2017

A Comparison of Honey Bee-Collected Pollen From Working Agricultural Lands Using Light Microscopy and ITS Metabarcoding

M. D. Smart
U.S. Geological Survey Northern Prairie Wildlife Research Center, msmart@usgs.gov

R. S. Cornman
U.S. Geological Survey Fort Collins Science Center, rcornman@usgs.gov

D. D. Iwanowicz
U.S. Geological Survey Leetown Science Center

M. McDermott-Kubeczko
University of Minnesota

J. S. Pettis
USDA-ARS Bee Research Laboratory, PettisJ@ba.ars.usda.gov

See next page for additional authors

Authors
M. D. Smart, R. S. Cornman, D. D. Iwanowicz, M. McDermott-Kubeczko, J. S. Pettis, M. S. Spivak, and C. R.V. Otto

Environmental Entomology, 2017, 1–12
doi: 10.1093/ee/nvw159
Research

A Comparison of Honey Bee-Collected Pollen From Working Agricultural Lands Using Light Microscopy and ITS Metabarcoding

M. D. Smart,[1,2] R. S. Cornman,[3] D. D. Iwanowicz,[4] M. McDermott-Kubeczko,[5] J. S. Pettis,[6] M. S. Spivak,[5] and C.R.V. Otto[1]

[1]U.S. Geological Survey Northern Prairie Wildlife Research Center, Jamestown, ND (msmart@usgs.gov; cotto@usgs.gov), [2]Corresponding author, e-mail: msmart@usgs.gov, [3]U.S. Geological Survey Fort Collins Science Center, Fort Collins, CO (rcornman@usgs.gov), [4]U.S. Geological Survey Leetown Science Center, Kearneysville, WV (diwanowicz@usgs.gov), [5]Department of Entomology, University of Minnesota, St. Paul, MN (mcde0099@umn.edu; spiva001@umn.edu), and [6]USDA-ARS Bee Research Laboratory, Beltsville, MD (jeff.pettis@ars.usda.gov)

Subject Editor: Gloria DeGrandi-Hoffman

Received 15 August 2016; Editorial decision 8 November 2016

Abstract

Taxonomic identification of pollen has historically been accomplished via light microscopy but requires specialized knowledge and reference collections, particularly when identification to lower taxonomic levels is necessary. Recently, next-generation sequencing technology has been used as a cost-effective alternative for identifying bee-collected pollen; however, this novel approach has not been tested on a spatially or temporally robust number of pollen samples. Here, we compare pollen identification results derived from light microscopy and DNA sequencing techniques with samples collected from honey bee colonies embedded within a gradient of intensive agricultural landscapes in the Northern Great Plains throughout the 2010–2011 growing seasons. We demonstrate that at all taxonomic levels, DNA sequencing was able to discern a greater number of taxa, and was particularly useful for the identification of infrequently detected species. Importantly, substantial phenological overlap did occur for commonly detected taxa using either technique, suggesting that DNA sequencing is an appropriate, and enhancing, substitutive technique for accurately capturing the breadth of bee-collected species of pollen present across agricultural landscapes. We also show that honey bees located in high and low intensity agricultural settings forage on dissimilar plants, though with overlap of the most abundantly collected pollen taxa. We highlight practical applications of utilizing sequencing technology, including addressing ecological issues surrounding land use, climate change, importance of taxa relative to abundance, and evaluating the impact of conservation program habitat enhancement efforts.

Key words: pollen identification, honey bee, land use, agriculture

Increased societal demand for pollination services, concurrent with recent declines in native and managed pollinators, has emphasized the need to accurately understand pollinator habitat and forage requirements (Aizen and Harder 2009, Vaudo et al. 2015, Koh et al. 2016). Information gleaned from pollinator forage studies can be useful for informing pollinator habitat enhancements and conservation efforts occurring throughout the United States (e.g., Gallant et al. 2014, Richardson et al. 2015a, Smart et al. 2016a, US Geological Survey, Northern Prairie Wildlife Research Center [USGS NPWRC] 2016). For example, the US Department of Agriculture recently initiated multiple land enhancement efforts directed toward improving forage for pollinators throughout the Upper Midwest (US Department of Agriculture, Farm Service Agency [USDA FSA] 2014). Furthermore, one of the primary goals set by the Pollinator Health Task Force (2015) is establishing 7 million acres of pollinator habitat by 2020.

Widespread changes in land use patterns have occurred over the past several decades across the Northern Great Plains (NGP) region of the United States (USDA 1984; USDA NASS 1994, 2004a, 2014a; Wright and Wimberly 2013). Changes in NGP land use are having negative impacts on managed honey bee colonies during the summer (Smart et al. 2016b) and wild bee populations (Koh et al. 2016). This region supports around 1 million honey bee colonies annually (~40% of the national commercial pool of honey bee colonies), sustained by the abundance of pollen- and nectar-producing flowers present on the landscape, coupled with an extended photoperiod during the growing season (USDA NASS 2004b, 2014b; Gallant et al. 2014). Although a critical part of the country for

commercial beekeepers, recent land use changes driven in part by rising commodity crop prices for corn and soybeans have eliminated grasslands and wetlands (Wright and Wimberly 2013), and specifically altered the habitat suitability for honey bees (Otto et al. 2016).

Taxonomic identification of bee-collected pollen has the potential to address specific questions related to plant–insect interaction dynamics, habitat use, and habitat and forage quality from both ecological and policy standpoints. This information may go on to influence decisions directed toward evaluating and enhancing pollinator habitat, thus contributing to the future security of plant and bee populations and pollination services (e.g., Olsen et al. 1979, Green 1983, Kleijn and Raemakers 2008). Rigorous assessments of overall habitat quality in intensively managed landscapes with the aim of linking specific floral resources over the foraging season to honey bee health, productivity, and survival have just recently been considered. Requier et al. (2015) and Smart et al. (2016a), for example, both found a strikingly high utilization and dependency of honey bees on volunteer and introduced species present in intensive agroecosystems. There is a growing need for methods that can quickly, accurately, and efficiently quantify honey bee foraging resources across landscapes.

Pollen is the primary protein source for colonies; its availability in the surrounding landscape and inside the hive enables colonies to grow and collect adequate nutritional resources throughout the season. Pollen availability affects many facets of colony functioning in addition to producing a robust population size for nectar foraging, such as physiology and gene expression related to nutrition and immunity (Pernal and Currie 2000, Alaux et al. 2010, Alaux et al. 2011, Huang 2012, Di Pasquale et al. 2013, Wheeler and Robinson 2014), susceptibility to diseases, parasites, and pesticides (De Grandi-Hoffman et al. 2010, Alaux et al. 2011), and overwintering success (Mattila and Otis 2007, Smart et al. 2016b).

Traditional techniques utilizing light microscopy to identify pollen grains (Erdtman 1966, Crompton and Wojtas 1993) have been useful in identifying pollen collected from honey bees and wild bees (Sawyer 1975, Olsen et al. 1979, Adams and Smith 1981, Larson et al. 2006). However, pollen identification via light microscopy can require substantial technical training and time to obtain high taxonomic resolution. More recently, DNA barcoding, including that of the ITS2 region, has been explored for its potential as a "universal plant and animal barcode" (Yao et al. 2010). Specific applications have included identification of medicinal plants and herbal materials (Chen et al. 2010, Pang et al. 2013), and for the identification of floral taxa in bee-collected pollen (Galimberti et al. 2014, Cornman et al. 2015, Richardson et al. 2015a). Genetic barcoding allows researchers to efficiently generate taxonomic profiles from multiple pollen samples without specialized palynological training; however, this technique requires continued field testing in working landscapes before results can be deemed reliable. A few previous studies have shown the potential of using ITS2 metabarcoding for pollen analysis (Keller et al. 2014; Richardson et al. 2015a,b) based on relatively small collections over a short period of time. Additional comparative studies are needed that include broader spatial and temporal sampling in working landscapes, with improved taxonomic resolution.

Here we utilize two methods to quantify pollen samples collected on 10 sample dates, among six apiary sites during the spring–autumn of 2010 and 2011. Samples were recovered from honey bee colonies located among six apiaries characterized by a gradient of intensive agricultural land use surrounding them in the Prairie Pothole Region of North Dakota (Supp. Fig. 1 [online only]).

Specifically, we use light microscopy and sequencing of nuclear ribosomal loci to determine: 1) Number and abundance of all flowering plant taxa assigned across two growing seasons, 2) Between-method phenological concordance of plant taxa, 3) Taxonomic resolution derived from each technique and site, 4) Indigenous status of assigned taxa, and 5) Pollen identity and diversity across a land-use gradient.

We identify the indigenous status of plant taxa because our study region is the focus of pollinator conservation and landscape enhancement efforts that may include planting native and nonnative seed mixes. Given the importance of our study region for the health of summering commercial honey bee colonies (Smart et al. 2016a,b) and recent land-use changes (Wright and Wimberly 2013, Morefield et al. 2016, Otto et al. 2016), our results have practical application toward land management and national pollinator conservation efforts.

Materials and Methods

Pollen Sample Preparation

Returning forager-collected pollen was recovered from two colonies at each of the six apiary locations varying in the intensity of surrounding agricultural land use (Smart et al. 2016a) over two years (2010–2011). The two colonies per apiary were fitted with pollen traps that, when opened, forced returning foraging honey bees to walk through the screens upon entering the hive (described in Delaplane et al. 2013). The screens dislodged the pollen loads from bee hind tibiae into a pollen collection drawer before the bee entered the colony. Traps were open for a 48-h period four to six times per summer (six in 2010, four in 2011), and subsequently, pollen was collected into a plastic bag and placed in a cooler on dry ice for shipping. Upon arrival at the USDA-ARS-Bee Research Lab in Beltsville, MD, samples were stored at − 20 °C until analysis.

Floral Pollen Source Identification via Light Microscopy

A 3-g fresh mixed pollen sample from each colony at each site and date was first narrowed down to limit taxonomic diversity by sorting similarly colored pollen pellets by eye, followed by their examination and identification using light microscopy. The average fresh weight of a honey bee pollen pellet is ∼7 mg (Roman 2006), and there were one to six pellet colors included in each sample from each colony on each date in our study. For each colony, date, and pollen color, seven fresh forager-collected pollen pellets were macerated and suspended in 75 µl glycerin to which 10 µl Calberla's stain was added. Therefore, light microscope taxonomic determinations were made from 49–294 mg of fresh sample material for each date by colony. Twenty microliters was placed on a microscope slide, topped with a coverslip, and sealed with acrylic paint. Pollen was allowed to absorb the stain for a minimum of 20 min before being visualized at 100× and 400×. In each case, 100 pollen grains in the field of view were counted and taxonomic identity was determined. A reference slide collection was not compiled for this specific project, though pollen reference slides from Minnesota and South Dakota forbs (Larson et al. 2014), in conjunction with published reference materials, were consulted (Crompton and Wojtas 1993, Palynological Database [PalDat] 2000, University of Arizona 2001, Kapp et al. 2007). Attempts were made to identify pollen to the lowest taxonomic level possible, though in many cases certain pollens could only be identified to genus or family, or were not able to be identified but were nevertheless counted. Data collected from each light microscopy sample consisted of a total number of grains of

each taxon for each date–site–colony combination. This number of grains for each detected taxon per sample was divided by the total number of grains counted per sample (100) and multiplied by 10^6 to arrive at the number of grain counts-per-million (cpm). This conversion was done to facilitate comparison to DNA sequencing data (described below).

Floral Pollen Source Identification via DNA Sequencing

A separate aliquot of each pollen sample subjected to light microscope identification was also used for DNA sequencing analysis. Detailed methods are outlined in Cornman et al. (2015). Briefly, for each pollen collection date, a mixed subsample was removed from the larger unsorted bulk sample (mean weight of mixed sample was 1.77 g). Each sample was crushed using a mortar and pestle and dried at 60 °C for 60 h. On average, ~30% weight loss was attributed to the drying process. DNA extraction was carried out on 25–40 mg of dried pollen per colony by date (equivalent to 33–52 mg fresh pollen) using a modified Doyle's method (Doyle 1991, Cornman et al. 2015) at USGS Leetown Science Center, Kearneysville, WV. The internal transcribed spacer (ITS) of the nuclear ribosomal locus (Kress et al. 2005, Cornman et al. 2015) was amplified and sequenced on the Illumina MiSeq following Illumina's standard amplicon-sequencing protocol. Paired-end reads of 300 bp (before trimming) represented nonoverlapping sequence from the ITS1 and ITS2 regions. De novo operational taxonomic units (OTU) were selected by clustering scaffolded reads at 97%, and the number of occurrences of each OTU determined by the number of reads matching at 97% with at most five indels. OTUs were given taxonomic assignments using the lowest common ancestor approach for which the combined BLAST bit score was the similarity metric and the NCBI nucleotide (nt) database was the taxonomic reference. Counts from OTUs with the same taxonomic assignment were combined in order to express the total number of reads for each taxon for each date–site–colony combination (a library).

A total of 223 distinct plant taxa (Supp. Table 1 [online only]) were assigned overall via DNA sequencing and light microscope analyses. Taxa that had fewer than 50 reads within a given library were removed from subsequent analyses because differences in these taxa near the edge of detection may not be reliable and their detection was not relevant to the questions of which taxa are the dominant bee-foraged plants. They may, however, be relevant to subsequent questions surrounding the detection of rare plants, particularly if bees are used as bio-indicators. All analyzed taxa within each library were then converted to a cpm value by dividing each taxon's number of reads within each library by the total number of reads in that library and then multiplying this proportion by 10^6. This resulted in 150 unique taxa. Reads not ascribed to flowering plants, e.g., those of putatively microbial origin, and those remaining as unassigned, were removed from further analysis.

Land Use Influence on Pollen Collection and Identification

Because the study apiaries were embedded within varying degrees of intensively managed agroecosystems (Supp. Fig. 1 [online only]; Smart et al. 2016a), we were able to examine spatial and temporal honey bee forage patterns within this system. Sites were binned into two groups, those surrounded by "LOW" agricultural intensity and "HIGH" agricultural intensity. Apiaries in the low group (A, B, C) had between 34–70% of surrounding land use in potential bee forage land (grassland, pasture, fallow fields, conservation land, flowering trees and shrubs, alfalfa, canola, sunflower, wetlands), while apiaries in the high group (D, E, F) had between 11–28% potential bee forage land in the surrounding landscape within 3.2 km (2 mi) radius.

Statistical Analysis

The data were heavily zero-inflated because of the greater taxonomic resolution derived from DNA sequencing, (i.e., many taxa detected by sequencing were not detected using light microscopy). Transformation of the data was explored using log and square-root transformations, but failed to result in normality. Therefore, to address objectives 2 and 5, nonparametric analysis (Spearman rank correlation) was conducted to compare ranked taxa between the two techniques in the context of time (sample date, year, overall data) and intensity of agriculture.

To address objective 3, we performed paired t-tests to separately compare the number of families, genera, and species detected among all pollen samples (i.e., number of taxa detected from each apiary and sample date using either technique was considered a paired replicate).

To quantify diversity, richness, and similarity of study sites in objective 5, we calculated the overall Shannon-Weiner diversity index (H), Pielou's evenness (J), and the alpha parameter of Fisher's log series (α).

Results

Objective 1. Number and Abundance of All Flowering Plant Taxa Detected

A higher number of taxa at all taxonomic levels were assigned using the DNA sequencing technique compared to light microscopy (Table 1). More than twice as many families were assigned using sequencing. High taxonomic richness using either identification technique occurred at the genus level (Table 1), wherein a total of 66 and 27 genera were assigned utilizing DNA sequencing and light microscopy, respectively. Only one plant species, *Melilotus officinalis*, was detected with the light microscopy technique, whereas 69 plant species were detected with DNA sequencing (Table 1).

A number of both common and unique taxa were ascribed using the two techniques. Substantial overlap occurred between taxa identified, with a high degree of continuity among the 20 most commonly assigned taxa (at all levels) in either case (Fig. 1). The most commonly assigned taxa using both techniques included *Melilotus*, *Sonchus*, and Asteraceae (the tribe Astereae was not a taxonomic unit of identification for microscopy). Other commonly assigned taxa included the Brassicaceae and Fabaceae families (and generic members), and genera within Asteraceae: *Grindelia*, *Helianthus*, *Solidago*, *Cirsium*, *and Artemisia* (Fig. 1).

Because taxonomic resolution was low using light microscopy, and the genus level was the richest level for microscopy (Table 1), going forward we chose to primarily focus at the generic level. Substantial overlap occurred in the taxa identified between the two techniques, with a high degree of continuity among the main taxa. Although our analyses detected 66 and 27 unique genera by technique, respectively (Table 1), 62% of the total count assignments were attributed to just six genera (*Melilotus*, *Sonchus*, *Brassica*, *Grindelia*, *Helianthus*, and *Solidago*). Genera containing plant species considered native to the region, such as *Amorpha*, *Alisma*, *Anemone*, *Dalea*, and *Monarda* represented only 12% (DNA sequencing) to 20% (microscopy) of the total taxonomic assignments. Further, *Alisma*, *Anemone*, and *Sium* in particular, represent genera of aquatic and wetland-associated plants not previously

Table 1. Total number of taxa assigned in pollen using DNA sequencing and light microscopy techniques

Family	DNA sequencing				Light microscopy		
	Family detected Y/N	No. tribes	No. genera	No. species	Family detected Y/N	No. genera	No. species
Alismataceae	Y	–	1	1	N	–	–
Amaranthaceae	Y	–	2	2 (1)	Y	1	–
Amaryllidaceae	Y	–	1	3 (2)	N	–	–
Apiaceae	Y	–	2 (1)	2 (1)	Y	–	–
Asteraceae	Y	5	26 (8)	47 (16)	Y	10	–
Boraginaceae	Y	–	1 (1)	1 (1)	N	–	–
Brassicaceae	Y	1	5 (1)	13 (6)	Y	3	–
Caprifoliaceae	Y	–	3 (1)	5 (3)	N	–	–
Caryophyllaceae	N	–	–	–	Y	1	–
Convolvulaceae	Y	–	3 (2)	2 (1)	N	–	–
Cyperaceae	Y	1	2 (1)	1	N	–	–
Elaeagnaceae	Y	–	1	1	N	–	–
Euphorbiaceae	Y	–	1	1	N	–	–
Fabaceae	Y	2	13 (2)	16 (5)[a]	Y	9	1[a]
Lamiaceae	Y	1	2	1	Y	–	–
Linaceae	N	–	–	–	Y	1	–
Lythraceae	Y	–	1	–	N	–	–
Oleaceae	Y	–	2	1	N	–	–
Onagraceae	Y	–	2 (1)	2 (1)	N	–	–
Plantaginaceae	Y	–	2 (1)	–	Y	1	–
Poaceae	Y	2 (1)	8 (3)	6 (3)	Y	–	–
Polygonaceae	Y	–	1	–	Y	1	–
Ranunculaceae	Y	–	2	2 (1)	N	–	–
Rhamnaceae	Y	–	1	–	N	–	–
Rosaceae	Y	1 (1)	3	2	Y	–	–
Salicaceae	Y	–	2	3 (2)	N	–	–
Sapindaceae	Y	–	1	–	N	–	–
Scrophulariaceae	N	–	–	–	Y	–	–
Typhaceae	Y	–	–	–	N	–	–
Urticaceae	Y	–	2 (2)	1 (1)	N	–	–
Total	27	13 – (2) = 11	90 – (24) = 66	113 – (44) = 69	13	27	1

Numbers in parentheses are the number of taxa assigned by DNA sequencing but conservatively removed from analysis due to a low number of reads (<50 reads in a sample).

[a] *Melilotus officinalis* and *Melilotus albus* were considered a single species (USDA NRCS 2016).

known to comprise a significant portion of the honey bee diet. *Glycine*, the soybean (*G. max*) genus, was detected using both techniques (Supp. Table 1 [online only]).

Objective 2. Between-Method Concordance of Plant Taxa

Analysis of ranked generic level cpm indicated no relationship among all genera assigned (Fig. 2a: Spearman $\rho = 0.004$, $P = 0.97$); however, this was primarily due to the substantial number of taxa (even at the generic level) assigned using DNA sequencing without a corresponding assignment via microscopy. When examining only the 27 genera assigned via light microscopy, a positive correlation was revealed (Fig. 2b: Spearman $\rho = 0.60$, $P = 0.001$). For the top 10 most common taxa, the Spearman correlation was $\rho = 0.52$ ($P < 0.0001$, Fig. 2c). A Pearson correlation analysis of *Melilotus* cpm for all samples revealed a strong, positive correlation between methods (Fig. 2d: $t = 6.26$, df = 43, $r^2 = 0.48$, $P < 0.0001$, 95% CI: 0.50–0.82). *Melilotus* was the most commonly detected genus, and contributed more than a third of all counts by both methods.

Further, within most sampling dates, we detected a positive correlation between all ranked generic cpm (Table 2). Nonsignificant correlations were primarily found on 2011 dates, wherein pollen was recovered from a smaller number of study apiaries on most dates. Only taxa that were detected in at least one of the techniques were included. This was done to avoid artificially inflating the correlative relationship by including the many corresponding nondetections using both techniques on a given sample date. Overall, ranked data derived from cpm using each technique coincided well within each year and when data from all dates and years were analyzed together (Table 2).

The 40 most abundant genera assigned between techniques by date are graphically depicted in Fig. 3 (all 82 genera may be found in Supp. Fig. 2 [online only]). In addition to identifying many genera that were undetected using light microscopy (e.g., *Amorpha*, *Dalea*, *Elaeagnus*, *Heterotheca*, *Monarda*, *Salix*, *Symphotrichium*), DNA sequencing detected certain dually-identified taxa at both earlier (e.g. *Grindelia*, *Helianthus*, *Sonchus*) and later (e.g., *Astragalus*, *Medicago*, *Taraxacum*) time points (Fig. 3). Some taxa were assigned regularly via one technique and not assigned in the other (e.g., *Heterotheca*, *Spartina*, *Phaseolus*, *Tragopogon*, *Vicia*). As discussed above, *Melilotus* assignment was common and coincided well between techniques at most time points, as did *Brassica*, *Cirsium*, *Sonchus*, and *Trifolium*.

Objective 3. Taxonomic Resolution Derived From Each Technique

At all taxonomic levels, DNA sequencing assigned significantly more taxa than light microscopy, with the greatest difference in mean number of taxa assigned occurring at the species level (Table 3).

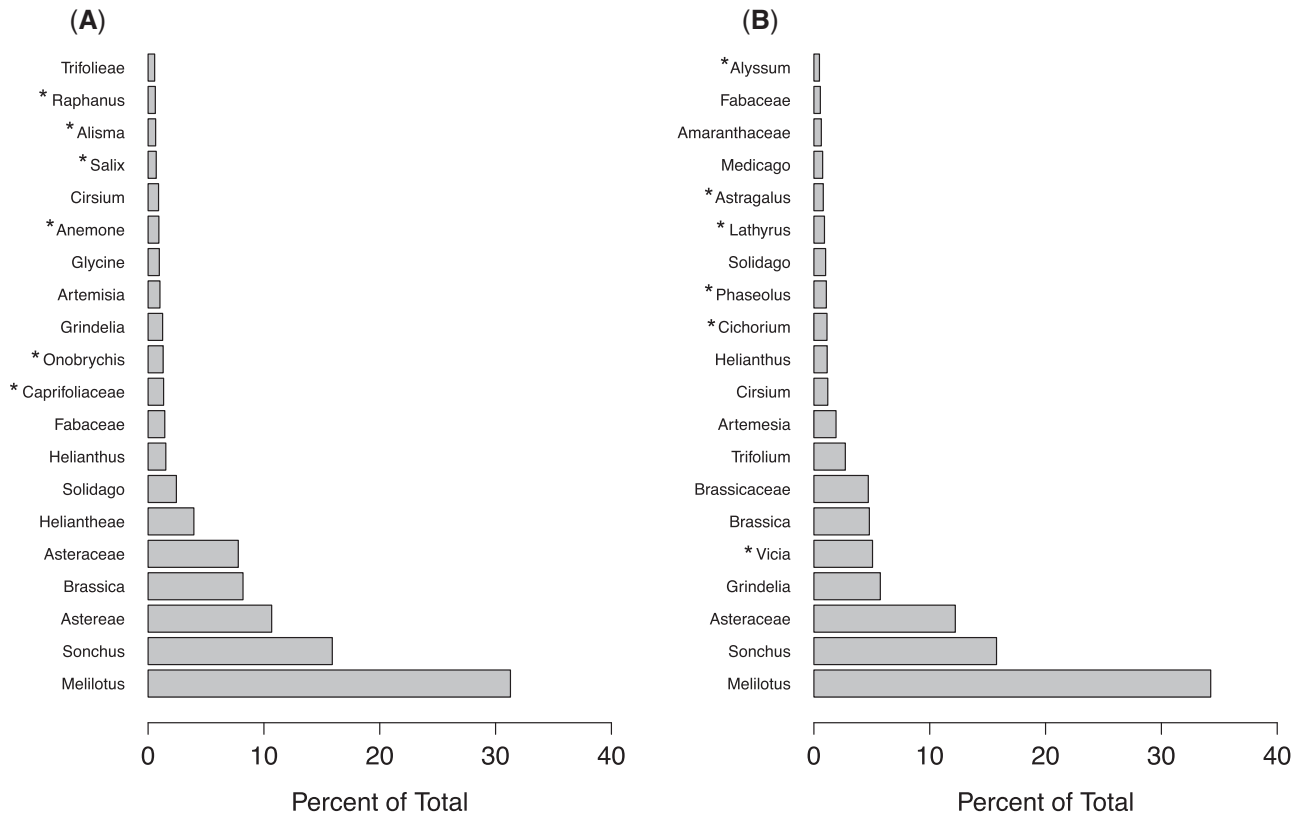

Fig. 1. Top 20 taxa (genus and higher taxonomic levels) identified using (**A**) DNA sequencing and (**B**) light microscopy. Taxa depicted are reported as the percent total counts per million (cpm) of all taxa assigned among all samples. Asterisked taxa were uniquely identified using either technique.

The number of taxa assigned at all taxonomic levels varied by technique (Table 3) and apiary site (Supp. Table 2 [online only]). *Melilotus albus/officinalis* was the only species identified via light microscopy. We assigned a greater total number of taxa, and average number of taxa per sample, with DNA sequencing when compared to light microscopy (Supp. Table 2 [online only]). In either technique, the greatest number of taxa (at all levels) per sample were yielded primarily from sites A and F. Interestingly, these were the two sites with the most dissimilar land use and also had the lowest total number of pollen samples overall. Site D, with the greatest number of samples (10), had the highest total number of taxa as determined by DNA sequencing but not microscopy (second to site F in families and genera).

Objective 4. Indigenous Status of Detected Taxa

We determined the indigenous, introduced and noxious status, and overall % composition, of all identified taxa using either technique (Supp. Table 3 [online only]). Many genera were ambiguously denoted as native or introduced depending on the identity of individual species contained with the genera identified. Plants strictly considered native to the region occurred at relatively low abundances, with *Helianthus* and *Grindelia* comprising the highest percentages within the native plant category. Several North Dakota state and county noxious weeds were assigned, including the genera: *Artemisia* (wormwood), *Centaurea* (knapweed, starthistle), *Cirsium* (thistle), and *Euphorbia* (spurge). Overall, pollen from flowering forbs predominated in the samples; however, several trees (*Acer*, *Populus*, *Salix*), woody shrubs (*Rhamnus*, *Symphoricarpos*, *Syringa*), wetland plants (*Alisma*, *Anemone*, *Cicuta*, *Sium*), vines (*Lonicera*) and sedges and grasses (*Bolboschoenus*, *Bromus*, *Spartina*) were detected. Additionally, several genera containing common agricultural row and forage crops were identified including *Brassica*, *Fagopyrum*, *Glycine*, *Helianthus*, *Medicago*, *Phaseolus*, *Raphanus*, and *Zea*.

Objective 5. Variation in Taxonomic Assignment and Diversity Relative to Land Use

Indices of generic richness, diversity, and evenness were determined to characterize and compare the six study sites because they existed along a gradient from high to low potential forage in the lands surrounding the study apiaries (Table 4). Indices included Shannon–Weiner diversity (*H'*), Fisher's α, and Pielou's evenness (J). Here, sites A, D, and F displayed the greatest diversity. All six sites were relatively even (J is constrained from 0 to 1, increasing as variation in counts of taxa decreases) in the plant community composition of forager-collected pollen at the generic level.

When grouped into LOW and HIGH agricultural intensity sites, identification techniques were positively correlated within each agricultural group (high: $\rho = 0.69$, $P = 0.002$; low: $\rho = 0.47$, $P = 0.04$), though the correlation was stronger for high intensity apiaries (Table 5). Because of the positive correlations between identification methods, we combined cpm from both methods and examined the relationship between agricultural intensity by individual sample date, years, and overall (Table 5). Rank-based analysis by sample date suggested distinct differences in pollen genera detected at LOW vs. HIGH agricultural intensity sites, though data were significantly correlated on 25 August 2010 and when all data were combined for all dates and years. This was due to a high degree of concordance of the most commonly assigned genera in the datasets.

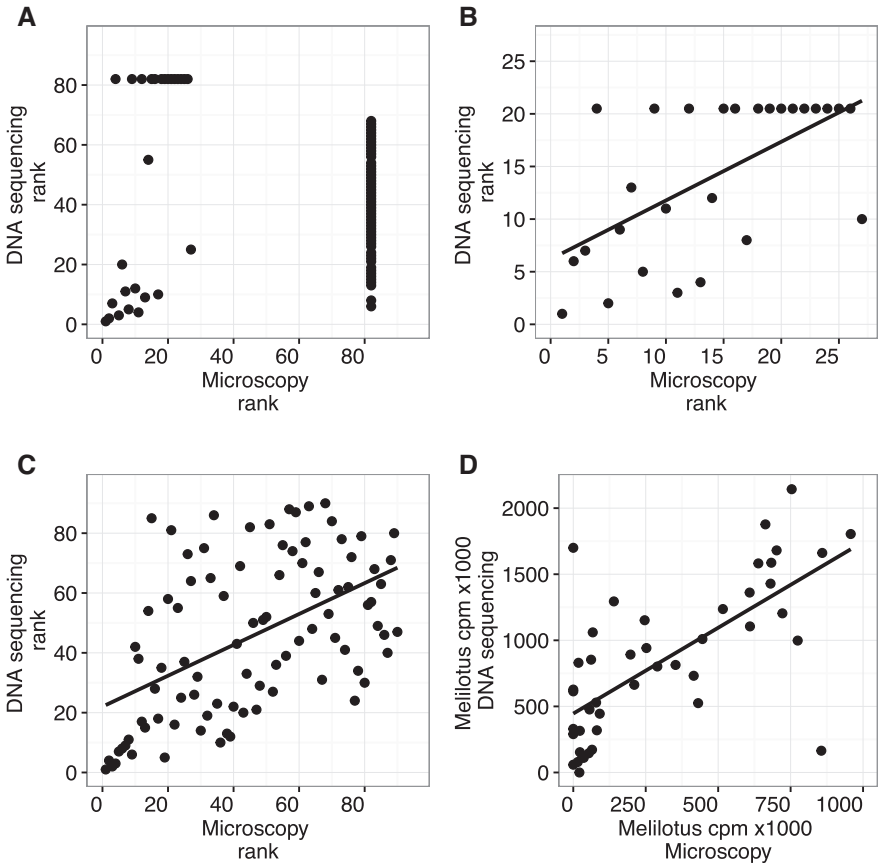

Fig. 2. DNA sequencing cpm (based on number of DNA reads) and microscope cpm (based on number of grains) ranked for all 82 overall assigned genera (**A**), all (27) ranked microscope-identified genera (**B**), top 10 ranked microscope-identified genera and associated DNA sequencing genera identified on each sample date (**C**), and (**D**) sweet clover (*Melilotus* spp.) cpm assigned on each sample date, 2010–2011. When genera were unassigned using a given technique, all zero detects were assigned the last rank for a given technique. Lines indicate a significant correlative relationship between techniques ($\alpha \leq 0.05$, using Spearman (2A–C) or Pearson (2D) correlations).

Table 2. Spearman's rho correlation of taxa (ranked genera) assigned via DNA sequencing vs. light microscopy

Sample date	No. apiaries sampled	ρ	*P*-value
21 June 2010	6	0.40	0.16
19 July 2010	6	0.60	0.002
01 Aug. 2010	4	0.48	0.03
17 Aug. 2010	4	0.66	0.001
25 Aug. 2010	6	0.56	0.0004
08 Sept. 2010	6	0.42	0.001
27 June 2011	4	0.89	<0.0001
19 July 2011	3	0.47	0.17
08 Aug. 2011	3	0.43	0.14
26 Aug. 2011	3	0.50	0.07
2010	6	0.56	<0.0001
2011	4	0.52	<0.0001
All dates	6	0.51	<0.0001

Pollen counts were ranked among apiaries for each sample date.

In both LOW and HIGH sites, a greater number of genera were assigned in the first half (June 21–August 1: LOW 35, HIGH 36) compared to the second half (August 8–September 8: LOW 23, HIGH 22) of the growing season (Fig. 4). When all 82 detected genera were considered, there were greater differences in plant richness across season and agricultural intensity (1st half: LOW 49, HIGH 59; 2nd half: LOW 37, HIGH 42). Further, for each half of the growing season there were 200 possible detections (40 genera by 5 dates). Interestingly, despite a similar number of genera assigned in the first half of the season between agricultural intensity, many more occurrences (or positive assignments) of those genera were found among the LOW apiaries (Fig. 4, LOW: 104 detections [52%], HIGH: 79 detections [40%]), suggesting there may have been differences in the persistence or abundance of pollen resources wherein genera found in LOW sites remained available or were collected for more sustained periods compared to HIGH sites. There was not a marked difference in the latter half of the season, however (LOW: 62 detections [31%], HIGH: 67 detections [34%]). This decreased number of genera in the late summer–autumn was largely due to the lack of discernment among the many members of the Asteraceae and Astereae (Supp. Table 3 [online only]) that occur during that time.

While overall there was continuity in the occurrence of common taxa by agricultural intensity (Fig. 4), the lack of correlation found relative to agricultural intensity (Table 5) was due to certain genera primarily identified from only LOW apiaries (e.g., *Amorpha*, *Monarda*, *Rosa*). Pollen from native grasses in the genus *Spartina* were also identified overwhelmingly in LOW apiaries. Interestingly, aquatic and wetland plants (*Alisma*, *Anemone*, *Sium*) were similarly assigned in HIGH and LOW sites. Likewise, weedy plants (*Artemisia*, *Cirsium*, *Euphorbia*) were found at similar time points and abundances regardless of landscape type. Pollen originating from agricultural fields, including soybean, field bean, and radish

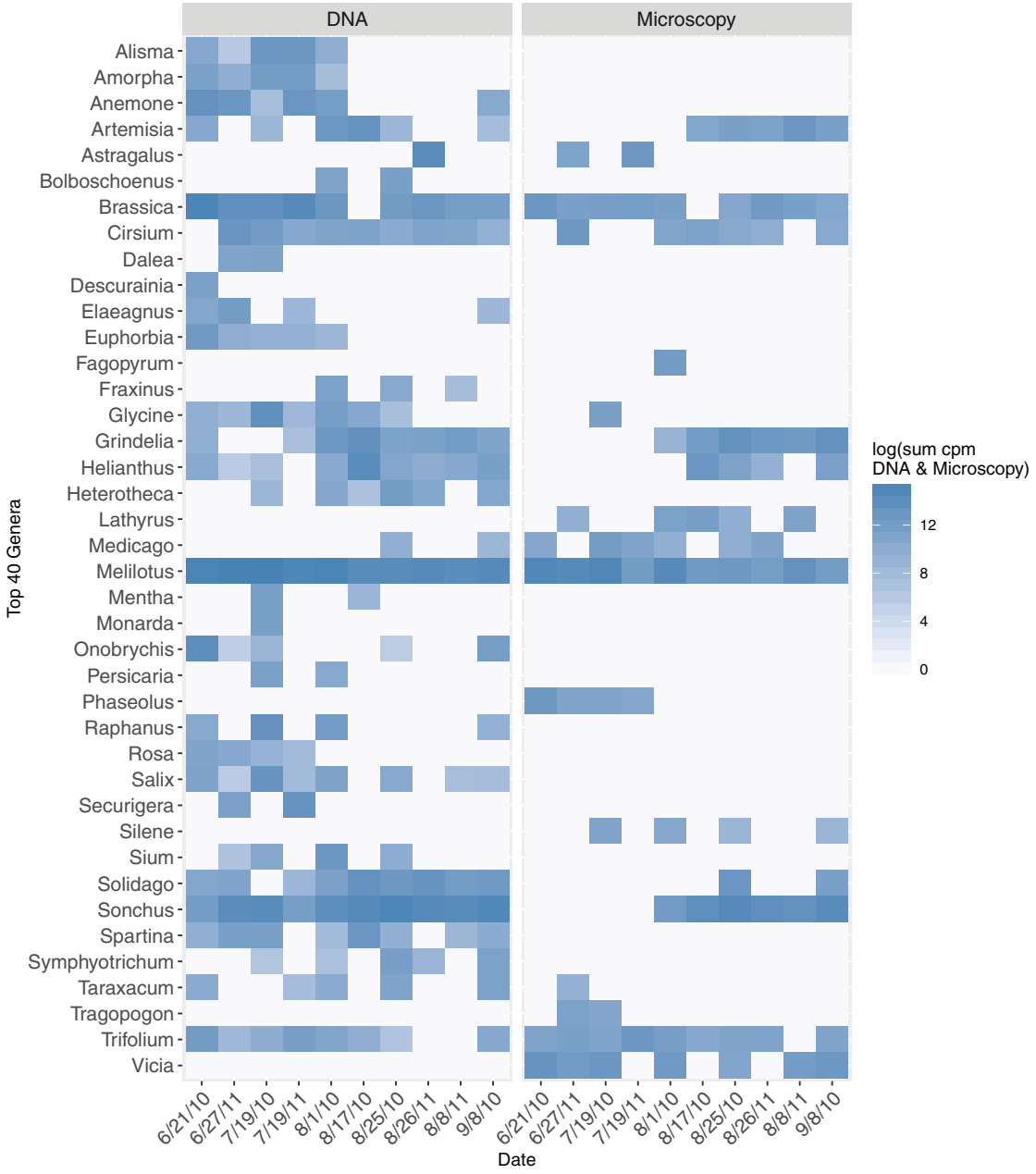

Fig. 3. Forty most commonly assigned genera (log sum cpm among all sample dates) between DNA (DNA) and light microscopy (Micro), 2010–2011.

Table 3. Paired *t*-tests comparing number of taxa between pollen identification techniques

Taxonomic level	*t*-statistic	df	*P*-value	Mean difference
Family	9.24	44	<0.0001	4.18
Genus	11.20	44	<0.0001	7.31
Species	14.13	44	<0.0001	8.16

The mean difference is relative to the number of taxa detected per sample.

(*Glycine*, *Phaseolus*, and *Raphanus*, respectively) tended to be found in LOW apiaries, while the opposite occurred in the case of buckwheat (*Fagopyrum*). Other pollen potentially originating from cultivated fields included the genera *Brassica* (e.g., rapeseed or canola) and *Helianthus* (sunflower), though other species within those two genera occur across the region and were assigned via DNA sequencing, including *B. juncea*, *B. nigra*, *B. oleracea*, *H. occidentalis*, *H. pauciflorus*, and *H. petiolaris*.

Discussion

We present evidence that a DNA sequencing technique utilizing ITS metabarcoding for the taxonomic identification of bee-collected pollen generates comparable, and finer detailed, results when compared to results derived from traditional light microscopy. We detected a high degree of continuity among methods, particularly for commonly occurring taxa (such as *Melilotus*, *Sonchus*, and *Brassica*). This verification is exciting because DNA sequencing required substantially less time and virtually no palynological expertise (though regional, phenological awareness of flora was necessary for

Table 4. Number of genera, diversity, and evenness of pollen samples originating from study apiaries using DNA sequencing and light microscopy techniques, 2010–2011

Site	No. genera	No. samples (DNA and Micro)	Genera per sample	H'	Fisher's α	J
A	48	12	4.00	3.56	28.95	0.92
B	36	14	2.57	3.31	18.63	0.92
C	36	18	2.00	3.22	16.74	0.9
D	54	20	2.70	3.46	28.65	0.87
E	34	16	2.13	3.07	16.40	0.87
F	45	10	4.50	3.58	32.22	0.94
LOW	62	22	2.82	3.34	18.85	0.81
HIGH	69	23	3.00	3.37	22.03	0.8

LOW includes sites A–C, HIGH includes sites D–F.

Table 5. Spearman's rho correlations for taxa (ranked cpm for detected genera) assigned in pollen via DNA sequencing and light microscopy among low and high agricultural intensity surrounding apiaries, 2010–2011

Comparison	Sample date	ρ	*P*-value
LOW vs. HIGH	21 June 2010	−0.24	0.19
LOW vs. HIGH	19 July 2010	0.08	0.60
LOW vs. HIGH	01 Aug. 2010	−0.07	0.65
LOW vs. HIGH	17 Aug. 2010	0.32	0.14
LOW vs. HIGH	25 Aug. 2010	0.44	0.005
LOW vs. HIGH	08 Sept. 2010	0.10	0.53
LOW vs. HIGH	27 June. 2011	0.14	0.45
LOW vs. HIGH	19 July. 2011	0.30	0.14
LOW vs. HIGH	08 Aug. 2011	0.18	0.50
LOW vs. HIGH	26 Aug. 2011	0.25	0.36
LOW vs. HIGH	2010	0.12	0.07
LOW vs. HIGH	2011	0.15	0.16
LOW vs. HIGH	All dates	0.11	0.05

LOW includes sites A–C, HIGH includes sites D–F.

assignment verification) to derive a greater number and finer overall resolution of taxa. Further, DNA sequencing detected taxa that we were not able to discern via light microscopy due to their low abundance in samples.

Because of the relatively few taxa assigned via microscopy compared to sequencing, we pared the data down to just the generic level for comparison (82 genera overall). Even then, we did not observe a significant correlation between techniques. Only when we examined the data from the initial perspective of genera assigned via light microscopy (27 genera) did a relationship emerge, thus demonstrating the overlap in results between the two techniques, but only for the most common, highly abundant taxa (in terms of pollen grains). However, comparing results at a single taxonomic rank is necessarily imperfect because of the hierarchical nature of taxonomy. That is, apparent discordance between methods for particular taxa may be due to relative resolution rather than actual nondetection of a pollen source. For example, four genera in Fig. 1 that were found by microscopy but not detected by sequencing (*Astraglaus*, *Lathyrus*, *Phaseolus*, *Vicia*) are within the family Fabaceae, which was in fact a common assignment by sequencing. It would require additional genetic analysis to confirm whether those four genera were present in the DNA isolates, and to determine whether additional database curation or an alternative barcode locus would aid in their recovery.

DNA sequencing offers powerful insight, and a much more complete picture, of honey bee – plant – land use interactions; and enables quantification of those relationships at a finer temporal resolution. However, sequencing methodology has yet to be employed using controlled proportions of mixtures of various pollens to determine whether the derived results are quantitatively supported. Comparing taxonomic assignments generated from two separate methods provides a way of validating DNA sequencing. However, using multiple methods to validate OTU assignments may not always be feasible, or may be impractical for an exceptionally large number of samples. In this case, plant species distributional and phenological data can be used to assess OTU assignment concordance with historical records and flowering times (Cornman et al. 2015), as noted above. It is also possible to include mock samples of single source or known mixed composition in a sequencing run to assess assignment accuracy and biased abundance.

Our study represents a logical next step to the work that has previously been conducted, and further, takes the methodology a step beyond to consider the influence of land use on the identification of bee-collected pollen. Compared to the proof of concept articles by Richardson et al. (2015a) and Richardson et al. (2015b), wherein only spring-collected pollen samples were quantified, our dataset provides a much broader sampling effort and a replicated design of six apiary sites over the majority of the growing season (June–September) for two years. The results of the two separate Richardson et al. (2015a,b) studies were equivocal, with the rank-based approach of Richardson et al. (2015a) suggesting a high degree of correspondence at the plant family level between sequencing and microscopy from pollen collected over a 6-day period in May, whereas Richardson et al. (2015b) did not find a strong relationship between techniques for a mixture of taxonomic levels (families, genera, and species) for four sample dates in April–May of a single year. Keller et al. (2014) collected pollen from a similarly narrow phenological window of time (~3 wk) in late July–early August in a single year and found abundance estimates were correlated when comparing sequencing to microscopy results.

Future Applications

Our results derived from ITS metabarcoding show promise for addressing ecological questions regarding the impact of land use on pollinator forage, pollinator host–plant interactions, and rapid detection of rare species and noxious weeds across large and multiple spatio-temporal scales. Given the relative ease of collecting pollen from a large number of honey bee colonies throughout multiple growing seasons, genetic identification of bee-collected pollen may also be used for long-term studies examining the effects of climate change on plant species diversity, richness, and phenology (Dunnell and Travers 2011).

Many flowering plant species included in conservation and enhancement seed mixes occur at relatively low spatial and temporal abundances on the landscape. ITS metabarcoding offers the

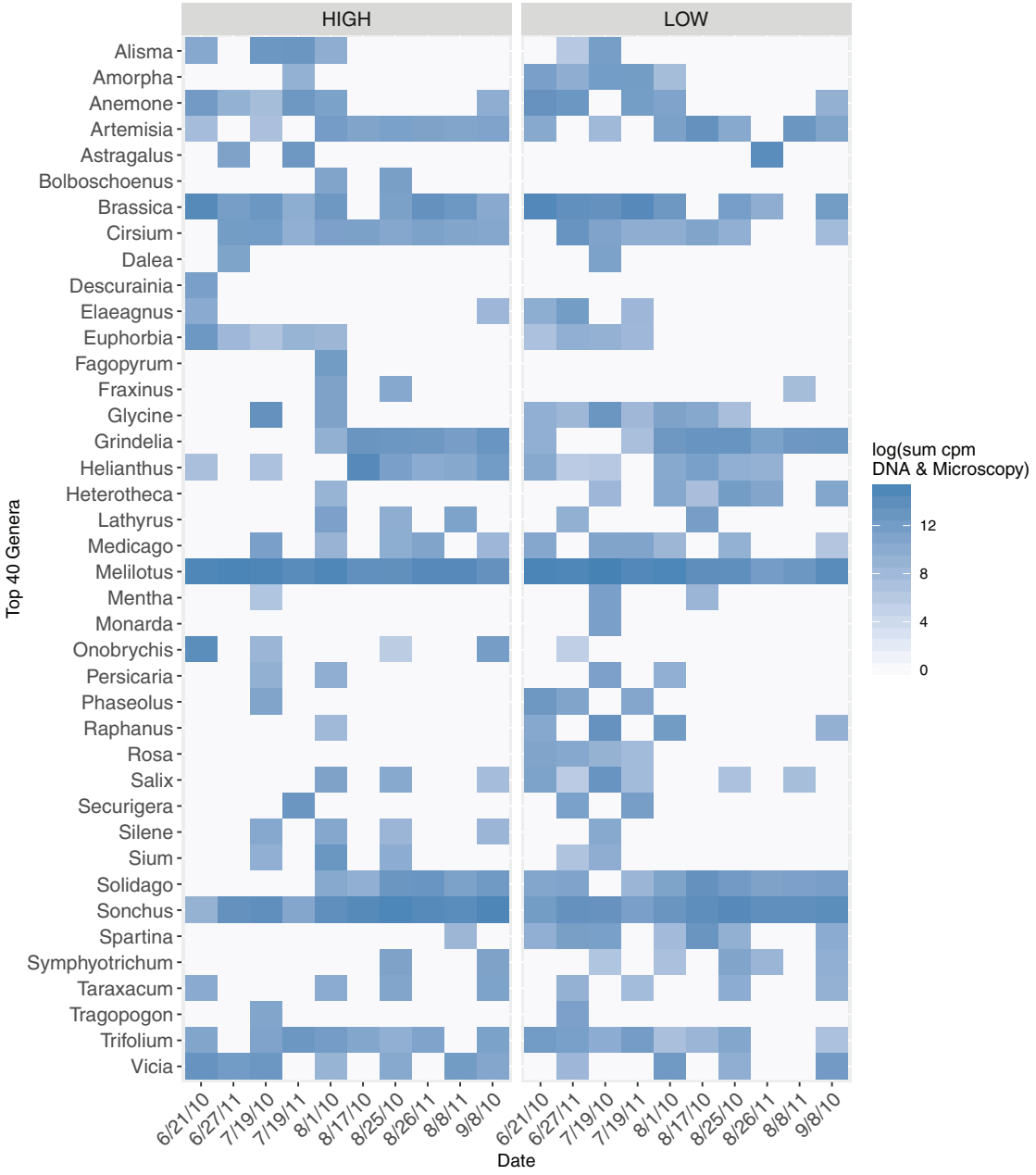

Fig. 4. Top 40 genera identified by date in apiaries surrounded by high and low intensity agriculture. DNA sequencing and light microscopy cpm were summed among all study apiaries on each sample date, 2010–2011. LOW includes sites A–C, HIGH includes sites D–F.

opportunity to quantitatively evaluate the impact of such low abundance species on the diet of honey bees and other wild pollinators located in varying habitats rather than painstakingly observing and documenting floral visitation and usage by individual bees (USGS NPWRC 2016). Managed honey bee colonies serve as an effective model organism to evaluate forage and habitat enhancement programs because they have a broad diet, extensive foraging range, and may be fitted with pollen traps to be open at any time.

Our study highlights the need for research that can differentiate honey bee use versus preference for native and introduced plant species in agricultural landscapes, similar to other studies conducted on native bees (Williams et al. 2011). Such studies would support the development of cost-effective seed mixes for habitat enhancement efforts and aid land management decisions regarding control of introduced plant species on public and private lands. Furthermore, such studies would be useful for identifying potential alternative plant species that would similarly meet the dietary demands of honey bee colonies. Foraging over a wide area, honey bees recruit nestmates to floral resource patches that are relatively large in size and high in quality and density in the surrounding environment (Seeley 1995, Dornhaus et al. 2005), while many wild pollinators operate on a much more local level (Gathmann and Tscharntke 2002). Typical NGP regional bee forage plants include perennial clovers and alfalfa (July–September), canola (early June), sunflower (late July–August), and native and nonnative wildflowers, including introduced species, throughout the summer (Gallant et al. 2014, Smart et al. in 2016a). Notably, *Melilotus* provides wild bees, as well as honey bees, with abundant and nutritious pollen and nectar resources (e.g., Campana and Moeller 1977, Van Riper and Larson 2009), supports native pollinator communities (Larson et al. 2014),

and may increase the abundance of native, wild bees under certain circumstances (Tepedino et al. 2008).

Our ability to identify pollen via light microscopy was a limiting factor and cause of the relatively low number of taxa detected with this technique. However, given the small pool of expert palynologists and palynology classes available, we suggest that our experience here reflects the reality of many researchers using pollen identification as a tool to investigate insect host-plant interactions. The initially greater laboratory costs per sample of barcode sequencing will generally be paid back in reduced human effort and reduced dependency on specialized taxonomic knowledge, and technical reproducibility appears high. Genetic sequencing of bee-collected pollen will present future opportunities for a diverse cohort of scientists to make novel contributions to pollinator research and address global pollinator declines.

Land Use

We found that overall pollen richness and diversity indices were not strongly related to the abundance of quality land use (along a gradient from high to low quality corresponding to sites A–F). While site A (and B and C in 2011), as predicted, did express high taxonomic richness and diversity, so too did sites D and F, and this trend was maintained when variation in sampling effort was considered. Interestingly, site F in particular, maintained persistently high metrics of diversity (though only data from 2010 were available at this site). Greater pollen taxonomic diversity using light microscopy from 2010–2012 was also found previously from site F (Smart et al. 2016a) and could be related to a relatively high amount of private land enrolled in the US Department of Agriculture-Conservation Reserve Program (similar in total area to site A) near the site.

When sites were binned by overall agricultural intensity (LOW vs. HIGH) and ranked abundances of plant genera were considered, differences were clearly distinguished between agricultural intensity among sample dates. Taken together, these results suggest that differences in honey bee colony pollen foraging exist in response to varying intensity of agriculture in general, but these large-scale differences interact with, and may be moderated by, more localized site-specific land-use features (e.g., presence of, and management practices on, conservation lands and wetlands).

The majority of commercially pollinating and honey-producing colonies in the United States are embedded in agricultural lands across the NGP and other parts of the country (USDA 2014b). Beekeepers primarily rely on permission from farmers, ranchers, and other landowners for physically positioning apiaries on the landscape. Therefore, beekeeping and agriculture are irrevocably entwined and agricultural lands, intermixed with uncultivated forage lands, may be some of the most productive areas for honey and other hive products, at least when compared to urban and forested landscapes in some regions (Sponsler and Johnson 2015).

Intensive agricultural practices across the study region may result in highly disturbed soils via tillage, grazing, mowing, etc.; it may be that the dominantly ascribed species such as *Melilotus* spp., *Sonchus* spp., and *Cirsium* spp. possess advantages over native species under such conditions (Boutin and Jobin 1998, Di Tomaso 2000, Larson 2002) and they may be more prevalent on the landscape as a result. Undesirable characteristics distinctly associated with some exotic plant species, and amplified in habitats with highly disturbed soils, may increase their attractiveness to pollinators (Larson et al. 2001, Whitney and Gabler 2008, Van Kleunen et al. 2009). Currently it is unclear whether pollen foraging patterns observed in our study are a result of honey bee preference for introduced plant species or are more related to the availability of these plants in agricultural landscapes. Regardless, volunteer species have been shown to be important targets for foraging honey bees in intensive agroecosystems in the United States and elsewhere (e.g., Bretagnolle and Gaba 2015; Requier et al. 2015, Smart et al. 2016a), thereby demonstrating the need for adequate forage lands for supporting pollinators in the NGP.

Recent evidence suggests that land use and reductions in floral resource availability play a role in honey bee and native bee declines (Gallant et al. 2014, Goulson et al. 2015, Otto et al. 2016, Smart et al. 2016a). In the future, linking quantified pollen identification with metrics of nutritional quality (e.g., pollen protein content) will help to establish the mechanistic relationship between variation in overall landscape quality and honey bee colony health. Such dietary differences may then contribute to differential outcomes in the health, productivity, and survival of honey bee colonies. Related, we have previously shown that the quantity or abundance of pollen available and collected, rather than pollen diversity *per se*, is important for honey bee colony survival (Smart et al. 2016a, b).

Limitations

Ascertainment biases can arise during laboratory processing (e.g., the target DNA is not recovered equimolar across pollen types or fails to amplify with equal efficiency) or during computational inference (uneven resolution or assignment errors). In the laboratory, a particular concern is partial or complete dropout of sequences that are divergent at the targeted priming sites or which have weaker annealing at degenerate positions. Preferential amplification of templates that have higher Tm might also occur. We did find that this latter concern was somewhat negated by our detection of correlated cpm by date of *Melilotus* pollen using both techniques (Fig. 2d), indicating that the dominance of this species at least is not due to amplification bias.

While greater numbers of taxa were discerned via DNA sequencing analysis, this technique is limited by the availability of appropriate sequences for comparison in reference databases. Cornman et al. (2015) tabulated summary similarity statistics for each assigned taxon, in order to identify taxonomic assignments that, while still the best match in the reference database, were likely incorrect and indicative of database gaps within a given phylogenetic lineage. Plant biologists and pollination ecologists should continue to support curated databases (e.g., BOLD and ITS2 databases) and work to supplement and enhance GenBank with additional voucher sequencing, working toward more complete genetic databases.

Database characteristics also have a profound effect on classification success. For example, the second most abundant taxonomic bin of read pairs after *Melilotus* was "unclassified", i.e., much of the DNA present in the sample was not matched to a known taxon. A significant portion of these unclassified read pairs were apparent chimeras (Cornman et al. 2015), the generation of which during PCR is likely promoted by the relatively high number of PCR cycles used and the presence of the highly conserved 5.8S sequence within the amplicon. However, apparent chimeras may also be a computational artifact of nonoverlapping read pairs when the representation of the two regions is taxonomically uneven. A disparity in ITS1 and ITS2 representation within GenBank, partly due to extensive sequencing of ITS2 in recent years (Chen et al. 2010, Sickel et al. 2015), may have contributed to unclassified assignment. These "computational" chimeras (paired sequences that appear to match to two separate taxa because the true taxon is not represented at both regions) may be rescued at a cost of reduced assignment scores

by mapping each read of a pair separately, or by demoting apparent chimeras to the next inclusive taxonomic rank. Alternatively, an ITS2-specific approach may be used (e.g., Chen et al. 2010), which achieves high taxonomic resolution (Sickel et al. 2015) and is likely less susceptible to chimeras.

In conclusion, for those lacking extensive pollen taxonomic knowledge and skill but nevertheless wishing to use pollen identification as a tool to address ecologically pertinent questions, identification via light microscopy may not be a viable option. DNA sequencing and quantification techniques offer a comparable method to quickly sample and analyze data on pollen collected from honey bee colonies. Going forward, ITS metabarcoding will be a valuable tool for addressing pertinent and timely ecological questions regarding pollinator forage abundance and diversity, the nutritional impacts of varying land use, and the influence of climate change on plant–pollinator networks. Our pollinator research efforts in the NGP region will continue to build on the data presented here to examine the nexus of land use, beekeeper apiary selection, bee-foraged plant species, and resulting honey bee colony health conditions.

Acknowledgments

The full dataset used for analysis can be found in Smart and Otto (2016). We thank Dan Cariveau and two anonymous reviewers for their thoughtful contributions. We also thank University of Maryland technician, Andrew Garivito, for color sorting pollen prior to light microscopy identification. This project was funded by USDA-NIFA grant number 2010-65615-20631, USDA-FSA grant number 131AMRECRTYA8, and USDA-NRCS grant number 673A7514178. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

References Cited

Adams, R. J., and M. V. Smith. 1981. Seasonal pollen analysis of nectar from the hive and of extracted honey. J. Apic. Res. 20: 243–248.

Aizen, M. A., and L. D. Harder. 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. 19: 915–918.

Alaux, C., F. Ducloz, D. Crauser, and Y. Le Conte. 2010. Diet effects on honeybee immunocompetence. Biol Lett. 6: 562–656. doi: 10.1098/rsbl.2009.0986.

Alaux, C., C. Dantec, H. Parrinello, and Y. Le Conte. 2011. Nutrigenomics in honey bees: Digital gene expression and analysis of pollen's nutritive effects on healthy and *Varroa*-parasitized bees. BMC Genomics 12: 496. [.CrossRef][10.1098/rsbl.2009.0986]

Boutin, C., and B. Jobin. 1998. Intensity of agricultural practices and effects on adjacent habitats. Ecol. Appl. 8: 544–557.

Bretagnolle, V., and S. Gaba. 2015. Weeds for bees? A review. Agron. Sustain. Dev. 35: 891-909.

Campana, B. J., and F. E. Moeller. 1977. Honey bees: Preference for and nutritive value of pollen from five plant sources. J. Econ. Entomol. 70: 39–41.

Chen, S., H. Yao, J. Han, C. Liu, J. Song, L. Shi, Y. Zhu, X. Ma, T. Gao, X. Pang, et al. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE 5: e8613.

Cornman, R. S., C.R.V. Otto, D. Iwanowicz, and J. S. Pettis. 2015. Taxonomic characterization of honey bee (*Apis mellifera*) pollen foraging based on non-overlapping paired-end sequencing of nuclear ribosomal loci. PLoS ONE 10: e0145365.

Crompton, C. W., and W. A. Wojtas. 1993. Pollen grains of Canadian honey plants. Publication 1892/E. Canada Communication Group, Ottawa, Canada, p. 228.

De Grandi-Hoffman, G., Y. Chen, E. Huang, and M. H. Huang. 2010. The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (*Apis mellifera* L.). J. Insect Physiol. 56: 1184–1191.

Delaplane, K.S., J. van der Steen, and E. Guzman-Novoa. 2013. Standard methods for estimating strength parameters of Apis mellifera colonies. J. Apic. Res. 52: 1-12.

Di Pasquale, G., M. Salignon, Y. LeConte, L. P. Belzunces, A. Decourtye, A. Kretzschmar, S. Suchail, J. C. Brunet, and C. Alaux. 2013. Influence of pollen nutrition on honey bee health: Do pollen quality and diversity matter? PLoS ONE 8: e72016.

Di Tomaso, J. M. 2000. Invasive weeds in rangelands: Species, impacts, and management. Weed Sci. 48: 255–265.

Dornhaus, A., F. Klugl, C. Oechslein, F. Puppe, and L. Chittka. 2005. Benefits of recruitment in honey bees: Effects of ecology and colony size in an individual-based model. Behav. Ecol. 17: 336–344.

Doyle, J. 1991. DNA protocols for plants, pp. 283–293. *In* G. M. Hewitt, A.W.B. Johnston, J.P.W. Yong (eds), Molecular techniques in taxonomy. Springer.

Dunnell, K. L., and S. E. Travers. 2011. Shifts in the flowering phenology of the northern Great Plains: Patterns over 100 years. Am. J. Bot. 98: 935–945.

Erdtman, G.1966. Pollen morphology and plant taxonomy. Angiosperms. Hafner, NewYork, NY, p. 496.

Galimberti, A., F. De Mattia, I. Bruni, D. Scaccabarozzi, A. Sandionigi, M. Barbuto, M. Casiraghi, and M. Labra. 2014. A DNA barcoding approach to characterize pollen collected by honeybees. PLoS ONE 9: e109363.

Gallant, A., N. H. Euliss, and Z. Browning. 2014. Mapping large-area landscape suitability for honey bees to assess the influence of land-use change on suitability of national pollination services. PLoS ONE 9: e99268.

Gathmann, A., and T. Tscharntke. 2002. Foraging ranges of solitary bees. J. Anim. Ecol. 71: 757–764.

Goulson, D., E. Nicholls, C. Botias, and E. L. Rotheray. 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347: Doi: 10.1126/science.1255957.

Green, D. G. 1983. The ecological interpretation of fine resolution pollen records. New Phytol. 94: 459–477.

Huang, Z. 2012. Pollen nutrition affects honey bee stress resistance. Terr. Arthropod Rev. 5: 175–189.

Kapp, R. O., O. K. Davis, and J. E. King. 2007. Guide to pollen and spores, 2nd ed. American Association of Stratigraphic Palynologists. ISBN: 10: 0931871050.

Keller, A., N. Danner, G. Grimmer, M. Ankenbrand, K. von der Ohe, W. van der Ohe, S. Rost, S. Hartel, and I. Steffan-Dewenter. 2014. Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples. Plant Biol. doi: 10.1111/plb.12251, in press.

Kleijn, D., and I. Raemakers. 2008. A retrospective analysis of pollen host plant use by stable and declining bumble bee species. Ecology 89: 1811–1823.

Koh, I., E. V. Lonsdorf, N. M. Williams, C. Brittain, R. Isaacs, J. Gibbs, and T. H. Ricketts. 2016. Modeling the status, trends, and impacts of wild bee abundance in the United States. Proc. Natl. Acad. Sci. USA 113: 140–145.

Kress, W. J., K. J. Wurdack, E. A. Zimmer, L. A. Weigt, and D. H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. USA 102: 8369–8374.

Larson, D. L., P. J. Anderson, and W. Newton. 2001. Alien plant invasion in mixed-grass prairie: Effects of vegetation type and anthropogenic disturbance. Ecol. Appl. 11: 128–141.

Larson, D. L. 2002. Native weeds and exotic plants: Relationships to disturbance in mixed grass prairie. Plant Ecol. 169: 317–333.

Larson, D. L., R. A. Royer, and M. R. Royer. 2006. Insect visitation and pollen deposition in an invaded prairie plant community. Biol. Conserv. 130: 148–159.

Larson, D. L., S. Droege, P. A. Rabie, J. L. Larson, J. Devalez, M. Haar, and M. McDermott- Kubeczko. 2014. Using a network modularity analysis to inform management of a rare endemic plant in the northern Great Plains. USA. J. Appl. Ecol. 51: 1024–1032.

Lym, R.G. 2015. A guide to North Dakota noxious and troublesome weeds. North Dakota State University. Pub. no. W1691.

Mattila, H. R., and G. W. Otis. 2007. Dwindling pollen resources trigger the transition to broodless populations of long-lived honeybees each autumn. Ecol. Entomol. 32: 496–505.

Morefield, P. E., S. D. LeDuc, C. M. Clark, and R. Iovanna. 2016. Grasslands, wetlands, and agriculture: the fate of land expiring from the Conservation Reserve Program in the Midwestern United States. Environ. Res. Lett. 11: 094005.

(NDDA) North Dakota Department of Agriculture 2015. Noxious Weeds. (http://www.nd.gov/ndda/program/noxious-weeds) (accessed 11 August 2015).

Olsen, L. G., R. Hoopingarner, and E. C. Martin. 1979. Pollen preferences of honeybees sited on four cultivated crops. J. Apic. Res. 18: 196–200.

Otto, C.R.V., C. Roth, B. Carlson, and M. D. Smart. 2016. Land use change reduces habitat suitability for supporting managed honey bee colonies in the Northern Great Plains. Proc. Natl. Acad. Sci. USA. 113: 10430–10435.

(PalDat) Palynological Database 2000. An online publication on recent pollen. [Updated 2016 Jun 21]. (https://www.paldat.org/) (accessed 2011-2013).

Pang, X., L. Shi, J. Song, X. Chen, and S. Chen. 2013. Use of the potential DNA barcode ITS2 to identify herbal material. J. Nat. Med. 67: 571–575.

Pernal, S. F., and R. F. Currie. 2000. Pollen quality of fresh and 1-year-old pollen diets for worker honey bees (*Apis mellifera* L.). Apidologie 31: 387–409.

Pollinator Health Task Force 2015. National strategy to promote the health of honey bees and other pollinators. The White House, Washington, DC. (https://www.whitehouse.gov/sites/default/files/microsites/ostp/Pollinator%20Health%20Strategy%202015.pdf) (accessed 3 January 2016).

Requier, F., J. F. Odoux, T. Tamic, N. Moreau, M. Henry, A. Decourtye, and V. Bretagnolle. 2015. Honey bee diet in intensive farmland habitats reveals an unexpectedly high flower richness and a major role of weeds. Ecol. Appl. 25: 881–890.

Richardson, R. T., C. H. Lin, D. B. Sponsler, J. O. Quijia, K. Goodell, and R.,M. Johnson. 2015a. Application of ITS2 metabarcoding to determine the provenance of pollen collected by honey bees in an agroecosystem. Appl. Plant Sci. 3: 1–6.

Richardson, R. T., C. H. Lin, J. O. Quijia, N. S. Riusech, K. Goodell, and R. M. Johnson. 2015b. Rank-based characterization of pollen assemblages collected by honey bees using a multi-locus metabarcoding approach. Appl. Plant Sci. 3: 1500043.

Roman, A. 2006. Effect of pollen load size on the weight of pollen harvested from honeybee colonies (*Apis mellifera* L.). J. Apicul. Sci. 50: 47–57.

Sawyer, R. W. 1975. Melissopalynology in the determination of the geographic and floral origin of honey. J. Assoc. Publ. Anal. 13: 64–71.

Seeley, T. D. 1995. The wisdom of the hive: The social physiology of honey bee colonies. Harvard University Press.

Sickel, W., M.J. Ankenbrand, G. Grimmer, A. Holzschuh, S. Hartel, J. Lanzen, I. Steffan-Dewenter, and A. Keller. 2015. Increased efficiency in identifying mixed pollen samples by meta-barcoding with a dual-indexing approach. BMC Ecol. 15: 1-9.

Smart, M. D., and C. R. V. Otto. 2016. A comparison of honey bee-collected pollen from working agricultural lands using light microscopy and ITS metabarcoding datasets: U.S. Geological Survey Data Release, http://dx.doi.org/10.5066/F7RF5S61.

Smart, M. D., J. S. Pettis, N. Euliss, and M. S. Spivak. 2016a. Land use in the Northern Great Plains region of the US influences the survival and productivity of honey bees. Agric., Ecosyst. Environ. 230: 139–149.

Smart, M., J. Pettis, N. Rice, Z. Browning, and M. Spivak. 2016b. Linking measures of colony and individual honey bee health to survival among apiaries exposed to varying agricultural land use. PLoS ONE 11: e0152685.

Sponsler, D. B., and R. M. Johnson. 2015. Honey bee success predicted by landscape composition in Ohio, USA. PeerJ. 3: e838.

Tepedino, V. J., B. A. Brosi, and T. L. Griswold. 2008. Might flowers of invasive plants increase native bee carrying capacity? Intimations from Capitol Reef National Park, Utah. Nat. Areas J. 28: 44–50.

University of Arizona 2001. Catalog of internet pollen and spore images. [Update 2011Nov]. (http://www.geo.arizona.edu/palynology/polonweb.html) (Accessed 2011–2013).

(USDA) US Department of Agriculture 1984. Agricultural Statistics 1984. U.S. Government Printing Office, Washington, DC.

(USDA NASS) US Department of Agriculture, National Agricultural Statistics Service 1994. Agricultural Statistics 1994. U.S. Government Printing Office, Washington, DC.

(USDA NASS) US Department of Agriculture, National Agricultural Statistics Service 2004a. Agricultural Statistics 2004. U.S. Government Printing Office, Washington, DC.

(USDA NASS) US Department of Agriculture, National Agricultural Statistics Service 2004b. Honey. (http://usda.mannlib.cornell.edu/usda/nass/Hone//2000s/2004/Hone-02-27-2004.pdf)

(USDA NASS) US Department of Agriculture, National Agricultural Statistics Service 2014a. Agricultural Statistics 2014. U.S. Government Printing Office, Washington, DC.

(USDA NASS) US Department of Agriculture, National Agricultural Statistics Service 2014b. Honey. US Department of Agriculture, Washington, DC. (http://usda.mannlib.cornell.edu/usda/current/Hone/Hone-03-22-2016.pdf) (accessed 16 August 2016). ISSN: 1949-1492.

(USDA FSA) US Department of Agriculture, Farm Service Agency 2014. Notice CRP-775. Washington, DC. (https://www.fsa.usda.gov/Internet/FSA_Notice/crp_775.pdf) (accessed 16 August 2016).

(USDA NRCS) US Department of Agriculture, Natural Resources Conservation Service 2016. The PLANTS Database. National Plant Data Team, Greensboro, NC, USA. (http://plants.usda.gov) (accessed 22 June 2016).

(USGS NPWRC) US Geological Survey, Northern Prairie Wildlife Research Center 2016. Pollinator Library. (http://www.npwrc.usgs.gov/pollintor/home) (accessed 16 August 2016).

Vaudo, A. D., J. F. Tooker, C. M. Grozinger, and H. M. Patch. 2015. Bee nutrition and floral resource restoration. Curr. Opin. Insect Sci. 10: 133–141.

Van Kleunen, M., E. Weber, and M. Fischer. 2009. A meta-analysis of trait differences between invasive and non-invasive plant species. Ecol. Lett. 13: 235–245.

Van Riper, L. C., and D. L. Larson. 2009. Role of invasive *Melilotus officinalis* in two native plant communities. Plant Ecol. 200: 129–139.

Wheeler, M. M., and G. E. Robinson. 2014. Diet-dependent gene expression in honey bees: Honey vs. sucrose or high fructose corn syrup. Sci. Rep. doi: 10.1038/srep05726, in press.

Whitney, K. D., and C. A. Gabler. 2008. Rapid evolution in introduced species, 'invasive traits' and recipient communities: Challenges for predicting invasive potential. Diversity Distrib. 14: 569–580.

Williams, N. M., D. Cariveau, R. Winfree, and C. Kremon. 2011. Bees in disturbed habitats use, but do not prefer, alien plants. Basic Appl. Ecol. 12: 332–341.

Wright, C. K., and M. C. Wimberly. 2013. Recent land use change in the Western Corn Belt threatens grasslands and wetlands. Proc. Natl. Acad. Sci. USA. 110: 4134–4139.

Yao, H., J. Song, C. Liu, K. Luo, J. Han, Y. Li, X. Pang, H. Xu, Y. Zhu, P. Xiao, et al. 2010. Use of the ITS2 region as the universal DNA barcode for plants and animals. PLoS ONE 5: e13102.