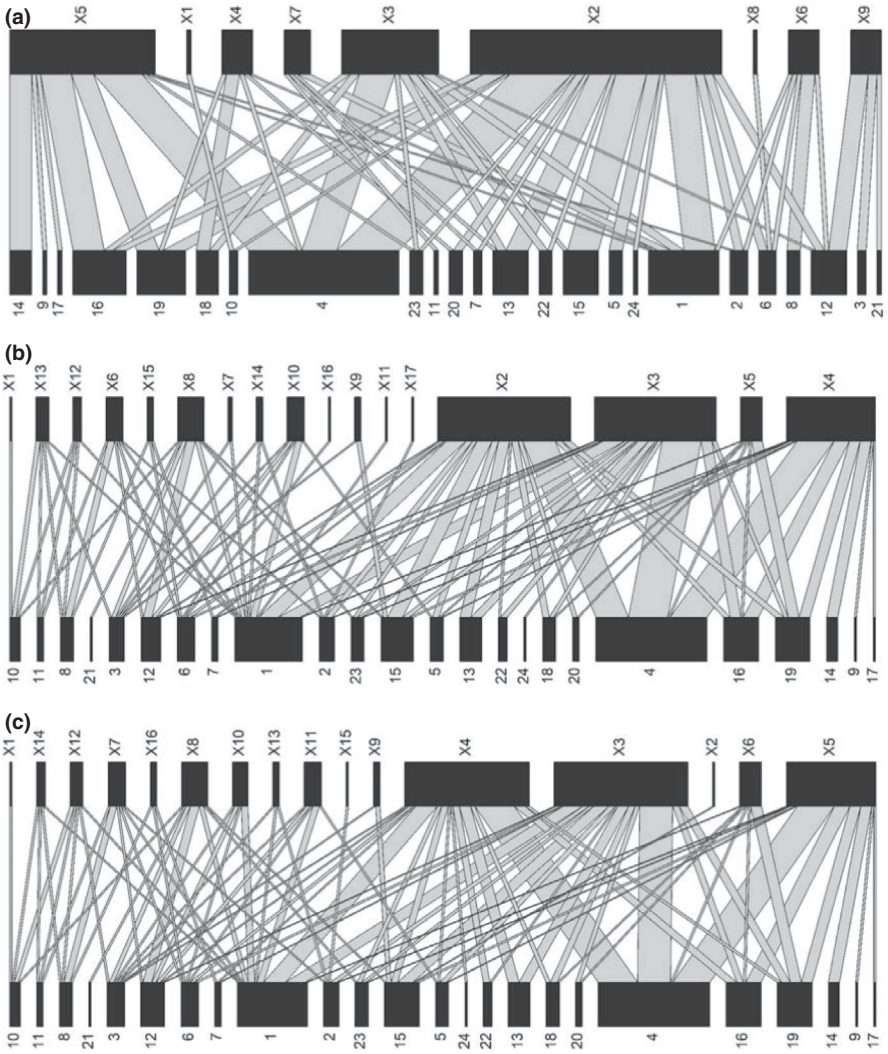

FIGURE 2 Plant–pollinator networks at Threemile Canyon Farms created from (a) bee foraging observations, (b) DNA metabarcoding data using a reference library of regional plants and (c) DNA metabarcoding data using a reference library of local plants only. In each network, the top row represents plant species and the bottom row represents bee species. Thickness of the lines represents the frequency of the interactions. Complete species listings for bees and plants can be found in Appendix 4

Three of these species (diffuse knapweed, hoary tansyaster and yellow star-thistle) were detected by both MB-RDB and MB-LDB, but tall tumblemustard was not detected using MB-RDB.

Of the 26 plant species that bees were observed visiting at Starkey, three made up 52% of observations: slender cinquefoil (*Potentilla gracilis* Douglas ex Hook.) (22%), Missouri goldenrod (*Solidago missouriensis* Nutt.) (21%) and mountain monardella (*Monardella odoratissima* Benth.) (9%) (Table 3). Slender cinquefoil was not detected using MB-RDB, and Missouri goldenrod was not detected using either MB-RDB or MB-LDB (Appendix 3). Mountain monardella was detected using both MB-RDB and MB-LDB, but it was not included in the 10 plant species that were most commonly detected in the pollen samples (Table 3).

Of the 23 plant species that bees were observed visiting at Zumwalt, four made up 43% of behavioural observations: slender cinquefoil (12%), shaggy fleabane (*Erigeron pumilus* Nutt.) (10%), silky lupine (*Lupinus sericeus* Pursh) (10%) and whitestem frasera (*Frasera albicaulis* Douglas ex Griseb.) (9%) (Table 3). Whitestem frasera was detected using MB-RDB and MB-LDB. Slender cinquefoil, shaggy fleabane and silky lupine were not detected using either MB-RDB or MB-LDB (Appendix 3).

4 | DISCUSSION

Bees provide food security and are vital to sustaining functioning ecosystems and biodiversity. Considering recent declines of many bee populations, a better understanding of bee–flower relationships is needed. Here, we illustrate some of the potential strengths and limitations of using plant species assignments derived from DNA metabarcoding data to study plant–pollinator interactions. With 73 species identified, this study contains the greatest native bee species richness of any pollen DNA metabarcoding study to date. We found that plant–pollinator networks created using plant species identifications derived from DNA metabarcoding data have significantly lower specialization and are more complex than those based on observations. On average, DNA metabarcoding identified twice as many plant species per individual bee.

We found that a substantial proportion of the bee species included in the diet breadth analysis (up to 21%) that were described as oligolectic using observational data were classified as polylectic when using metabarcoding data. Observers are limited by their field of vision and the amount of area that can be observed in a reasonable amount of time. Observers are also more likely to pay more

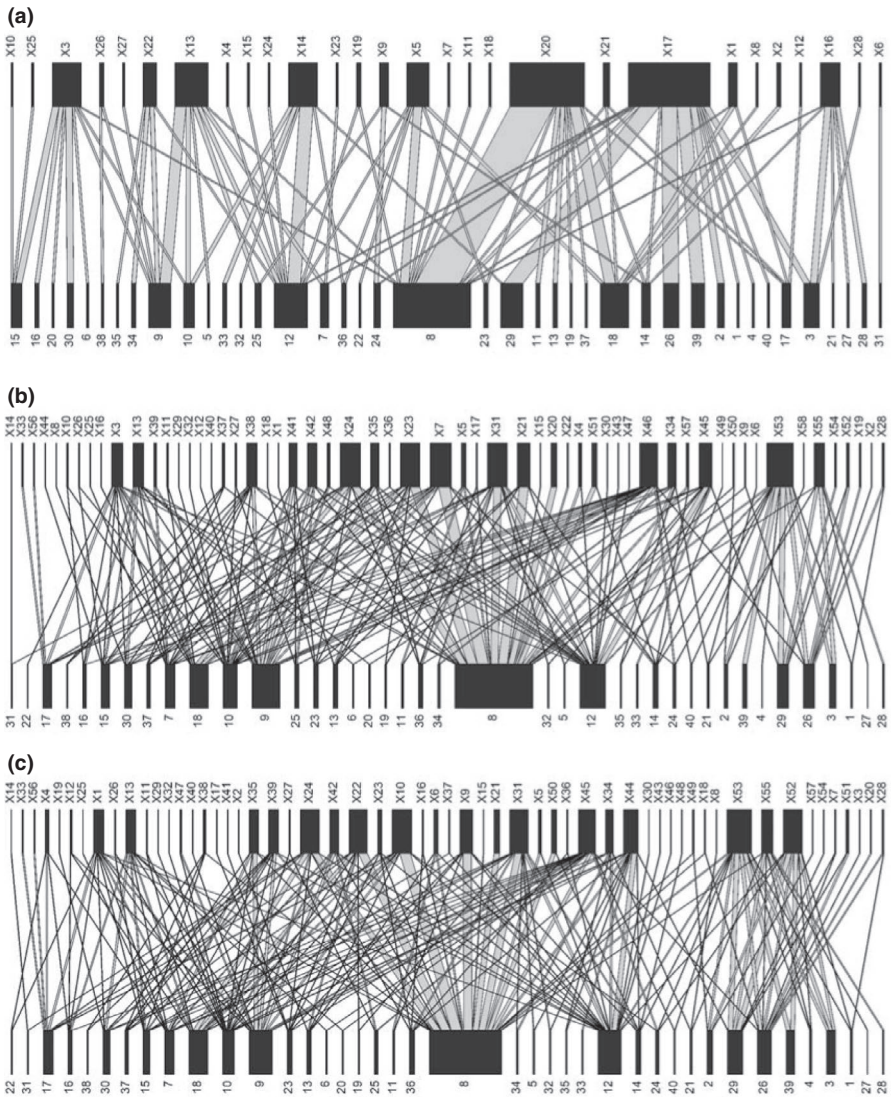

FIGURE 3 Plant–pollinator networks at Starkey Experimental Forest and Range created from (a) bee foraging observations, (b) DNA metabarcoding data using a reference library of regional plants and (c) DNA metabarcoding data using a reference library of local plants only. In each network, the top row represents plant species and the bottom row represents bee species. Thickness of the lines represents the frequency of the interactions. Complete species listings for bees and plants can be found in Appendix 5

attention to large, noticeable flowers, as opposed to small, inconspicuous flowers. These biases can lead to a higher percentage of bee species being classified as oligolectic. We found that many of the plant species detected in pollen loads with DNA metabarcoding but with no observed bee visitation were small in stature or rare at the study sites, (e.g., black locust [*Robinia pseudoacacia* L.], black medick [*Medicago lupulina* L.], varileaf phacelia [*Phacelia heterophylla* Pursh]). This key finding suggests that DNA metabarcoding may help to document interactions that are not easy to observe in the field. We chose not to include grasses, sedges or rushes in our database because we have not observed bees in our study systems visiting these species over more than five years of observations at each location. However, researchers using metabarcoding to study bees in other systems should consider including them because bees can collect pollen from grasses and other wind-pollinated plants (Saunders, 2018).

We also found that 12% of bee species that were described as polylectic when using observational data were classified as oligolectic when using metabarcoding data regardless of whether plant family or genus was used for characterization. This pattern may be due to the fact that it is often difficult to determine whether a bee is feeding on nectar or collecting pollen; in fact, many researchers collecting floral visitation records include any bee that comes into contact with a plant's reproductive parts (Steffan-Dewenter and Tscharntke, 2001; Roof et al., 2018). This leads to the potential for false positive detections of pollen collection, which could result in inaccurate diet breadth characterizations. DNA metabarcoding only detects interactions where pollen was collected, resulting in more accurate descriptions of polylectic and oligolectic diet breadth.

While observing all plant visitations from the moment a bee leaves its nest to when it returns can be difficult if not impossible, DNA metabarcoding allows us to easily determine which individual bees exhibit floral constancy. This could be especially helpful for bee species with long lifespans that are characterized as polylectic as a whole but may exhibit constancy in relation to different plant species throughout the growing season. On average, at all three locations, 44% of females exhibited floral constancy. These findings could be used to inform restoration projects where multiple forage resources with differing phenologies are planted to sustain bee communities throughout the entire growing season. Using DNA metabarcoding of

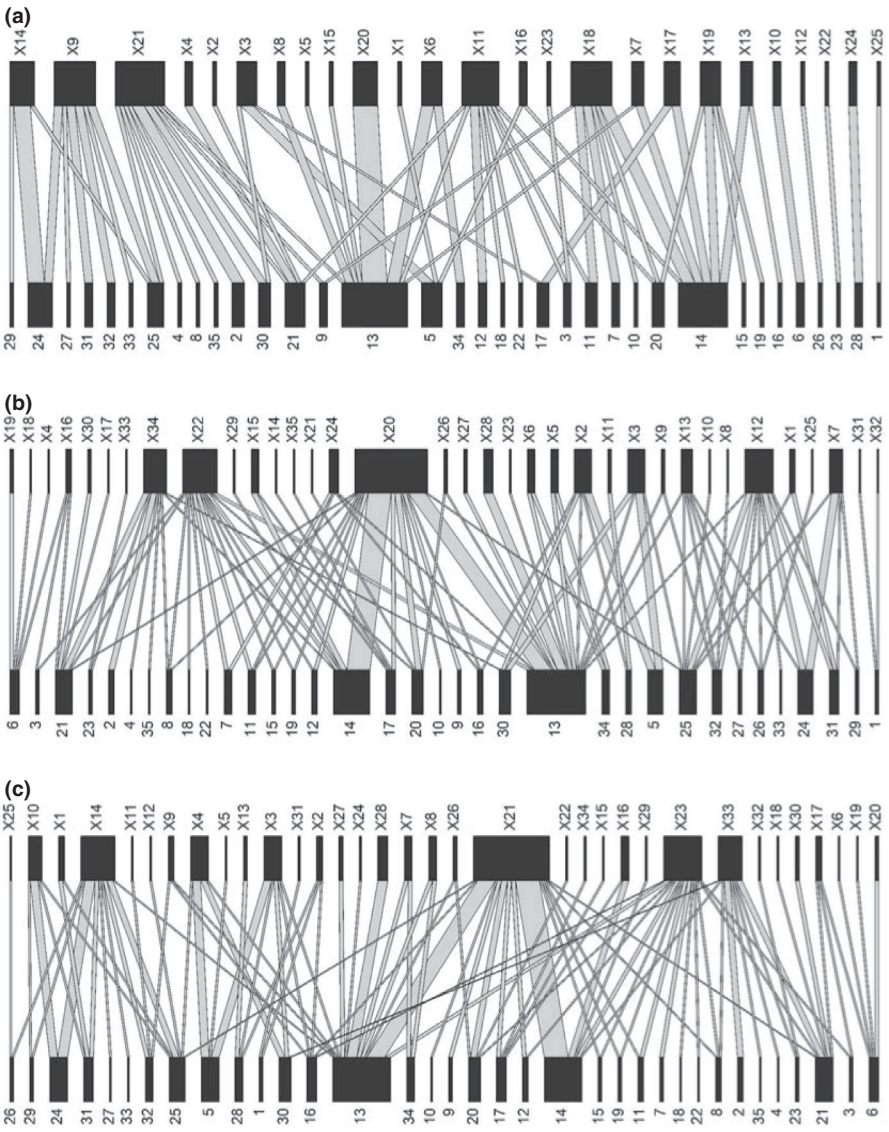

FIGURE 4 Plant–pollinator networks at Zumwalt created from (a) bee foraging observations, (b) DNA metabarcoding data using a reference library of regional plants and (c) DNA metabarcoding data using a reference library of local plants only. In each network, the top row represents plant species and the bottom row represents bee species. Thickness of the lines represents the frequency of the interactions. Complete species listings for bees and plants can be found in Appendix 6

bee pollen to determine whether a bee species is polylectic is also a potentially faster and less labour-intensive approach because the likelihood of detecting additional plant species in a bee's pollen load using metabarcoding is probably higher than that of observing a single bee species visiting multiple flower species.

Although a large proportion of the female bees in this study exhibited floral constancy, our results suggest that bees forage on a wider variety of resources than indicated by bee foraging observations alone. Specialization was significantly lower, and linkage density and bee generality, though not statistically different between methods, were greater for metabarcoding networks than observation networks. This increased complexity could be attributed, in part, to flowers containing not only their own pollen but also pollen from other species. Pollen can be carried through the environment by wind or animals (Janzen, 1983; Latta et al., 1998; Whitehead, 1983). Thus, when a foraging bee visits a flower to collect pollen or nectar, it may incidentally collect pollen from other plant species brought there by wind or other organisms, resulting in pollen from multiple plant species collected during a single visit. Even if the pollen was acquired in this way, it could still be useful as food for the bee, and its detection is informative. Future research could test this hypothesis by using DNA metabarcoding techniques on pollen isolated from individual flowers collected in the wild to approximate the baseline number of plant species that reside on a flower prior to or after pollinator visits. This could help to determine the number of interactions in a network that can be directly explained by pollinator foraging patterns vs. those explained by other mechanisms.

It is reasonable to assume that the plant species on which a bee was foraging when sampled would be detected in its pollen load using plant species assignments from DNA metabarcoding data. However, there are several reasons why this may not occur. We sampled bees that were observed coming into contact with plant reproductive parts, but we did not determine whether pollen was collected from the flower before the bee was sampled. Therefore, the bee may not have had time to collect pollen from the observed plant species before it was sampled. Furthermore, some of the bees sampled in this study may have been foraging for nectar at the time

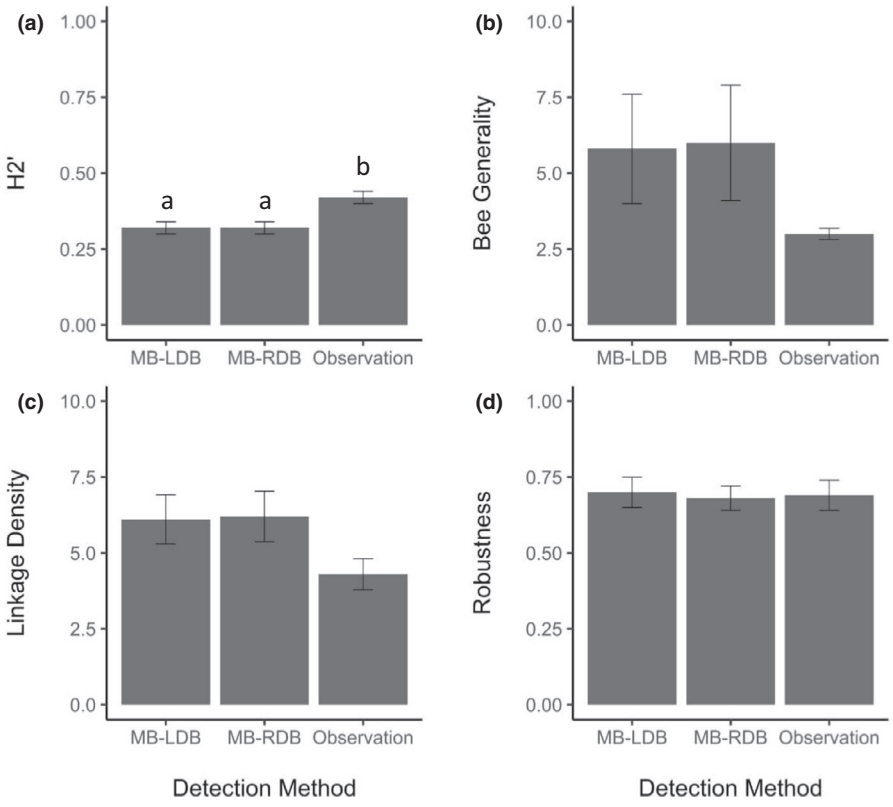

FIGURE 5 Bar plots of (a) H2', (b) bee generality, (c) linkage density and (d) robustness for bee foraging observations, DNA metabarcoding using a regional reference database (MB-RDB) and DNA metabarcoding using a local reference database (MB-LDB). Different letters denote averages that differ significantly according to a *post hoc* Tukey's HSD test. Error bars are ±*SE*

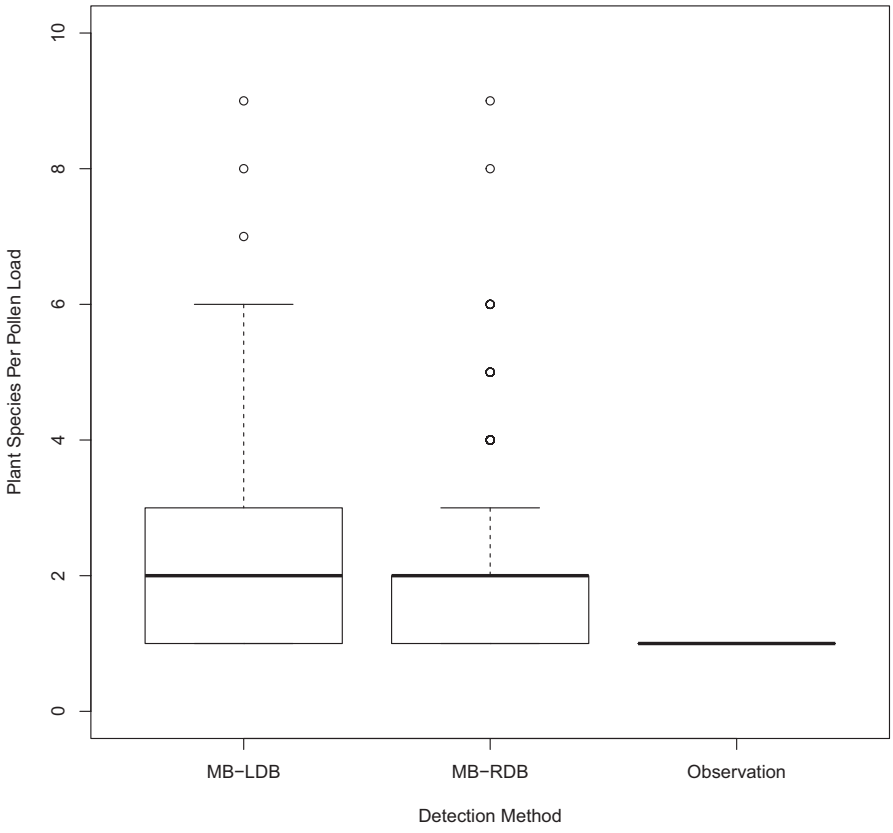

FIGURE 6 Number of plant species detected in a single pollen load for each detection method. MB-LDB, DNA metabarcoding using a local reference database; MB-RDB, DNA metabarcoding using a regional reference database

of sampling and may not have actively collected any pollen from the flower they were visiting. Male bees and cleptoparasitic bees also do not actively collect pollen from the flowers that they visit. However, male bees passively collect small amounts of pollen on their bodies and can play a substantial role in pollination in certain situations (Ostevik et al., 2010). Lastly, we used a fairly conservative sequence count removal threshold to remove potential contamination, so some legitimate rare interactions may have been removed

TABLE 2 Bee species characterized as monolectic (M), oligolectic (O) and polylectic (P) based on bee foraging observations (BFO) and plant species assignments obtained using DNA metabarcoding using a regional database (MB-RDB) and a local database (MB-LDB) [Colour table can be viewed at wileyonlinelibrary.com]

			Diet characterization by plant family			Diet characterization by plant genus		
Bee species	Location	Number of females	BFO	MB-RDB	MB-LDB	BFO	MB-RDB	MB-LDB
Agapostemon femoratus	Threemile	10	O	P	P	P	P	P
Agapostemon texanus	Threemile	3	O	P	P	P	P	P
Andrena melanochroa	Starkey	7	P	O	O	P	P	P
	Zumwalt	3	M	M	M	M	M	M
Andrena pallidifovea	Zumwalt	5	P	O	O	P	P	P
Anthophora curta	Threemile	24	O	O	O	P	P	P
Bombus appositus	Zumwalt	3	P	P	P (FC)	P	P	P (FC)
Bombus bifarius	Starkey	22	P	P	P	P	P	P
Bombus californicus	Starkey	5	P	P	P	P	P	P
	Zumwalt	16	P	P	P	P	P	P
Bombus centralis	Starkey	3	P	P	P	P	P	P
	Zumwalt	12	P	P	P	P	P	P
Bombus flavifrons	Starkey	12	P	P	P	P	P	P
Bombus griseocollis	Threemile	4	P	P	P	P	P	P
Bombus huntii	Zumwalt	3	P	P	P	P	P	P
Bombus mixtus	Starkey	4	P	P	P	P	P	P
Bombus nevadensis	Threemile	3	O	P	P	O	P	P
	Zumwalt	3	P	P	P	P	P	P
Bombus rufocinctus	Zumwalt	5	P	P	P	P	P	P
Bombus vosnesenskii	Starkey	3	P	P	P	P	P	P
Halictus ligatus	Starkey	13	O	P	P	P	P	P
	Zumwalt	4	O	O	O	P	P (FC)	P
Lasioglossum laevissimum	Starkey	3	P	P	P	P	P	P
Lasioglossum nevadense	Starkey	3	P	P	P	P	P	P
Lasioglossum olympiae	Starkey	7	O	P	P	O	P	P
Lasioglossum sp. 2	Starkey	10	O	O	P (FC)	O	P	P
Lasioglossum titusi	Zumwalt	3	P	P	P	P	P	P
Megachile apicalis	Threemile	8	P	O	O	P	O	O
Megachile onobrychidis	Threemile	5	O	P	P	O	P	P
Melissodes bimatris	Threemile	12	O	O	O	P	P	P
Melissodes lutulentus	Threemile	5	O	O	O	P	P	P
Melissodes pallidisignatus	Threemile	11	O	O	O	P	P	P
Melissodes subagilis	Threemile	3	O	O	O	P	P (FC)	P (FC)
Svastra obliqua	Threemile	3	O	O	O	P	O	O

Notes: Floral constancy (FC) is noted for polylectic species in which at least 90% of individuals collected pure pollen loads. Yellow shading indicates a species that was characterized as oligolectic using observational data and polylectic using metabarcoding data. Blue shading indicates a species that was characterized as polylectic using observational data and oligolectic using metabarcoding data.

(e.g., tiny trumpet [*Collomia linearis* Nutt.], Missouri goldenrod, silky lupine, pinkfairies [*Clarkia pulchella* Pursh]).

Unlike at Starkey and Zumwalt, a large percentage of bee pollen loads from Threemile contained the flower species on which the bees were sampled. We believe this is due to the low diversity of available forage resources at these sites. Bees were observed visiting only nine plant species at Threemile as opposed to 26 at Starkey and 23 at Zumwalt. Additionally, only three plant species make up 80% of the total observations at Threemile. Therefore, the likelihood of a bee carrying pollen from one of these species would be higher than for the bees sampled from Starkey or Zumwalt, where the blooming plant abundance and diversity were much higher. At Threemile, about 20% of individuals that were observed visiting non-native plant species had pollen from native plant species in their

TABLE 3 Ten most common plant species identified in plant–pollinator networks at Threemile Canyon Farms (Threemile), the USFS Starkey Experimental Forest and Range (Starkey), and The Nature Conservancy's Zumwalt Prairie Preserve (Zumwalt) using behavioural observations and DNA metabarcoding with a regional (MB-RDB) and local (MB-LDB) reference library

Observations	MB-RDB	MB-LDB
Threemile (140)		
Centaurea diffusa (57) ‡	*Centaurea diffusa* (61) ‡	*Centaurea diffusa* (61) ‡
Dieteria canescens (33) ‡	*Centaurea solsitialis* (56) ‡	*Centaurea solsitialis* (57) ‡
Centaurea solstitialis (22) ‡	*Dieteria canescens* (41) ‡	*Dieteria canescens* (41) ‡
Gutierrezia sarothrae (7) ‡	*Gutierrezia sarothrae* (10) ‡	*Sisymbrium altissimum* (12) †
Onopordum acanthium (7) ‡	*Onopordum acanthium* (8) ‡	*Gutierrezia sarothrae* (10) ‡
Sisymbrium altissimum (7) †	*Kali turgidum* (8) ‡	*Onopordum acanthium* (8) ‡
Ladeania lanceolata (6) ‡	*Ladeania lanceolata* (6) ‡	*Kali turgidum* (8) ‡
Achillea millefolium (1) ‡	*Gleditsia triacanthos* (4)	*Ladeania lanceolata* (6) ‡
Kali turgidum (1) ‡	*Sisymbrium loeselii* (3) †	*Robinia pseudoacacia* (4)
--	*Medicago sativa* (3) †; *Solanum dulcamara* (3)	*Sisymbrium loeselii* (3) †; *Medicago sativa* (3) †; *Solanum triflorum* (3) †
Starkey (168)		
Potentilla gracilis (37) †	*Potentilla flabellifolia* (35)	*Potentilla recta* (35)
Solidago missouriensis (35)	*Achillea millefolium* (28) †	*Anthemis cotula* (28)
Monardella odoratissima (15) ‡	*Symphyotrichum spathulatum* (27) †	*Symphyotrichum ascendens* (27)
Cirsium vulgare (13) ‡	*Solidago canadensis* (26) †	*Potentilla gracilis* (27) †
Penstemon procerus (13) †	*Lupinus leucophyllus* (26) †	*Solidago canadensis* (26) †
Erigeron corymbosus (10)	*Hypericum perforatum* (24) ‡	*Lupinus leucophyllus* (26) †
Potentilla arguta (9)	*Penstemon procerus* (17) †	*Hypericum perforatum* (24) ‡
Thermopsis montana (6) ‡	*Lomatium triternatum* (15)	*Penstemon confertus* (21)
Achillea millefolium (4) †	*Cirsium vulgare* (14) ‡	*Rosa gymnocarpa* (16) †
Hypericum perforatum (4) ‡	*Trifolium wormskioldii* (14)‡; *Rosa gymnocarpa* (14) †	*Cirsium vulgare* (14) ‡
Zumwalt (96)		
Potentilla gracilis (12)	*Lupinus leucophyllus* (38) ‡	*Lupinus leucophyllus* (38) ‡
Erigeron pumilus (10)	*Frasera albicaulis* (18) ‡	*Frasera albicaulis* (19) ‡
Lupinus sericeus (10)	*Symphyotrichum ascendens* (15)	*Symphyotrichum spathulatum* (17) †
Frasera albicaulis (9) ‡	*Potentilla flabellifolia* (12) †	*Potentilla flabellifolia* (12) †
Arnica sororia (5) ‡	*Achillea millefolium* (9) ‡	*Achillea millefolium* (9) ‡
Grindelia nana (6)	*Arnica sororia* (9) ‡	*Arnica sororia* (9) ‡
Orthocarpus tenuifolius (6) ‡	*Grindelia squarrosa* (7) †	*Grindelia squarrosa* (7) †
Cirsium brevifolium (5)	*Symphyotrichum spathulatum* (6) †	*Orthocarpus tenuifolius* (5) ‡
Lupinus leucophyllus (4) ‡	*Gentianella amarella* (5)	*Cirsium foliosum* (4) †
Geranium viscosissimum (3)	*Orthocarpus tenuifolius* (5) ‡	*Phacelia heterophylla* (4)

Notes: Numbers in parentheses following location names indicate total pollen loads sampled at that location. Numbers in parentheses following plant species names indicate number of pollen loads in which that plant species was identified. A "†" indicates a plant species that was identified using two detection methods. A "‡" indicates a plant species that was identified using all three detection methods. In the case of ties for the tenth most common species, all species included in the tie are listed.

pollen loads. We also found native plant species in the pollen loads of every bee species that was deemed oligolectic using observations but polylectic based on metabarcoding data at Threemile. Therefore, despite the low native plant species diversity in this disturbed landscape, the bee species at Threemile appear to be less reliant on nonnatives than observations alone suggest.

Inconsistencies between bee foraging observations and species assignments derived from DNA metabarcoding data may also be due to plant misidentification in the field. Certain plant species are difficult to differentiate due to similar morphology and other features. One example is longleaf fleabane (*Erigeron corymbosus* Nutt.) and western mountain aster (*Symphyotrichum spathulatum* [Lind.] G.L.

TABLE 4 Percentage of samples in which metabarcoding detected the plant species from which the corresponding bee was collected

	Threemile		Starkey		Zumwalt	
	MB-RDB	MB-LDB	MB-RDB	MB-LDB	MB-RDB	MB-LDB
Family	96.4	96.4	91.1	91.1	77.1	76.0
Genus	88.6	92.9	71.4	67.3	64.6	63.5
Species	80.7	85.7	38.7	39.9	31.3	31.3

Note: MB-RDB, DNA metabarcoding using a regional reference database; MB-LDB, DNA metabarcoding using a local reference database.

TABLE 5 Mismatches of regional database metabarcoding

Location	Regional mismatches
Threemile	*Gleditsia triacanthos* ‡
	Medicago arabica ‡
	Rhaponticum repens †
	Solanum dulcamara §
Starkey	*Ambrosia acanthicarpa* †
	Centaurea diffusa †
	Cirsium foliosum †
	Grindelia squarrosa †
	Lomatium triternatum †
	Mentha spicata †
	Micranthes integrifolia ‡
	Potentilla flabellifolia §
	Senecio hydrophiloides †
	Trifolium hybridum †
	Vicia villosa §
Zumwalt	*Cryptantha affinis* †
	Gentianella amarella ‡
	Hieracium scouleri †
	Moehringia lateriflora †
	Phacelia hastata †
	Symphyotrichum ascendens †
	Veratrum viride ‡

Notes: Mismatches for the regional database are species that were identified with DNA metabarcoding that are not known to occur in the area. See Appendix 3 for more detail. A "†" indicates a plant species assigned using the ITS2 gene region, a "‡" indicates a plant species assigned using the *rbcL* gene region, and a "§" indicates a plant species assigned using both ITS2 and *rbcL* gene regions.

Nesom). Bees were commonly observed visiting longleaf fleabane at Zumwalt, but this species was never detected using DNA metabarcoding. However, western mountain aster was commonly detected in the pollen loads using MB-LDB, and it was detected in every pollen sample on which the bee was collected on longleaf fleabane. Sequence data for both the ITS2 and *rbcL* gene regions of longleaf fleabane were included in the reference library, and western mountain aster shares less than 96% of its ITS2 and *rbcL* DNA sequences with longleaf fleabane. Therefore, it is unlikely that this was an erroneous species assignment, and it is possible that we misidentified western mountain aster as longleaf fleabane, causing the mismatch in our data. Although we believe occurrences such as this were rare in our study, this is an example of how DNA metabarcoding may help improve our understanding of foraging behaviour by flagging plant species that may have been misidentified in the field.

In this study, we used multiple barcode markers for taxonomic identification (i.e., ITS2 and *rbcL*). It is well known that different loci and primer pairs have their own taxonomic biases with respect to detection and using two different loci allows us to resolve different taxonomic groups (Cowart et al., 2015; Deagle et al., 2014; Elbrecht & Leese, 2015; Leray & Knowlton, 2017; Piñol et al., 2015). Other pollen metabarcoding studies have shown that plastid barcode markers such as *trnL* and *trnH-pbsA* also perform well in plant identification (Bruni et al., 2015; Galimberti et al., 2014; Kraaijeveld et al., 2015; Pornon et al., 2016). We chose to use the combination of ITS2 and *rbcL* because ITS2 has been identified as a successful universal DNA barcode for plant identification (Chen et al., 2010) and *rbcL* is recognized internationally as a core DNA barcode for plants (CBOL Plant Working Group, 2009). Furthermore, ITS2 and *rbcL* are commonly used in pollen metabarcoding studies (Bell et al., 2017; Lucas et al., 2018b; Richardson et al., 2018), and curated reference libraries have been published for each of these barcode markers (Bell et al., 2017; Sickel et al., 2015). We determined that, when compared to other plastid barcode markers, a greater number of *rbcL* DNA sequences were available for the plant species that are known to occur in our study system, making *rbcL* the most appropriate plastid marker for this study.

ITS2 sequence data for certain plant species (e.g., mountain monardella, oceanspray [*Holodiscus discolor* (Pursh) Maxim.]) were not available to add to our reference library, so these species could not be identified using the ITS2 region. However, we did have *rbcL* sequence data for these plant species in our reference library, allowing for the identification of these species by *rbcL* alone. The NCBI nucleotide database is far from complete, and using multiple loci allows us to obtain additional plant species assignments. We can also be more confident in plant species that were detected using both loci (e.g, whitestem frasera, common St. Johnswort [*Hypericum perforatum* L.], thinleaved owl's-clover [*Orthocarpus tenuifolius* (Pursh) Benth.]) because the probability of a false-positive identification occurring at both gene regions is smaller than the probability of one occurring at just one gene region (Richardson, Lin, Quijia, et al., 2015).

Here we used a multiplex approach in which both primer sets were added to the same PCR. The majority of plant species assignments were made using the ITS2 region, with 98% of the raw reads being ITS2 sequences. Only 2% of the raw reads were *rbcL* sequences,

suggesting that amplification bias was occurring in favour of ITS2. *rbcL* primer sets typically require an annealing temperature of 50°C (Bruni et al., 2012; Hawkins et al., 2015; Potter et al., 2019), while the annealing temperature of ITS2 primer sets can range from 50°C to 60°C (Bálint et al., 2014; Richardson, Lin, Sponsler, et al., 2015). In our multiplex PCR, we used an annealing temperature of 55°C, which may have favoured amplification of the ITS2 region. Future research is needed to compare amplification of the *rbcL* barcode marker in a separate PCR to amplification of the *rbcL* barcode marker when it is multiplexed with another barcode marker (e.g., ITS2) to determine whether it performs better in a separate PCR.

The diet breadth of bee species was often characterized differently based on DNA metabarcoding data compared to observational data. When using foraging observations to describe bee diets, polylectic species can be deemed oligolectic when interactions are missed due to spatial and temporal constraints, and oligolectic species can be deemed polylectic when there is no distinction between pollen collecting and nectar feeding during floral visitations. With DNA metabarcoding techniques, we can determine which bee species are polylectic quickly and with lower sampling effort and describe floral fidelity in individual bees with minimal effort compared to the amount required for observing floral constancy in the field. While researchers should exercise caution when characterizing diet breadth based on small sample sizes because of the increased risk of polylectic species being mischaracterized as oligolectic, our study found that even for species with relatively few observations, the use of metabarcoding showed that species characterized as oligolectic with foraging observations were actually polylectic.

Using a local reference database reduced the potential for regional mismatches that can occur when using a regional reference database. However, we believe that both sets of plant species assignments obtained from DNA metabarcoding data (i.e., MB-RDB and MB-LDB) provide a better representation of plant species use by native bees at these locations than do bee foraging observations despite some erroneous species assignments that we suspect occurred. Here, we had the advantage of having well-described flora for our study sites, but that is often not the case. Those who wish to use plant species assignments derived from DNA metabarcoding of pollen to study the plant–pollinator interactions in locations that are not well described should err on the side of caution, and the chosen method for describing plant–bee interactions will depend on the types of questions being asked. Past work has shown that for questions that can be answered by family- or genus-level identification, DNA metabarcoding of pollen works very well (Lucas et al., 2018a; Potter et al., 2019). However, if plant species identification is important, extra steps may need to be taken (e.g., additional loci, plant surveys, observations, microscopy) to confirm that plant species identifications based on DNA metabarcoding data are accurate.

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AUTHOR CONTRIBUTIONS

K.A.A., S.J.D., K.F., M.M.R. and D.E.W. designed the study. K.A.A. conducted the research. K.F., S.J.D. and D.E.W. contributed laboratory space, equipment and materials. S.B. identified the bees in the study. K.A.A., X.L. and S.J.D. analysed the data. K.A.A. wrote the manuscript. All authors helped edit the manuscript.

DATA AVAILABILITY STATEMENT

Sequencing results have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA604079. The trained databases, command line arguments and Python code have been uploaded to https://github.com/KAArsting/NativeBeePollenMetabarcoding. Data associated with this paper have been deposited in the Dryad repository: 10.5061/dryad.xwdbrv1dr.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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APPENDIX 1

Number of individuals of each taxon observed visiting flowers at Threemile Canyon Farms (Threemile), United States Forest Service Starkey Experimental Forest and Range (Starkey), and The Nature Conservancy's Zumwalt Prairie Preserve (Zumwalt), northeastern Oregon, USA. Subspecies names are in parentheses.

Bee species (subgenus)	Threemile	Starkey	Zumwalt	Total
Agapostemon (*Agapostemon*) *femoratus*	15	0	0	15
Agapostemon (*Agapostemon*) *texanus*	4	0	0	4
Andrena (*Euandrena*) *astragali*	0	0	1	1
Andrena (*Leucandrena*) *barbilabris*	0	1	0	1
Andrena (*Trachandrena*) *cyanophila*	0	3	0	3
Andrena (*Micrandrena*) *melanochroa*	0	7	3	10
Andrena (*Euandrena*) *nigrocaerulea*	0	0	2	2
Andrena (*Melandrena*) *nivalis*	0	0	1	1
Andrena (*Simandrena*) *pallidifovea*	0	0	5	5
Andrena (*Plastandrena*) *prunorum*	2	0	2	4
Andrena (*Euandrena*) sp. 1	0	0	2	2
Andrena (*Thysandrena*) sp. A	0	1	0	1
Andrena (*Melandrena*) *transnigra*	0	0	1	1
Andrena (*Andrena*) *vicinoides*	0	0	2	2
Anthophora (*Melea*) *bomboides*	0	1	0	1
Anthophora (*Micranthophora*) *curta*	34	0	0	34
Anthophora (*Lophanthophora*) *pacifica*	0	0	1	1
Anthophora (*Mystacanthophora*) *urbana*	3	1	0	4
Bombus (*Subterraneobombus*) *appositus*	0	4	3	7
Bombus (*Pyrobombus*) *bifarius*	0	35	2	37
Bombus (*Fervidobombus*) *californicus*	0	10	16	26
Bombus (*Pyrobombus*) *centralis*	0	5	12	17
Bombus (*Psithyrus*) *fernaldae*	0	2	0	2
Bombus (*Fervidobombus*) *fervidus*	0	0	1	1
Bombus (*Pyrobombus*) *flavifrons*	0	15	1	16
Bombus (*Separatobombus*) *griseocollis*	4	0	0	4
Bombus (*Pyrobombus*) *huntii*	2	0	3	5
Bombus (*Psithyrus*) *insularis*	0	2	1	3
Bombus (*Pyrobombus*) *mixtus*	0	4	1	5
Bombus (*Bombias*) *nevadensis*	3	0	3	6
Bombus (*Cullumanobombus*) *rufocinctus*	0	0	5	5
Bombus (*Pyrobombus*) *vosnesenskii*	0	4	1	5
Coelioxys (*Boreocoelioxys*) *rufitarsis*	1	2	0	3
Colletes phaceliae	2	0	0	2
Diadasia nigrifrons	0	0	1	1
Halictus (*Nealictus*) *farinosus*	1	4	0	5
Halictus (*Odontalictus*) *ligatus*	0	14	6	20
Halictus (*Protohalictus*) *rubicundus*	0	1	4	5
Halictus (*Seladonia*) *tripartitus*	0	1	1	2
Hylaeus (*Hylaeus*) *verticalis*	0	1	0	1
Lasioglossum (*Dialictus*) *albipenne*	0	0	1	1
Lasioglossum (*Evylaeus*) *cooleyi*	0	1	0	1
Lasioglossum (*Dialictus*) *incompletum*	0	2	2	4

Bee species (subgenus)	Threemile	Starkey	Zumwalt	Total
Lasioglossum (*Dialictus*) *laevissimum*	0	3	0	3
Lasioglossum (*Dialictus*) *nevadense*	0	3	0	3
Lasioglossum (*Lasioglossum*) *olympiae*	0	7	0	7
Lasioglossum (*Evylaeus*) *ovaliceps*	0	0	1	1
Lasioglossum (*Dialictus*) *sedi*	0	1	0	1
Lasioglossum (*Dialictus*) sp.	8	2	0	10
Lasioglossum (*Hemihalictus*) sp. 2	0	10	0	10
Lasioglossum (*Lasioglossum*) *titusi*	0	0	3	3
Megachile (*Eutricharaea*) *apicalis*	8	0	0	8
Megachile (*Megachiloides*) *nevadensis*	5	0	0	5
Megachile (*Litomegachile*) *onobrychidis*	8	0	2	10
Megachile (*Xanthosarus*) *perihirta*	0	3	2	5
Melissodes (*Eumelissodes*) *bimatris*	12	0	0	12
Melissodes (*Callimelissodes*) *lupinus*	1	0	1	2
Melissodes (*Eumelissodes*) *lutulentus*	5	0	0	5
Melissodes (*Eumelissodes*) *microstictus*	0	1	0	1
Melissodes (*Eumelissodes*) *pallidisignatus*	11	0	0	11
Melissodes (*Heliomelissodes*) *rivalis*	0	0	2	2
Melissodes (*Eumelissodes*) *subagilis*	3	0	0	3
Osmia (*Melanosmia*) *atrocyanea*	0	1	0	1
Osmia (*Melanosmia*) *brevis*	0	2	0	2
Osmia (*Melanosmia*) *bucephala*	0	2	0	2
Osmia (*Acanthosmioides*) *integra*	0	1	0	1
Osmia (*Melanosmia*) *juxta*	0	2	0	2
Osmia (*Melanosmia*) *raritatis*	0	1	0	1
Osmia sp. 2	0	1	0	1
Panurginus sp. A	0	6	1	7
Perdita (*Pygoperdita*) *wyomingensis*	0	1	0	1
Sphecodes sp.	1	0	0	1
Svastra (*Epimelissodes*) *obliqua*	3	0	0	3
Triepeolus paenepectoralis	3	0	0	3
Xeromelecta (*Melectomorpha*) *californica*	1	0	0	1

APPENDIX 2

Monolectic and oligolectic bee species, as determined by metabarcoding of pollen, the plant families and genera they use, and the plant species on which individuals exhibited floral constancy.

Bee species (subgenus)	Location/ detection method	Diet breadth characterization	Family	Genus	Species	Proportion of females using one plant species
Andrena (*Micrandrena*) *melanochroa*	Starkey/ MB-RDB	Oligolectic (Family)	Rosaceae	*Potentilla*; *Rosa*	*Potentilla flabellifolia*; *Rosa gymnocarpa*	0.7
	Starkey/ MB-LDB	Oligolectic (Family)	Rosaceae	*Potentilla*; *Rosa*	*Potentilla gracilis*; *Potentilla recta*; *Rosa gymnocarpa*	0.4
	Zumwalt/ MB-RDB	Monolectic (Species)	Rosaceae	*Potentilla*	*Potentilla flabellifolia*	1.0
	Zumwalt/ MB-LDB	Monolectic (Species)	Rosaceae	*Potentilla*	*Potentilla flabellifolia*	1.0
Andrena (*Simandrena*) *pallidifovea*	Zumwalt/ MB-LDB	Oligolectic (Family)	Asteraceae	*Achillea*; *Arnica*; *Artemisia*	*Achillea millefolium*; *Arnica sororia*	0.4
	Zumwalt/ MB-RDB	Oligolectic (Family)	Asteraceae	*Achillea*; *Arnica*	*Achillea millefolium*; *Arnica sororia*	0.4
Anthophora (*Micranthophora*) *curta*	Threemile/ MB-RDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Dieteria*	*Centaurea diffusa*; *Centaurea solstitialis*	0.3
	Threemile/ MB-LDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Dieteria*	*Centaurea diffusa*; *Centaurea solstitialis*	0.3
Halictus (*Odontalictus*) *ligatus*	Zumwalt/ MB-RDB	Oligolectic (Family)	Asteraceae	*Grindelia*; *Symphyotrichum*	*Grindelia squarrosa*; *Symphyotrichum ascendens*	1.0
	Zumwalt/ MB-LDB	Oligolectic (Family)	Asteraceae	*Grindelia*; *Symphyotrichum*	*Grindelia squarrosa*; *Symphyotrichum spathulatum*	0.5
Lasioglossum (*Hemihalictus*) *sp. 2*	Starkey/ MB-RDB	Oligolectic (Family)	Plantaginaceae; Rosaceae	*Penstemon*; *Physocarpus*; *Potentilla*; *Rosa*	*Penstemon procerus*; *Potentilla flabellifolia*; *Rosa* spp.; *Rosa gymnocarpa*	0.6
Megachile (*Eutricharaea*) *apicalis*	Threemile/ MB-RDB	Oligolectic (Genus)	Asteraceae	*Centaurea*	*Centaurea diffusa*; *Centaurea solstitialis*	0.75
	Threemile/ MB-LDB	Oligolectic (Genus)	Asteraceae	*Centaurea*	*Centaurea diffusa*; *Centaurea solstitialis*	0.75
Melissodes (*Eumelissodes*) *bimatris*	Threemile/ MB-RDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Dieteria*; *Gutierrezia*	*Centaurea diffusa*; *Centaurea solstitialis*; *Dieteria canescens*	0.7
	Threemile/ MB-LDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Dieteria*; *Gutierrezia*	*Centaurea diffusa*; *Centaurea solstitialis*; *Dieteria canescens*	0.7
Melissodes (*Eumelissodes*) *lutulentus*	Threemile/ MB-RDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Gutierrezia*	*Centaurea diffusa*; *Centaurea solstitialis*	0.8
	Threemile/ MB-LDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Gutierrezia*	*Centaurea diffusa*; *Centaurea solstitialis*	0.8
Melissodes (*Eumelissodes*) *pallidisignatus*	Threemile/ MB-RDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Dieteria*; *Gutierrezia*	*Centaurea diffusa*; *Dieteria canescens*; *Gutierrezia sarothrae*	0.6
	Threemile/ MB-LDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Dieteria*; *Gutierrezia*	*Centaurea diffusa*; *Dieteria canescens*; *Gutierrezia sarothrae*	0.6

Bee species (subgenus)	Location/ detection method	Diet breadth characterization	Family	Genus	Species	Proportion of females using one plant species
Melissodes (Eumelissodes) subagilis	Threemile/ MB-RDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Gutierrezia*	*Centaurea diffusa*; *Gutierrezia sarothrae*	1.0
	Threemile/ MB-LDB	Oligolectic (Family)	Asteraceae	*Centaurea*; *Gutierrezia*	*Centaurea diffusa*; *Gutierrezia sarothrae*	1.0
Svastra (Epimelissodes) obliqua	Threemile/ MB-RDB	Oligolectic (Genus)	Asteraceae	*Centaurea*	*Centaurea diffusa*; *Centaurea solstitialis*	0.7
	Threemile/ MB-LDB	Oligolectic (Genus)	Asteraceae	*Centaurea*	*Centaurea diffusa*; *Centaurea solstitialis*	0.7

APPENDIX 3

Plant taxa identified at Threemile Canyon Farms (Threemile), United States Forest Service Starkey Experimental Forest and Range (Starkey), and The Nature Conservancy's Zumwalt Prairie Preserve (Zumwalt) using behavioural observations (BO), DNA metabarcoding using a regional reference database (MB-RDB) and DNA metabarcoding using a local reference database (MB-LDB). A "†" indicates a plant species that was detected using the ITS2 gene region, a "‡" indicates a plant taxon that was detected using the *rbcL* gene region, a "§" indicates a plant taxon that was identified using both ITS2 and *rbcL* gene regions, and a "¶" indicates a plant taxon that was identified using behavioural observations. Grey shading indicates a regional mismatch (i.e., a plant species assigned using the regional reference database that is not known to occur in the area).

	Threemile			Starkey			Zumwalt		
Plant species	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB
Achillea millefolium	¶	†	†	¶	†		¶	†	†
Agastache urticifolia								‡	‡
Agoseris heterophylla			‡						
Allium acuminatum					†	†			
Allium validum					†	†			
Ambrosia acanthicarpa					†				
Angelica arguta					†	†			
Anthemis cotula						†			
Apiaceae						†			
Arceuthobium campylopodum					†	†			
Arnica sororia							¶	†	†
Artemisia sp.									‡
Asteraceae		‡			‡	§		‡	‡
Blepharipappus scaber					†			†	†
Brassicaceae		†						†	†
Calochortus eurycarpus							¶		
Calochortus macrocarpus								‡	‡
Caryophyllaceae								‡	
Castilleja cusickii								†	†
Castilleja lutescens							¶		
Centaurea diffusa	¶	†	†		†				
Centaurea solstitialis	¶	†	§						
Chenopodium sp.			‡						
Cirsium sp.								†	†

	Threemile			Starkey			Zumwalt		
Plant species	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB
Cirsium arvense				¶	†	†			
Cirsium brevifolium							¶		
Cirsium scariosum						†			
Cirsium foliosum					†			†	†
Cirsium vulgare				¶	†	†			
Clarkia pulchella							¶		
Collomia linearis				¶					
Cornus canadensis						‡			
Cornus sericea					‡				
Crataegus sp.					‡				
Crataegus monogyna					†	§			
Crepis occidentalis									†
Cryptantha affinis								†	
Cryptantha ambigua									†
Delphinium nuttallianum							¶		
Dieteria canescens	¶	†	†						
Eregomone congesta							¶		
Erigeron sp.					†	†			
Erigeron corymbosus				¶					
Erigeron pumilus							¶		
Erigeron speciosus				¶					
Eriogonum flavum								†	†
Eriogonum heracleoides				¶			¶		
Eriophyllum lanatum					†	†			
Erythranthe sp.					†				
Fabaceae						‡			
Frasera albicaulis							¶	†	§
Gentiana affinis					†	†		†	†
Gentianella amarella								‡	
Geranium pusillum								†	†
Geranium viscosissimum							¶		
Geum sp.					‡				
Geum macrophyllum					†	§			
Gleditsia triacanthos		‡							
Goodyera oblongifolia					‡	‡			
Grindelia nana				¶			¶		
Grindelia squarrosa					†			†	†
Gutierrezia sarothrae	¶	†	†						
Helianthus annuus								†	†
Heracleum maximum					†	†			
Heuchera cylindrica						‡			
Hieracium cynoglossoides							¶		
Hieracium scouleri					†	†		†	

	Threemile			Starkey			Zumwalt		
Plant species	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB
Holodiscus discolor					‡	‡			
Hypericum perforatum				¶	§	§			
Kali turgidum	¶	§	†						
Lactuca serriola					†	†		†	†
Ladeania lanceolata	¶	†	†						
Lomatium gormanii						†			
Lomatium triternatum					†			†	†
Lupinus sp.					‡	‡		‡	
Lupinus caudatus							¶		
Lupinus leucophyllus					†	†	¶	†	†
Lupinus sericeus							¶		
Lupinus sulphureous				¶					
Madia sp.						†			
Madia gracilis						†			
Malva neglecta						†			
Medicago arabica		‡							
Medicago lupulina						†			
Medicago sativa		†	†		†				
Melilotus sp.					†				
Melilotus officinalis						†			
Mentha arvensis				¶		†			
Mentha spicata					†				
Micranthes integrifolia					‡				
Microseris nutans					†	†			
Moehringia lateriflora								†	
Monardella odoratissima				¶	‡	‡			
Myosurus minimus						†			
Nemophila breviflora						†			
Onopordum acanthium	¶	†	†						
Orthocarpus tenuifolius							¶	§	§
Packera sp.					‡				
Packera pseudaurea						‡			
Pedicularis groenlandica					§	§			
Penstemon sp.					‡				
Penstemon confertus						§			
Penstemon procerus				¶	†			†	†
Phacelia hastata					†			†	
Phacelia heterophylla									†
Philadelphus lewisii								§	§
Physocarpus malvaceus					§	§			
Polemonium occidentale				¶	§	§			
Polygonum douglasii					‡				

	Threemile			Starkey			Zumwalt		
Plant species	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB	BO	MB-RDB	MB-LDB
Polygonum minimum						‡			
Potentilla arguta				¶					
Potentilla flabellifolia					§			§	§
Potentilla gracilis				¶		‡	¶		
Potentilla recta						†			
Purshia tridentata			‡						
Ranunculaceae					†				
Rhaponticum repens		†							
Robinia pseudoacacia			‡						
Rosa sp.					§	‡		§	§
Rosa gymnocarpa					†	†			
Sedum stenopetalum					†				
Senecio sp.					†				
Senecio hydrophiloides					†				
Senecio serra				¶					
Sidalcea oregana				¶	†		¶	§	§
Silene sp.									‡
Silene scouleri									†
Sisymbrium altissimum	¶		†				¶		
Sisymbrium loeselii		†	†						
Solanum dulcamara		§							
Solanum triflorum		†	§						
Solidago canadensis					†	†		†	†
Solidago missouriensis				¶			¶		
Symphoricarpos albus					‡	‡			
Symphyotrichum sp.						†			
Symphyotrichum ascendens						†		†	
Symphyotrichum spathulatum				¶	†			†	†
Thermopsis montana				¶	§	§			
Toxicoscordion venenosum							¶	†	†
Tragopogon dubius				¶	†	†			
Tribulus terrestris		§							
Trifolium sp.						†			
Trifolium eriocephalum						†			
Trifolium hybridum					†				
Trifolium pratense				¶	§	§			
Trifolium repens					†	†			
Trifolium wormskioldii				¶	§	§			
Triteleia grandiflora				¶		‡			‡
Veratrum viride								‡	
Vicia cracca				¶					
Vicia villosa					§			§	§

APPENDIX 4

Legends for Threemile Canyon Farms plant–pollinator networks created from behavioural observations, DNA metabarcoding using a regional reference database (MB-RDB) and DNA metabarcoding using a local reference database (MB-LDB) (Figure 2).

Network ID	Bee species (subgenus)
1	*Agapostemon* (*Agapostemon*) *femoratus*
2	*Agapostemon* (*Agapostemon*) *texanus*
3	*Andrena* (*Plastandrena*) *prunorum*
4	*Anthophora* (*Micranthophora*) *curta*
5	*Anthophora* (*Mystacanthophora*) *urbana*
6	*Bombus* (*Separatobombus*) *griseocollis*
7	*Bombus* (*Pyrobombus*) *huntii*
8	*Bombus* (*Bombias*) *nevadensis*
9	*Coelioxys* (*Boreocoelioxys*) *rufitarsis*
10	*Colletes phaceliae*
11	*Halictus* (*Nealictus*) *farinosus*
12	*Lasioglossum* (*Dialictus*) sp.
13	*Megachile* (*Eutricharaea*) *apicalis*
14	*Megachile* (*Megachiloides*) *nevadensis*
15	*Megachile* (*Litomegachile*) *onobrychidis*
16	*Melissodes* (*Eumelissodes*) *bimatris*
17	*Melissodes* (*Callimelissodes*) *lupinus*
18	*Melissodes* (*Eumelissodes*) *lutulentus*
19	*Melissodes* (*Eumelissodes*) *pallidisignatus*
20	*Melissodes* (*Eumelissodes*) *subagilis*
21	*Sphecodes* sp.
22	*Svastra* (*Epimelissodes*) *obliqua*
23	*Triepeolus paenepectoralis*
24	*Xeromelecta* (*Melectomorpha*) *californica*

Behavioral observations (Figure 2a)	
Network ID	**Plant species**
X1	*Achillea millefolium*
X2	*Centaurea diffusa*
X3	*Centaurea solstitialis*
X4	*Gutierrezia sarothrae*
X5	*Dieteria canescens*
X6	*Onopordum acanthium*
X7	*Psoralidium lanceolatum*
X8	*Salsola kali*
X9	*Sisymbrium altissimum*

MB-RDB (Figure 2b)	
Network ID	**Plant species**
X1	*Achillea millefolium*
X2	*Centaurea diffusa*
X3	*Centaurea solstitialis*
X4	*Dieteria canescens*
X5	*Gutierrezia sarothrae*
X6	*Onopordum acanthium*
X7	*Rhaponticum repens*
X8	Brassicaceae
X9	*Sisymbrium loeselii*
X10	*Kali turgidum*
X11	*Medicago arabica*
X12	*Gleditsia triacanthos*
X13	*Ladeania lanceolata*
X14	*Medicago sativa*
X15	*Solanum dulcamara*
X16	*Solanum triflorum*
X17	*Tribulus terrestris*

MB-LDB (Figure 2c)	
Network ID	**Plant species**
X1	*Achillea millefolium*
X2	*Agoseris heterophylla*
X3	*Centaurea diffusa*
X4	*Centaurea solstitialis*
X5	*Dieteria canescens*
X6	*Gutierrezia sarothrae*
X7	*Onopordum acanthium*
X8	*Sisymbrium altissimum*
X9	*Sisymbrium loeselii*
X10	*Chenopodium* sp.
X11	*Kali turgidum*
X12	*Ladeania lanceolata*
X13	*Medicago sativa*
X14	*Robinia pseudoacacia*
X15	*Purshia tridentata*
X16	*Solanum triflorum*

APPENDIX 5

Legends for the United States Forest Service Starkey Experimental Forest and Range plant–pollinator networks created from behavioural observations, DNA metabarcoding using a regional reference database (MB-RDB) and DNA metabarcoding using a local reference database (MB-LDB) (Figure 3).

Network ID	Bee species (subgenus)
1	*Andrena* (*Leucandrena*) *barbilabris*
2	*Andrena* (*Trachandrena*) *cyanophila*
3	*Andrena* (*Micrandrena*) *melanochroa*
4	*Andrena* (*Thysandrena*) sp. A
5	*Anthophora* (*Melea*) *bomboides*
6	*Anthophora* (*Mystacanthophora*) *urbana*
7	*Bombus* (*Subterraneobombus*) *appositus*
8	*Bombus* (*Pyrobombus*) *bifarius*
9	*Bombus* (*Fervidobombus*) *californicus*
10	*Bombus* (*Pyrobombus*) *centralis*
11	*Bombus* (*Psithyrus*) *fernaldae*
12	*Bombus* (*Pyrobombus*) *flavifrons*
13	*Bombus* (*Psithyrus*) *insularis*
14	*Bombus* (*Pyrobombus*) *mixtus*
15	*Bombus* (*Pyrobombus*) *vosnesenskii*
16	*Coelioxys* (*Boreocoelioxys*) *rufitarsis*
17	*Halictus* (*Nealictus*) *farinosus*
18	*Halictus* (*Odontalictus*) *ligatus*
19	*Halictus* (*Protohalictus*) *rubicundus*
20	*Halictus* (*Seladonia*) *tripartitus*
21	*Hylaeus* (*Hylaeus*) *verticalis*
22	*Lasioglossum* (*Evylaeus*) *cooleyi*
23	*Lasioglossum* (*Dialictus*) *incompletum*
24	*Lasioglossum* (*Dialictus*) *laevissimum*
25	*Lasioglossum* (*Dialictus*) *nevadense*
26	*Lasioglossum* (*Lasioglossum*) *olympiae*
27	*Lasioglossum* (*Dialictus*) *sedi*
28	*Lasioglossum* (*Dialictus*) sp.
29	*Lasioglossum* (*Hemihalictus*) sp. 2
30	*Megachile* (*Xanthosarus*) *perihirta*
31	*Melissodes* (*Eumelissodes*) *microstictus*
32	*Osmia* (*Melanosmia*) *atrocyanea*
33	*Osmia* (*Melanosmia*) *brevis*
34	*Osmia* (*Melanosmia*) *bucephala*
35	*Osmia* (*Acanthosmioides*) *integra*
36	*Osmia* (*Melanosmia*) *juxta*
37	*Osmia* (*Melanosmia*) *raritatis*
38	*Osmia* sp. 2
39	*Panurginus* sp. A
40	*Perdita* (*Pygoperdita*) *wyomingensis*

Behavioural observations (Figure 3a)

Network ID	Plant species
X1	*Achillea millefolium*
X2	*Cirsium arvense*
X3	*Cirsium vulgare*
X4	*Collomia linearis*
X5	*Erigeron corymbosus*
X6	*Erigeron speciosus*
X7	*Eriogonum heracleoides*
X8	*Grindelia nana*
X9	*Hypericum perforatum*
X10	*Lupinus sulphureus*
X11	*Lupinus* sp.
X12	*Mentha arvensis*
X13	*Monardella odoratissima*
X14	*Penstemon procerus*
X15	*Polemonium occidentale*
X16	*Potentilla arguta*
X17	*Potentilla gracilis*
X18	*Senecio serra*
X19	*Sidalcea oregana*
X20	*Solidago missouriensis*
X21	*Symphyotrichum spathulatum*
X22	*Thermopsis montana*
X23	*Tragopogon dubius*
X24	*Trifolium pratense*
X25	*Trifolium* sp.
X26	*Trifolium wormskioldii*
X27	*Triteleia grandiflora*
X28	*Vicia cracca*

MB-RDB (Figure 3b)

Network ID	Plant species
X1	*Angelica arguta*
X2	*Heracleum maximum*
X3	*Lomatium triternatum*
X4	*Allium acuminatum*
X5	*Allium validum*
X6	*Goodyera oblongifolia*
X7	*Achillea millefolium*
X8	*Ambrosia acanthicarpa*
X9	*Blepharipappus scaber*
X10	*Centaurea diffusa*
X11	*Cirsium arvense*
X12	*Cirsium foliosum*

MB-RDB (Figure 3b)	
Network ID	**Plant species**
X13	*Cirsium vulgare*
X14	*Erigeron* sp.
X15	*Eriophyllum lanatum*
X16	*Grindelia squarrosa*
X17	*Hieracium scouleri*
X18	*Lactuca serriola*
X19	*Microseris nutans*
X20	*Packera* sp.
X21	*Senecio* sp.
X22	*Senecio hydrophiloides*
X23	*Solidago canadensis*
X24	*Symphyotrichum spathulatum*
X25	*Tragopogon dubius*
X26	*Phacelia hastata*
X27	*Polygonum douglasii*
X28	*Cornus sericea*
X29	*Symphoricarpos albus*
X30	*Polemonium occidentale*
X31	*Lupinus leucophyllus*
X32	*Medicago sativa*
X33	*Melilotus* sp.
X34	*Thermopsis montana*
X35	*Trifolium hybridum*
X36	*Trifolium pratense*
X37	*Trifolium repens*
X38	*Trifolium wormskioldii*
X39	*Vicia villosa*
X40	*Gentiana affinis*
X41	*Mentha spicata*
X42	*Monardella odoratissima*
X43	*Pedicularis groenlandica*
X44	*Erythranthe* sp.
X45	*Penstemon procerus*
X46	*Hypericum perforatum*
X47	*Sidalcea oregana*
X48	Ranunculaceae
X49	*Crataegus monogyna*
X50	*Geum macrophyllum*
X51	*Holodiscus discolor*
X52	*Physocarpus malvaceus*
X53	*Potentilla flabellifolia*
X54	*Rosa* sp.
X55	*Rosa gymnocarpa*
X56	*Arceuthobium campylopodum*
X57	*Sedum stenopetalum*
X58	*Micranthes integrifolia*

MB-LDB (Figure 3c)	
Network ID	**Plant species**
X1	Apiaceae
X2	*Angelica arguta*
X3	*Heracleum maximum*
X4	*Lomatium gormanii*
X5	*Allium acuminatum*
X6	*Allium validum*
X7	*Triteleia grandiflora*
X8	*Goodyera oblongifolia*
X9	Asteraceae
X10	*Anthemis cotula*
X11	*Cirsium arvense*
X12	*Cirsium scariosum*
X13	*Cirsium vulgare*
X14	*Erigeron* sp.
X15	*Eriophyllum lanatum*
X16	*Hieracium scouleri*
X17	*Lactuca serriola*
X18	*Madia* sp.
X19	*Madia gracilis*
X20	*Microseris nutans*
X21	*Packera pseudaurea*
X22	*Solidago canadensis*
X23	*Symphyotrichum* sp.
X24	*Symphyotrichum ascendens*
X25	*Tragopogon dubius*
X26	*Nemophila breviflora*
X27	*Polygonum minimum*
X28	*Cornus canadensis*
X29	*Symphoricarpos albus*
X30	*Polemonium occidentale*
X31	*Lupinus leucophyllus*
X32	*Medicago lupulina*
X33	*Melilotus officinalis*
X34	*Thermopsis montana*
X35	*Trifolium* sp.
X36	*Trifolium eriocephalum*
X37	*Trifolium pratense*
X38	*Trifolium repens*
X39	*Trifolium wormskioldii*
X40	*Gentiana affinis*
X41	*Mentha arvensis*
X42	*Monardella odoratissima*
X43	*Pedicularis groenlandica*
X44	*Penstemon confertus*
X45	*Hypericum perforatum*
X46	*Malva neglecta*

MB-LDB (Figure 3c)	
Network ID	**Plant species**
X47	*Myosurus minimus*
X48	*Crataegus monogyna*
X49	*Geum macrophyllum*
X50	*Holodiscus discolor*
X51	*Physocarpus malvaceus*
X52	*Potentilla gracilis*
X53	*Potentilla recta*
X54	*Rosa* sp.
X55	*Rosa gymnocarpa*
X56	*Arceuthobium campylopodum*
X57	*Heuchera cylindrica*

APPENDIX 6

Legends for The Nature Conservancy's Zumwalt Prairie Preserve plant–pollinator networks created from behavioural observations, DNA metabarcoding using a regional reference database (MB-RDB) and DNA metabarcoding using a local reference database (MB-LDB) (Figure 4).

Network ID	Bee species (subgenus)
1	*Andrena* (*Euandrena*) *astragali*
2	*Andrena* (*Micrandrena*) *melanochroa*
3	*Andrena* (*Euandrena*) *nigrocaerulea*
4	*Andrena* (*Melandrena*) *nivalis*
5	*Andrena* (*Simandrena*) *pallidifovea*
6	*Andrena* (*Plastandrena*) *prunorum*
7	*Andrena* (*Euandrena*) sp. 1
8	*Andrena* (*Melandrena*) *transnigra*
9	*Andrena* (*Andrena*) *vicinoides*
10	*Anthophora* (*Lophanthophora*) *pacifica*
11	*Bombus* (*Subterraneobombus*) *appositus*
12	*Bombus* (*Pyrobombus*) *bifarius*
13	*Bombus* (*Fervidobombus*) *californicus*
14	*Bombus* (*Pyrobombus*) *centralis*
15	*Bombus* (*Fervidobombus*) *fervidus*
16	*Bombus* (*Pyrobombus*) *flavifrons*
17	*Bombus* (*Pyrobombus*) *huntii*
18	*Bombus* (*Psithyrus*) *insularis*
19	*Bombus* (*Pyrobombus*) *mixtus*
20	*Bombus* (*Bombias*) *nevadensis*
21	*Bombus* (*Cullumanobombus*) *rufocinctus*
22	*Bombus* (*Pyrobombus*) *vosnesenskii*
23	*Diadasia nigrifrons*
24	*Halictus* (*Odontalictus*) *ligatus*
25	*Halictus* (*Protohalictus*) *rubicundus*
26	*Halictus* (*Seladonia*) *tripartitus*
27	*Lasioglossum* (*Dialictus*) *albipenne*
28	*Lasioglossum* (*Dialictus*) *incompletum*
29	*Lasioglossum* (*Evylaeus*) *ovaliceps*
30	*Lasioglossum* (*Lasioglossum*) *titusi*
31	*Megachile* (*Litomegachile*) *onobrychidis*
32	*Megachile* (*Xanthosarus*) *perihirta*
33	*Melissodes* (*Callimelissodes*) *lupinus*
34	*Melissodes* (*Heliomelissodes*) *rivalis*
35	*Panurginus* sp. A

Behavioral observations (Figure 4a)	
Network ID	**Plant species**
X1	*Achillea millefolium*
X2	*Arenaria congesta*
X3	*Arnica sororia*
X4	*Calochortus eurycarpus*
X5	*Castilleja lutescens*
X6	*Cirsium brevifolium*
X7	*Clarkia pulchella*
X8	*Delphinium nuttallianum*
X9	*Erigeron pumilus*
X10	*Eriogonum heracleoides*
X11	*Frasera albicaulis*
X12	*Geranium* sp.
X13	*Geranium viscosissimum*
X14	*Grindelia nana*
X15	*Hieracium cynoglossoides*
X16	*Lupinus caudatus*
X17	*Lupinus leucophyllus*
X18	*Lupinus sericeus*
X19	*Lupinus* sp.
X20	*Orthocarpus tenuifolius*
X21	*Potentilla gracilis*
X22	*Sidalcea oregana*
X23	*Sisymbrium altissimum*
X24	*Solidago missouriensis*
X25	*Toxicoscordion venenosum*

MB-RDB (Figure 4b)	
Nework ID	**Plant species**
X1	*Lomatium triternatum*
X2	*Achillea millefolium*
X3	*Arnica sororia*
X4	*Blepharipappus scaber*
X5	*Cirsium* sp.

MB-RDB (Figure 4b)

Nework ID	Plant species
X6	*Cirsium foliosum*
X7	*Grindelia squarrosa*
X8	*Helianthus annuus*
X9	*Hieracium scouleri*
X10	*Lactuca serriola*
X11	*Solidago canadensis*
X12	*Symphyotrichum ascendens*
X13	*Symphyotrichum spathulatum*
X14	*Cryptantha affinis*
X15	*Phacelia hastata*
X16	Brassicaceae
X17	*Moehringia lateriflora*
X18	*Eriogonum flavum*
X19	*Philadelphus lewisii*
X20	*Lupinus leucophyllus*
X21	*Vicia villosa*
X22	*Frasera albicaulis*
X23	*Gentiana affinis*
X24	*Gentianella amarella*
X25	*Geranium pusillum*
X26	*Agastache urticifolia*
X27	*Castilleja cusickii*
X28	*Orthocarpus tenuifolius*
X29	*Penstemon procerus*
X30	*Calochortus macrocarpus*
X31	*Toxicoscordion venenosum*
X32	*Veratrum viride*
X33	*Sidalcea oregana*
X34	*Potentilla flabellifolia*
X35	*Rosa* sp.

MB-LDB (Figure 4c)

Network ID	Plant species
X6	*Blepharipappus scaber*
X7	*Cirsium* sp.
X8	*Cirsium foliosum*
X9	*Crepis occidentalis*
X10	*Grindelia squarrosa*
X11	*Helianthus annuus*
X12	*Lactuca serriola*
X13	*Solidago canadensis*
X14	*Symphyotrichum spathulatum*
X15	*Cryptantha ambigua*
X16	*Phacelia heterophylla*
X17	Brassicaceae
X18	*Silene scouleri*
X19	*Eriogonum flavum*
X20	*Philadelphus lewisii*
X21	*Lupinus leucophyllus*
X22	*Vicia villosa*
X23	*Frasera albicaulis*
X24	*Gentiana affinis*
X25	*Geranium pusillum*
X26	*Agastache urticifolia*
X27	*Castilleja cusickii*
X28	*Orthocarpus tenuifolius*
X29	*Penstemon procerus*
X30	*Calochortus macrocarpus*
X31	*Toxicoscordion venenosum*
X32	*Sidalcea oregana*
X33	*Potentilla flabellifolia*
X34	*Rosa* sp.

MB-LDB (Figure 4c)

Network ID	Plant species
X1	*Lomatium triternatum*
X2	*Triteleia grandiflora*
X3	*Achillea millefolium*
X4	*Arnica sororia*
X5	*Artemisia* sp.