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Metabarcoding Analysis of Pollen Species Foraged by *Osmia excavata* Alfken (Hymenoptera: Megachilidae) in China

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To meet the pollination need of economic crops, *Osmia excavata* has been successfully used to improve the pollination efficiency of Rosaceae and Brassicaceae plants. As a widely used pollinator of economic crops, a systematic study of flower-visiting species and diversities of *O. excavata* stocked in China was not found. To investigate the foraging pollen species and diversities of *O. excavata*, beebread from 20 experimental plots in China was collected by the trap-nesting method and analyzed by DNA metabarcoding technology. A total of 26 pollen plants in 14 genera and nine families were identified. A further analysis showed that the richness and abundance of the wild flowering plants in orchards and farmlands were lower than those in the nearby semi-natural habitats. The favorite pollen comes from economic crops apple and rape and wild flowering plants *Juncus interior*, *Rosa gymnocarpa*, and *Rosa laevigata*. Through a diversity index analysis, it was found that the Anhui region has the highest pollen plant diversity, while the Liaoning region has the lowest. Our results can provide a basis for flower-visiting species and diversities of *O. excavata*.

Keywords: *Osmia excavata*, metabarcoding method, pollen plants, diversity, species conservation

INTRODUCTION

Pollinators are of great importance in ecosystems and contribute about $136 billion annually to agricultural systems in China (Ouyang et al., 2019). Due to the intensification and expansion of agriculture, the diversity of pollinators has been declining rapidly for decades, which has led to the loss, fragmentation, and degradation of pollinator habitats and a severe decline in global pollination (Foley et al., 2011; Féon et al., 2013; Belsky and Joshi, 2019). In the past 50 years, the dependence of global agriculture on pollinators has increased by more than 300%, while the abundance of pollinators worldwide has continued to decline (Potts et al., 2016). Previous studies have shown that planting of pollen plants in pollinator habitats can significantly increase the number and species of pollinators (Jönsson et al., 2015; M'Gonigle et al., 2016; Warzecha et al., 2018; Williams and Lonsdorf, 2018; Nichols et al., 2019). Therefore, it is necessary to clarify how to choose the best plants for different pollinators.

The internal transcribed spacer II (*ITS2*) barcode based on metabarcoding method can identify pollen plant species from mixed pollen and shows great potential in the analysis of beebread ingredients (Pornon et al., 2016; De-Vere et al., 2017; Lucas et al., 2018). Compared with traditional palynology, the molecular method not only has a higher sensitivity for the identification of mixed pollen but also effectively reduces time and cost (Lang et al., 2018; Tremblay et al., 2019). The *ITS2* region has proved to be a core barcode for pollen identification (Richardson et al., 2015; Gous et al., 2019; Tremblay et al., 2019). The barcode combination of *rbcL*, *trnH-psbA*, and *ITS2* successfully identified the main pollen plants, *Vitex negundo* and *Vitex quinata*, in the beebread of *Megachile strupigera* by He et al. (2020). Therefore, DNA metabarcode not only helps to identify species and determine the relative abundance of pollen plants in beebread but is also used to construct pollination networks, monitor plant pathogens, assess species diversity, and predict biodiversity patterns (Huang et al., 2020).

To meet the pollination demand of economic crops, pollinators with commercial potential have been found and developed (Ryder et al., 2020). Bumblebees have been successfully used as pollinators for eggplant, tomato, peach, and pear (Wu et al., 2006). *Megachile rotundata* was used for the pollination of alfalfa (Xu et al., 2009). In orchards, the bee of genus *Osmia* was considered as efficient pollinators of some Rosaceae plants, such as apricot, plum, cherry, peach, pear, and apple (Parker, 1981; Torchio et al., 1987; Wei et al., 1991; Xu et al., 1994). Importantly, *Osmia* bee can be artificially stocked due to its advantages such as easy management, low cost, and high pollination efficiency (Wei and Zhao, 1995; Wei et al., 2000b; Dai, 2004). In north and northwest of China, there are mainly five species of *Osmia* (*O. excavata*, *O. cornifrons*, *O. taurus*, *O. jacoti*, and *O. pedicornis*) that play important roles in enhancing pollination, increasing fruit diameter and the number of seeds per fruit, and decreasing the percentage of asymmetrical fruit (Wei et al., 2002). For *O. excavata*, it has many advantages, such as low-temperature tolerance, long daily activity time, high flower-visiting efficiency, centralized pollination range, and easy management (Wei and Yuan, 1997). Therefore, it was considered as an excellent and large-scale stocking pollinator. The pollination of *O. excavata* can create more than $27.8, $9.4, and $3.0 billion for cherries, apples, and rapes planting, respectively, each year (Liu et al., 2018, 2019a,b). Pollination can significantly improve the fruit set rate and fruit quality, and it has broad application prospects in the future of fruit planting industry (He and Zhou, 2009).

However, the short flowering period of fruit trees limits the nutritional requirements of *O. excavata* during the foraging period (Yan et al., 2018). Because of this limitation, survival and large-scale stocking of *O. excavata* face great challenges. A previous study showed that the diversity of pollen plants is the main factor affecting the diversity of wild bee species (Kovács-Hostyánszki et al., 2017). Therefore, the effective solution is to grow other pollen plants in the orchard. This will not only improve the biodiversity of plantations but also provide adequate food sources for bees (Boyle et al., 2020). So, it is necessary to understand the foraging preferences of bees. In this study, beebread of *O. excavata* was collected by trap-nesting from 20 experimental plots in China. The species and abundance of plants were determined by *ITS2* barcode based on metabarcoding method, and the dominant plants that meet the nutrition needs of bees were further analyzed.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Osmia excavata can build a nest in trap nests made by reed tubes and make beebread to breed its offspring (Wang et al., 2001; Huang et al., 2013). According to the main activity period (March–April) of *O. excavata*, trap nests were placed in similar habitats (e.g., flower composition, competitors, and predators, etc.) in each experimental plot. Trap nests of *O. excavata* were collected from 20 regions of China (March–May 2020), covering three types of land: orchards (monoculture or mixed-species plantation), farmlands, and semi-natural habitats (SNHs; agroforestry ecotone). Detailed geographic information of each sampling plot and surrounding main plant types are listed in **Table 1**. In the laboratory, mixed pollens within five groups of beebread from the trap nests of each site were stored at −80°C for later use. Mixed pollens were extracted using the E.Z.N.A.® Soil DNA Kit according to the manufacturer's protocol (Omega Bio-Tek, Norcross, GA, United States). The concentration and purity of purified DNA were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., United States).

PCR Amplification and Sequencing

The *ITS2* region was amplified using the forward primer ITSS2F: 5′ATGCGATACTTGGTGTGAAT3′ and the reverse primer ITSS4R: 5′TCCTCCGCTTATTGATATGC 3′. PCR reactions were performed in a 20-μl system, containing 4 μl FastPfu Buffer (5×), 2 μl dNTPs (2.5 mM/each), 0.8 μl each primer (5 μM), 0.4 μl FastPfu Polymerase (5 U/μl), 2 μl template DNA (5 ng/μl), and 10 μl ddH_2O. All reactions were performed in an ABI GeneAmp® 9700 (Applied Biosystems, Carlsbad, CA, United States) with the following parameters: an initial denaturation at 95°C for 5 min, followed by 29 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States). The DNA concentrations were measured with a Qubit® 3.0 fluorometer (Life Invitrogen, Waltham, MA, United States) and then paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) at the Biozeron Biological Technology Co. Ltd. (Shanghai, China).

Data Analysis

Raw sequencing data were filtered to delete the tail-base of reads ($Q < 20$) using Trimmomatic v0.38 (Bolger et al., 2014). The chimeric sequences were removed and effective sequences clustered into operational taxonomic units (OTUs; ≥97% similarity) by Usearch v10 (Edgar, 2013). Based on the Unite database v8.2,[1] the OTU representative sequences were analyzed taxonomically to obtain the community composition of each sample using the RDP classifier Bayes algorithm with default parameters (Wang et al., 2007).

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[1] http://unite.ut.ee/index.php

TABLE 1 | Geographic information of pollen plants in 20 experimental plots.

Sample	Location	Regions	Coordinates (Lat./Lon.)	Altitude (m)	Main types of plants	Main type of land use
H1	Chengguan, Huaiyuan	Anhui	32.947/117.221	64	Pomegranate	Farmlands
H2	Tangji, Huaiyuan	Anhui	32.866/117.221	24	Pomegranate	Farmlands
H3	Liugusi, Cangzhou	Hebei	38.606/116.273	7	Apple and pear	Mixed orchards
H4	Zishi, Jingzhou	Hebei	30.183/112.423	35	Rape	Farmlands
H5	Lianjiang, Ganzhou	Jiangxi	26.345/115.327	174	Mixed field[1]	SNHs
H6	Wuyun, Ganzhou	Jiangxi	25.993/114.828	142	Mixed field[1]	SNHs
H7	Liantang, Nanchang	Jiangxi	28.504/115.958	25	Rape	Farmlands
H8	Sanrunbao, Dalian	Liaoning	38.940/121.170	30	Multispecies of cherry	Mixed orchards
H9	Manduhu, Shenyang	Liaoning	41.567/122.629	15	Multispecies of apple	Mixed orchards
H10	Niuxintuo, Shenyang	Liaoning	41.571/122.524	13	Apple	Monoculture orchard
H11	Tangwang, Jinan	Shandong	36.819/117.276	26	Apple and pear	Mixed orchards
H12	Cuiping, Qixia	Shandong	37.279/120.844	174	Apple	Monoculture orchard
H13	Guandao, Qixia	Shandong	37.132/120.670	94	Apple	Monoculture orchard
H14	Taocun, Qixia	Shandong	37.179/121.193	90	Apple	Monoculture orchard
H15	Pingyao, Jinzhong	Shanxi	37.282/112.306	780	Apple and pear	Mixed orchards
H16	Nanan, Wenshui	Shanxi	37.492/112.229	754	Apple and pear	Mixed orchards
H17	Qunbake, Luntai	Xinjiang	41.968/84.155	1,498	Multispecies of pear	Mixed orchards
H18	Yiganqi, Aksu	Xinjiang	41.144/80.263	1,097	Multispecies of apple	Mixed orchards
H19	Towankebashi, Aksu	Xinjiang	41.034/80.353	1,081	Multispecies of apple	Mixed orchards
H20	Mongolian	Xinjiang	41.727/86.023	921	Pear	Monoculture orchard

[1] Mixed field, wild plants, and small-scale economic plants (pear tree and rape; planting area: 2 km^2) were planted. SNHs, semi-natural habitats.

Alpha diversity indexes (Shannon, Simpson, and Chao1) were calculated using Mothur v1.30 (Schloss et al., 2009). The Fisher's least significant difference (LSD) in SPSS v25.0 (SPSS, Inc., Chicago, IL, United States) was used to analyze the differences of diversities of beebread between different land types and experimental plots. Species accumulation curves (SACs), the abundance, and composition of beebread were prepared and visualized by the "ggplot2" package (Hadley, 2016) and the "pheatmap" package (Raivo, 2019).

RESULTS

Sequencing and Assembly

A total of 4,563,930 clean *ITS2* sequences from all experimental plots were obtained with an average length of 335 ± 9 bp and clustered into 64 OTUs (**Supplementary Table 1**). A statistical analysis showed that the SACs tend to be flat, indicating that the sample sizes reach the conditions that species are fully discovered. The species richness in the environment should not increase significantly with the increase of sample sizes (**Supplementary Figure 1**).

Species and Abundance Analysis of Pollen Plants

Based on OTU representative sequences, a total of nine families (14 genera and 26 species) were identified from all samples combined (**Supplementary Table 1**). The clustering results of plant species showed that the rape and apple pollens were rich in four samples (H1, H2, H4, and H7) and 11 samples (H3, H9–H16, H18, and H19), respectively. Wild plant pollens were dominated in samples H2 (*Juncus interior* and *Papaver rhoeas*), H5 (*J. interior*, *Rosa gymnocarpa*, *Rosa laevigata*, and *Rosa primula*), H6 (*R. gymnocarpa*, *R. laevigata*, and *R. primula*), H7 (*J. interior*), and H16 (*Prinsepia sinensis*; **Figure 1** and **Supplementary Table 2**). A further analysis revealed that plant species in beebread was dominated by economic crops, including apple (*Malus domestica*, *Malus pumila*, and *Malus* sp.), pear (*Pyrus betulifolia*, *Pyrus communis*, *Pyrus cordata*, and *Pyrus spinosa*), plum (*Prunus brachypoda*), cherry (*Cerasus avium*), and rape (*Brassica juncea* and *Brassica* sp.). In addition, nine types of wild plants were detected in beebread. Among them, the abundance of *J. interior*, *R. gymnocarpa*, and *R. laevigata* was higher than others (**Figure 1** and **Supplementary Table 2**).

Impacts of Different Types of Land on the Composition of Beebread

The composition and abundance of pollen in beebread from different types of land use were compared (**Table 1**). The results showed that economic crop pollens mainly came from the single- or mixed-fruit plantations and intensive farmland, and wild plant pollens mainly came from the agroforestry ecotone (**Figure 2A**). Pollen plants in beebread from semi-natural habitat (SNH; agroforestry ecotone) samples have the highest species abundance (4.7 ± 0.95, $n = 10$), then followed by that from mixed plantations (3.33 ± 0.76, $n = 45$), farmlands (3.05 ± 0.94, $n = 20$), and single-fruit plantations (2.08 ± 0.56, $n = 25$; **Figure 2B**). A further analysis found that the species abundance of pollen plants in beebread from farmlands and mixed-fruit plantations did not differ significantly ($p = 0.36$), but there were significant differences between other experimental plots ($p < 0.05$; **Supplementary Table 3**).

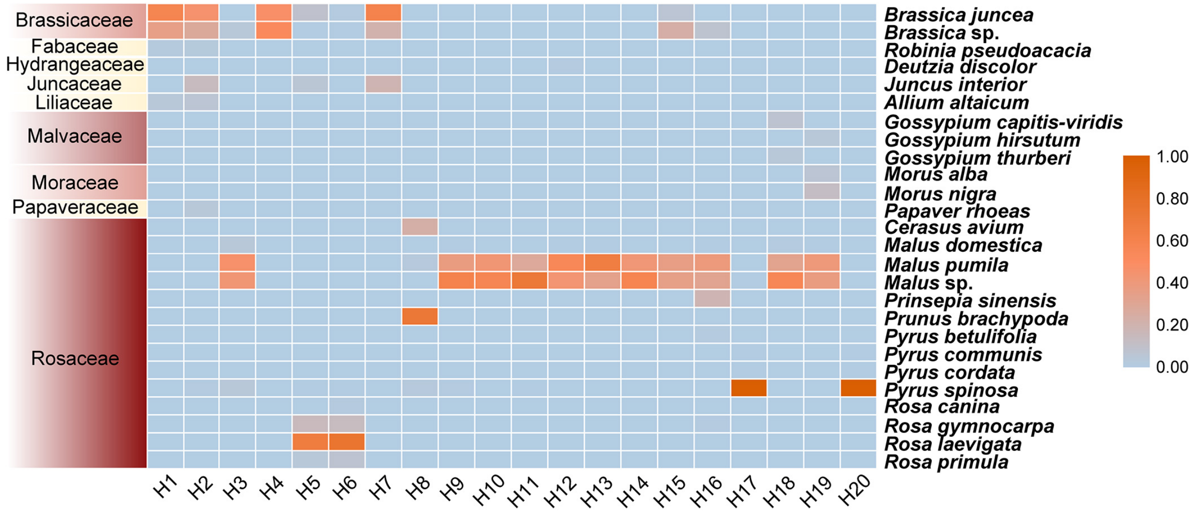

FIGURE 1 | Composition and relative abundance of pollen plants. The left and right of the *Y*-axis and the *X*-axis represent the family and species of pollen plants and sampling plot, respectively. The legend in the upper right represents the proportional abundance of pollen plants.

Diversity of Pollen Plants in Beebread

To better understand the richness and diversity of pollen plants in beebread, 20 experimental plots were divided into seven regions according to the province where *O. excavata* is attracted (**Table 1**). The results showed that the Chao1 index of Anhui (9.40 ± 2.72, $n = 10$) is the highest, then followed by that of Jiangxi (7.15 ± 2.11, $n = 20$), Hebei (5.60 ± 2.97, $n = 5$), Shanxi (5.40 ± 1.07, $n = 10$), Xinjiang (4.80 ± 0.89, $n = 20$), Shandong (2.55 ± 1.10, $n = 20$), and Liaoning (2.33 ± 0.98, $n = 15$; **Table 2** and **Supplementary Table 4**). The Shannon index of Anhui (1.82 ± 0.24, $n = 10$), Shanxi (1.37 ± 0.14, $n = 10$), Jiangxi (1.29 ± 0.38, $n = 20$), and Hebei (1.06 ± 0.31, $n = 5$) is significantly higher than that of Xinjiang (0.74 ± 0.45, $n = 20$), Shandong (0.67 ± 0.10, $n = 20$), and Liaoning (0.62 ± 0.18, $n = 15$; all $p < 0.05$), which indicated that the diversity of pollen plants in Anhui, Shanxi, Jiangxi, and Hebei is high (**Table 2** and **Supplementary Table 4**). A large Chao1 and Shannon indexes or a small Simpson index can explain the high species richness and diversity in beebread. Therefore, it can be concluded that the abundance of pollen plants in the beebread from Anhui was significantly higher than that in other regions. In general, the richness and diversity of pollen plants in different regions can be divided into three levels from high to low: Anhui → Hebei, Shanxi, and Jiangxi → Xinjiang, Shandong, and Liaoning (**Table 2**).

DISCUSSION

Wild bees are important pollinators for economic crops and wild plants. They rely on different landscapes to obtain flowering resources and nesting habitats (Kratschmer et al., 2019). In this study, 20 experimental plots in China were set up to collect the beebread of *O. excavata*. A total of 26 pollen plant species were identified, belonging to 14 genera of nine families and including 17 economic crops and nine wild plant species (*Robinia pseudoacacia*, *Deutzia discolor*, *J. interior*, *P. rhoeas*, *P. sinensis*, *Rosa canina*, *R. gymnocarpa*, *R. laevigata*, and *R. primula*). Based on the composition and relative abundance of pollens (**Figures 1**, **2**), *O. excavata* is an effective pollinator for economic crops (*B. juncea*, *Brassica* sp., *M. pumila*, *Malus* sp., *P. brachypoda*, and *P. spinosa*).

Previous studies indicated that the larvae of *O. excavata* released at the early flowering stages of economic crops will grow into effective pollinators, especially Rosaceae and Brassicaceae plants (Yang et al., 1997; Wei et al., 2000a; Hawkins et al., 2015; Gresty et al., 2018). However, the flowering period (7–15 days; **Supplementary Table 5**) (Lech et al., 2008; Liu et al., 2008, 2010; Wang et al., 2012; Lu et al., 2013; Lin et al., 2019; Zhang et al., 2019, 2020; Li et al., 2020) of most economic crops is short and cannot cover the entire adult stage of *O. excavata* (40–55 days; Wei et al., 2002; Men et al., 2018). Through the observation of professional beekeepers and our field work on the stocking of *O. excavata*, when *O. excavata* had no diverse food sources except economic crops, this gap seriously affects the bees to reserve beebread for their offspring, which causes a low recovery rate after artificial stocking. The number of reduced populations needs to be supplemented every year, excluding the situation of unnatural death, human activities, and population migration.

Although when the beebread from H5/H6 experimental plots were collected, the economic crops (pear and rape trees) in the plantation were in the late flowering stage, which led to the lack of pollen resources, *O. excavata* could still obtain pollens from wild plants (*J. interior*, *R. gymnocarpa*, *R. laevigata*, and *R. primula*; **Figure 1**). This means that in order to reduce the impact of the lack of pollen resources of economic crops, *O. excavata* can use the pollen of wild plants as their main source of nutrition for meeting survival needs. Plantations (orchards and farmlands) were dominated by a single type of economic crops and lack of flowering resources of wild plants (**Figure 2A**). A further analysis revealed that more wild plants in beebread were from SHNs (**Figure 2A**). Papanikolaou et al. (2017) found that increasing

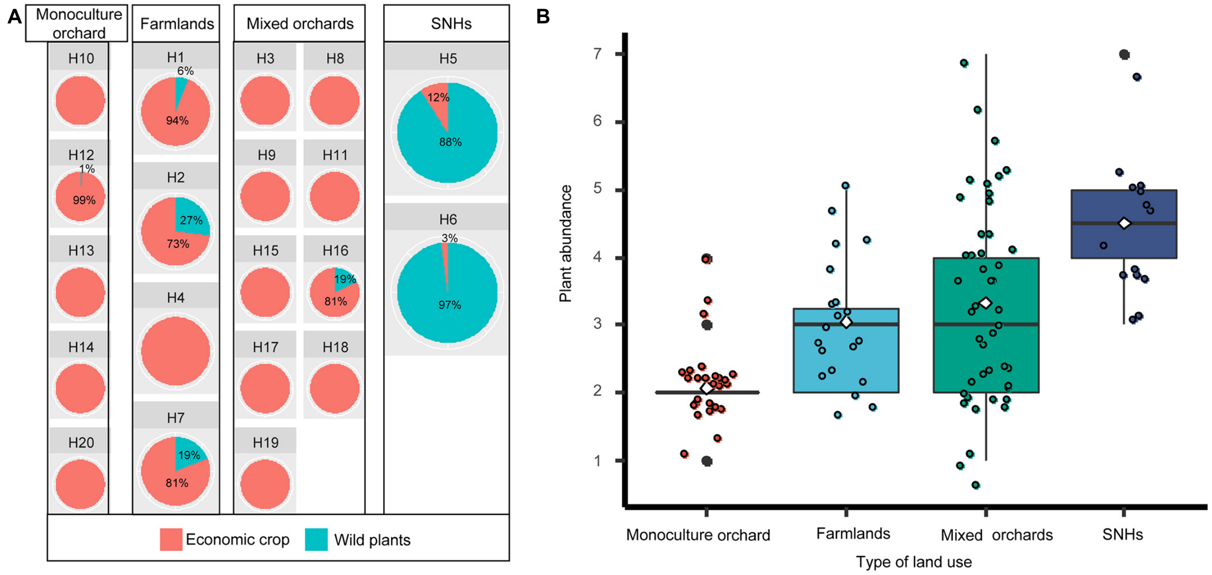

FIGURE 2 | The composition **(A)** and abundance **(B)** of beebread in different types of land use. **(A)** The proportion of economic crops and wild plants in each experimental plot. **(B)** Plant abundance under different land-use types. The *X*-axis and *Y*-axis represent land-use types and plant abundance, respectively.

the richness of plants in the habitat would significantly affect the abundance and diversity of bees. When the pollen resources of economic crops cannot meet the needs of the entire adult stage of *O. excavata*, wild flowering plants can be planted in plantations or introduced into SNHs. The transplanting of wild flowering plants into the plantation helps to attract *O. excavata* and increase food sources. Ideal type of wild flowering plants can be selected from nine wild plants identified in beebread, especially *J. interior*, *R. laevigata*, and *R. gymnocarpa*.

Piko et al. (2021) concluded that the mixed flower field model can effectively provide attractive food resources, thereby greatly increasing the abundance and diversity of bumblebee. Bihaly et al. (2020) also emphasized that planting a variety of flowering herbaceous plants on a small scale could significantly increase the number of bee nests in the orchard. In response to concerns about the food source of the *O. excavata*, combing the previous results, a conjecture is proposed: a mixed flower field model suitable for *O. excavata* (MFF-OE model). We can plant a variety of favorite wild flowering plants around the plantation to meet the needs of pollen resources during the entire adult stage of *O. excavata*. However, the model needs to be further explored in terms of variety allocation, optimum planting area selection, and planting scale, etc. The comprehensive analysis of Chao1 and Shannon indexes reflected lower species richness and diversity in Liaoning. The Liaoning region can be used as a pilot for the exploration and implementation of the MFF-OE model. Taken together, we look forward that the implementation of the MFF-OE model can effectively solve the shortage of pollen resources of economic crops.

TABLE 2 | Alpha diversity indexes of pollen plants.

Region	Chao1 index[1]	Shannon index[1]	Simpson index[1]
Anhui	9.40 ± 2.72	1.82 ± 0.24	0.16 ± 0.04
Jiangxi	7.15 ± 2.11	1.29 ± 0.38	0.38 ± 0.17
Hebei	5.60 ± 2.97	1.06 ± 0.31	0.20 ± 0.23
Shanxi	5.40 ± 1.07	1.37 ± 0.14	0.28 ± 0.04
Xinjiang	4.80 ± 0.89	0.74 ± 0.45	0.62 ± 0.26
Shandong	2.55 ± 1.10	0.67 ± 0.10	0.54 ± 0.07
Liaoning	2.33 ± 0.98	0.62 ± 0.18	0.55 ± 0.14

[1] *Index data format refer to "mean ± SD."*

CONCLUSION

Osmia excavata has been successfully used for pollination in commercial orchards because it can significantly improve fruit setting rate and fruit quality. In this study, beebread of *O. excavata* was collected from various habitats by trap-nesting and analyzed by DNA metabarcoding technology. A total 26 pollen plants in 14 genera and nine families were identified, including 17 economic crops and nine wild plant species. The abundances of wild flowering plants in the plantation were lower than those in SNHs. According to the composition and abundance of pollen plants, *O. excavata* is regarded as an effective pollinator for economic crops in Rosaceae and Brassicaceae from the molecular level.

DATA AVAILABILITY STATEMENT

The sequence data that support the findings of this study are openly available in GenBank of NCBI at https://www.ncbi.nlm.nih.gov/. The associated BioProject, SRA, and

BioSample numbers are PRJNA742556, SRR15011441, and SAMN19967841, respectively.

ETHICS STATEMENT

All experiments procedures for this study complied with the current animal ethics guidelines and did not involve any protected animals.

AUTHOR CONTRIBUTIONS

AL and DH were responsible for the conceptualization and supervision of the study. HL, FD, and YH were responsible for the methodology and writing – original draft preparation. HL, FD, YL, KZ, and HZ were responsible for the software. ZZ, CZ, AL, and DH performed the validation. HL and FD conducted the formal analysis and visualization. FD, YL, KZ, HZ, and DH conducted the investigation. FD and DH were responsible for the resources. HL, FD, YL, KZ, HZ, and DH were responsible for data curation. YH, ZZ, CZ, AL, and DH were responsible for writing – review and editing. DH was responsible for project administration. HL, ZZ, AL, and DH were responsible for funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.730549/full#supplementary-material

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