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RESEARCH ARTICLE - BEES

Diversity of Plants Foraged by *Apis cerana* Fabricius Around Qinling Mountains (Central China) Based on Honey Pollen Samples

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Abstract
To illustrate the essential role of *Apis cerana* Fabricius in plant pollination, a survey was conducted using PCR, DNA barcoding, and high-throughput sequencing technologies to assess the diversity of plants foraged by *A. cerana* at five sites around Qinling Mountains. A fragment of the *rbcL* gene in pollen isolated from honey collected at each site was amplified and sequenced. The results show that samples BJ and TC, samples FP and AK have a high correlation, respectively. According to the information of a total of 64 plant species identified in the five sites, belonging to 53 genera, 37 families, and 23 orders, it is found that there is a relatively high diversity and complex composition of nectar and pollen plants and that most of the plants are sporadically available. The identified species show higher occurrence in some orders, such as Sapindales, Rosales, etc. There are significantly more plants of temperate origin than those of tropical origin, 58.5% and 34.0%, respectively. Some species of Rosales or Sapindales are more visited by *A. cerana*, with *Toxicodendron vernicifluum* (Stokes) being the dominant species. These results provide a basis for assessing the role of *A. cerana* in plant pollination and maintaining biodiversity.

Introduction

Pollinators are essential for the reproductive success of plants and play an important role in maintaining plant communities (Blitzer et al., 2016; Katumo et al., 2022). As one of the most important pollinators in China, the native bee species *Apis cerana* Fabricius (Hymenoptera: Apidae) has developed characteristics adapted to mountain environments during its evolutionary history and has a significant advantage in exploiting sporadic nectar plants (Yang, 2005; Radloff et al., 2010; Zhang et al., 2019). In addition to providing abundant bee products, *A. cerana* has long been pollinating numerous native crops and wild plants in China. Due to the influence of environmental factors and the introduction of *Apis mellifera* L., the population of *A. cerana* has declined dramatically over the past decade (Chen et al., 2017; Liu et al., 2018a). However, the impact of declining *A. cerana* populations on the reproduction and survival of various plants pollinated primarily by *A. cerana* remains unclear. By studying the composition and distribution characteristics of plants foraged by *A. cerana,* we can understand the potential impact of the decline of *A. cerana* on plant diversity.

DNA barcoding and high-throughput sequencing technologies can be used to quickly and accurately identify species (von Cräutlein et al., 2011; Kennedy et al., 2020; Chen et al., 2021), which has opened up the opportunity to identify plant species from a wide variety of samples such as pollen and honey (Lang et al., 2018; Nagarajan et al., 2020). To a certain extent, these new technologies can break through the bottlenecks caused by the lack of expertise in pollen identification in previous studies.

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We analyzed the pollen composition of honey samples collected around the Qinling area in this study to clarify the diversity and compositional characteristics of the nectar and pollen plants foraged by *A. cerana*. The results will provide a theoretical basis for understanding the importance of *A. cerana* in the pollination process of some plants and for understanding the coevolutionary relationships between pollinating insects and nectar and pollen plants, thus providing a basis for *A. cerana* conservation.

Materials and methods

Study area

As the dividing line between China's northern and southern climates and the biogeography of the Oriental and Palaearctic, Qinling Mountains are one of the most popular areas for biodiversity research. Protecting its ecological environment has attracted the attention of governments and scientific research institutions. In Shaanxi Province, the Qinling area is located between 105.50°-110.08° E and 32.67°-34.58° N, and its altitude range is 195-3771.2 m. The climatic factors of the Qinling Mountains vary significantly with the increase in altitude, and the vegetation shows a well-defined vertical zonality. From bottom to top, there are different vegetation types, such as evergreen broad-leaved forest (only on the southern slope), deciduous broad-leaved forest, coniferous and broad-leaved mixed forest, coniferous forest and subalpine meadow (Zhao et al., 2023). Five sites, including Foping (FP), Ankang (AK), Baoji (BJ), Mianxian (MX), and Tongchuan (TC), were selected around this area for collecting samples of comb honey (Table 1, Fig 1). Overall, of the five sites, FP, AK and MX on the southern slope of the Qinling Mountains are located in the subtropical monsoon climate zone. In contrast, BJ is located in the temperate monsoon climate zone on the northern slope of the Qinling Mountains, and TC is farther north. The vegetation at the five sites is mainly a deciduous broad-leaved forest with abundant woody and herbaceous plants (He et al., 2022).

Table 1. Geographic information on the source locations of honey samples in the Qiling area.

Sample	Longitude & Latitude of Collecting Sites		Average Elevation (m)
	E°	N°	
FP	107.92	33.61	2100
AK	109.47	32.98	920
BJ	107.04	34.18	1500
MX	106.69	33.30	1800
TC	109.06	35.42	1395

Sample processing and high-throughput sequencing

The samples of comb honey were provided by beekeepers. We collected comb honey from multiple hives at one site in the autumn of 2020, consistent with the regular collection season, and mixed it as a sample, with three replicates for each sample. We obtained the sample solution by weighing 50 g of comb honey from each sample and adding 100 mL of distilled water. Then, we added 10 mL of sample solution to the centrifugal tube and centrifuged for 10 min at 3000 rpm. After removing the supernatant, we added distilled water and centrifuged a second time. We discarded the upper liquid and kept the bottom residue to obtain a pollen sample.

Fig 1. A map of sampling sites around the Qinling Mountains area in the Shaanxi Province of China.

We used E.Z.N.A™ Mag-Bind Soil DNA Kit to extract pollen DNA. We determined the appropriate amount of DNA for the PCR reaction based on the accurate quantitative results of genomic DNA obtained with a Qubit3.0 DNA detection kit. The primers used for PCR amplification of the target fragment (*rbcL*) were F (CTTACCAGYCTTGATCGTTACAAAGG) and R (GTAAAATCAAGTCCAACCRCG) (Erickson et al., 2017; Liu et al., 2018b).

Sangon Biotech (Shanghai) Co., Ltd completed constructing the library of samples and high-throughput sequencing.

Data processing

We clustered the resulting sequences to obtain representative sequences of OTUs (operational taxonomic units) (Edgar, 2013). Based on the number of sequences in each OTU, correlation coefficients and P-values between samples were calculated using SparCC. We drew a sample correlation heatmap using the R language gplots package. The sequences of OTUs were compared to the data in GenBank via BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to screen the best alignment results and obtain the related plant species. We classified plants and analyzed floristic characteristics based on the Scientific Database of China Plant Species (http://db.kib.ac.cn/), the Subject Database of China Plant (http://www.plant.csdb.cn/), and Wu (2010).

Analysis of evolutionary relationships of nectar and pollen plants

Phylogenetic and molecular evolutionary analysis based on the partial sequence of *rbcL* gene was conducted using MEGA11 (Tamura et al., 2021). The resulting evolutionary relationships between plants were compared with APG IV (THE ANGIOSPERM PHYLOGENY GROUP, 2016).

Results

OTUs and species composition of pollen in honey samples

We obtained a total of 103 groups of OTUs. At the OTUs level, the correlations among samples are shown in Fig 2. The samples BJ and TC are clustered into one branch with a high correlation. Similarly, FP and AK are clustered into a highly correlated branch. There is also some correlation between BJ and FP. The correlation between samples is, to some extent, related to the sampling location.

According to the alignment results, 64 species of nectar and pollen plants foraged by *A. cerana* around the Qinling area were identified (including 16 species that were identified to the correct genera only), belonging to 23 orders, 37 families, and 53 genera (Table 2).

Of all the samples, the largest number of plant species identified was in sample MX, with 41 species, while only ten were identified in sample AK. The identified species are mainly concentrated in several orders, with ten species belonging to the order Sapindales, six to Rosales, six to Poales, and six to Ranunculales.

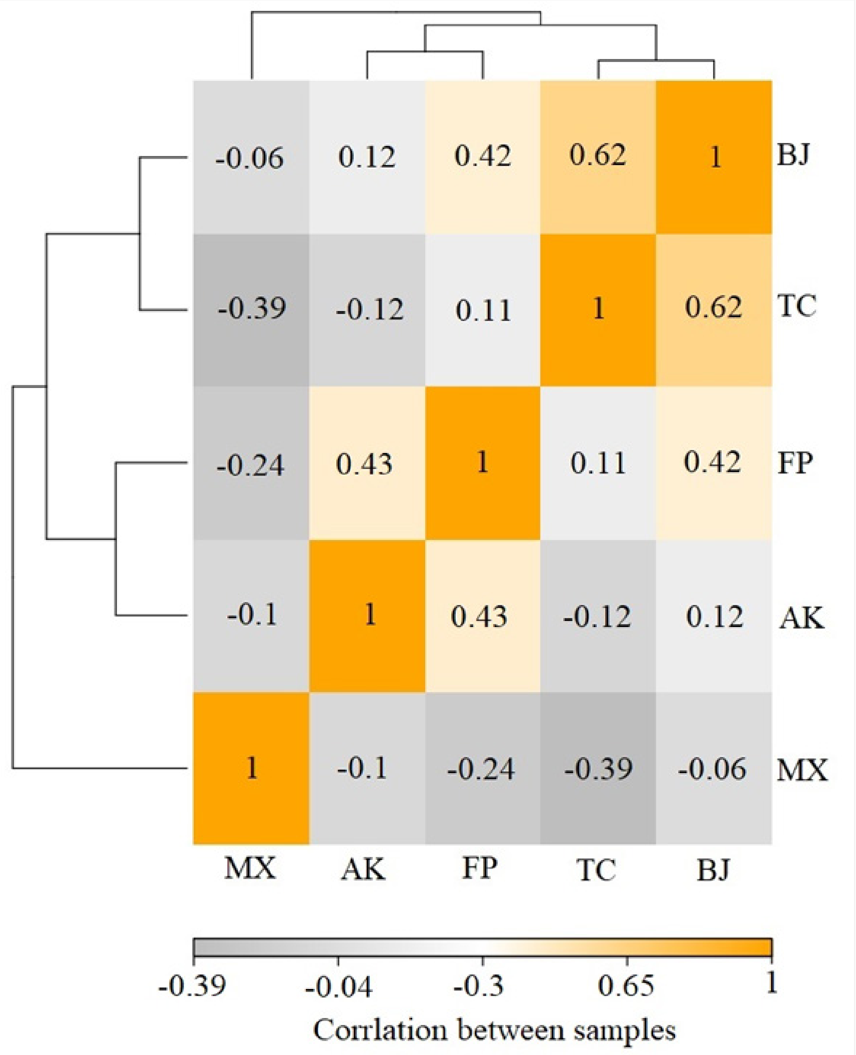

Fig 2. Heatmap of samples correlation at the OTUs level. The colored blocks indicate the values of the correlation coefficients.

A total of 21 species co-occurred in three or more samples, representing 32.8% of the total, including five species belonging to Rosales. Among them, four species or 6.3% of total species, including *Bothrocaryum controversum* (Hemsl.) (order Cornales), *Actinidia arguta* (Sieb. et Zucc.) (order Ericales), *Quercus phillyraeoides* A. Gray (order Fagales) and *Toxicodendron vernicifluum* (Stokes) (order Sapindales) appeared in all samples. A total of 27 species were only present in one sample.

Among the plants visited by *A. cerana*, there are 25 species of herbs and 39 species of woody plants (including trees, shrubs, woody vines, etc.). Except for a few solitary flowers (such as *Citrullus lanatus* (Thunb.), *Codonopsis pilosula* (Franch.)), the flowers of most other plants form inflorescences, including spikes (such as *Elsholtzia ciliata* (Thunb.)), racemes (such as *Saccharum spontaneum* L.), spadices (such as *Amorphophallus konjac* K. Koch), umbels (such as *Spiraea blumei* G. Don) and panicles (such as *Thalictrum minus* L.). We did not find an obvious preference for inflorescence selection in the present study.

The floristic characteristics of nectar and pollen plants foraged by *A. cerana* are complex, as shown in Tables 2 and 3.

Table 2. Composition characteristics of nectar and pollen plants around the Qinling area.

No.	Order	Family	Species	Sample					Areal-types of genera
				FP	AK	BJ	TC	MX	
1	Alismatales	Araceae	*Amorphophallus konjac*	+	+	-	-	-	Tropical Asia to Topical Africa
2	Apiales	Apiaceae	*Heracleum yungningense*	+	-	+	-	-	North Temperate
3		Araliaceae	*Aralia continentalis*	+	-	+	+	+	East Asia and North America disjunct
4			*Panax sp.*	-	-	-	-	+	East Asia and North America disjunct
5	Asterales	Asteraceae	*Artemisia selengensis*	-	-	-	-	+	North Temperate
6			*Helianthus annuus*	+	-	-	-	-	East Asia and North America disjunct
7		Campanulaceae	*Codonopsis pilosula*	-	-	-	-	+	East Asia
8	Caryophyllales	Polygonaceae	*Rumex japonicus*	-	-	-	-	+	Cosmopolitan
9	Celastrales	Celastraceae	*Celastrus orbiculatus*	+	+	+	+	-	Pantropic
10			*Celastrus sp.*	-	-	-	+	-	
11	Cornales	Cornaceae	*Bothrocaryum controversum*	+	+	+	+	+	East Asia and North America disjunct
12	Cucurbitales	Cucurbitaceae	*Citrullus lanatus*	+	-	-	-	+	Tropical Asia to Topical Africa
13	Dipsacales	Caprifoliaceae	*Lonicera maackii*	-	-	+	+	+	North Temperate
14	Ericales	Actinidiaceae	*Actinidia arguta*	+	+	+	+	+	East Asia
15			*Actinidia sp.*	+	-	-	-	-	
16		Ebenaceae	*Diospyros lotus*	+	-	-	+	-	Pantropic
17		Theaceae	*Camellia sinensis*	-	-	-	+	-	Tropical Asia
18	Fabales	Fabaceae	*Glycine max*	-	-	-	-	+	Tropical Asia to Topical Africa
19			*Phaseolus vulgaris*	-	-	-	-	+	Pantropic
20			*Robinia pseudoacacia*	+	-	+	+	-	East Asia and North America disjunct
21			*Senna occidentalis*	+	-	+	+	-	Pantropic
22	Fagales	Fagaceae	*Quercus phillyreoides*	+	+	+	+	+	North Temperate
23			*Quercus sp.*	+	-	+	-	+	
24			*Quercus sp.*	+	-	-	-	+	
25			*Lithocarpus sp.*	+	-	+	-	+	East Asia and North America disjunct
26	Garryales	Eucommiaceae	*Eucommia ulmoides*	+	+	-	-	-	Endemic to China
27	Gentianales	Rubiaceae	*Paederia scandens*	+	-	-	-	-	Tropical Asia

Table 2. Composition characteristics of nectar and pollen plants around the Qinling area. (Continuation)

No.	Order	Family	Species	Sample					Areal-types of genera
				FP	AK	BJ	TC	MX	
28	Lamiales	Lamiaceae	*Elsholtzia ciliata*	-	-	-	-	+	Old World Temperate
29	Malpighiales	Euphorbiaceae	*Discocleidion rufescens*	-	-	-	-	+	East Asia (SJ)
30	Malvales	Malvaceae	*Tilia amurensis*	+	-	+	-	-	North Temperate
31	Poales	Poaceae	*Brachypodium sylvaticum*	-	-	-	-	+	North Temperate
32			*Hordeum vulgare*	+	+	+	-	+	North Temperate
33			*Oryza sativa*	+	+	-	-	+	Pantropic
34			*Panicum sp.*	-	-	-	-	+	Cosmopolitan
35			*Saccharum spontaneum*	+	-	-	-	+	Pantropic
36			*Zea mays*	+	-	-	-	+	Tropical Asia and Tropical America disjunct
37	Ranunculales	Berberidaceae	*Epimedium brevicornu*	-	-	-	+	-	Old World Temperate
38			*Epimedium sp.*	-	-	-	+	-	
39		Lardizabalaceae	*Akebia trifoliata*	-	-	+	-	-	East Asia
40		Papaveraceae	*Dicranostigma leptopodum*	-	-	-	+	-	East Asia
41		Ranunculaceae	*Clematis heracleifolia*	-	-	+	+	-	Cosmopolitan
42			*Thalictrum minus*	-	+	+	-	+	North Temperate
43	Rosales	Cannabaceae	*Humulus scandens*	-	-	+	-	+	North Temperate
44		Moraceae	*Broussonetia papyrifera*	-	-	-	+	-	Tropical Asia
45		Rhamnaceae	*Berchemia sp.*	+	-	+	-	+	East Asia and North America disjunct
46			*Rhamnella franguloides*	+	-	+	+	+	Tropical Asia and Tropical Australasia
47		Rosaceae	*Rosa hugonis*	-	-	+	+	+	North Temperate
48			*Sorbus setschwanensis*	-	-	+	+	+	North Temperate
49			*Spiraea blumei*	+	-	+	+	-	North Temperate
50			*Spiraea sp.*	-	-	+	+	-	
51	Sapindales	Anacardiaceae	*Toxicodendron vernicifluum*	+	+	+	+	+	East Asia and North America disjunct
52			*Toxicodendron sp.*	-	-	-	+	+	
53		Rutaceae	*Tetradium daniellii*	-	-	+	-	+	Old World Tropic
54			*Tetradium sp.*	-	-	-	-	+	
55			*Tetradium sp.*	-	-	-	-	+	
56			*Zanthoxylum sp.*	-	-	-	-	+	Pantropic
57			*Zanthoxylum sp.*	-	-	-	-	+	
58			*Zanthoxylum sp.*	-	-	-	-	+	
59		Sapindaceae	*Acer henryi*	+	-	+	+	+	North Temperate
60		Simaroubaceae	*Ailanthus altissima*	+	-	+	+	+	Tropical Asia and Tropical Australasia
61	Saxifragales	Crassulaceae	*Phedimus aizoon*	+	-	-	-	-	North Temperate
62		Paeoniaceae	*Paeonia lactiflora*	-	-	-	+	-	North Temperate
63	Solanales	Solanaceae	*Capsicum annuum*	+	-	-	-	+	Tropical Asia and Tropical America disjunct
64	Vitales	Vitaceae	*Vitis vinifera*	+	-	-	-	+	North Temperate

"+" means found in the sample, "-" means not found in the sample

There are three Cosmopolitan (Type 1) genera, including *Rumex*, *Panicum,* and *Clematis*, and 18 genera of tropical distribution types (Type 2-Type 7), of which the genus belonging to Pantropic (Type 2) is the most, 31 genera of temperate distribution types (Type 8-Type 14) of which the genus belonging to North Temperature (Type 8) is the most. One genus (*Eucommia*) classifies as Endemic to China (Type 15). Based on the floristic components of the genera, the plants belonging to types of temperate distribution significantly outnumber those of tropical distribution, accounting for 58.5% and 34.0%, respectively.

Analysis of the dominant species visited by A. cerana

The most abundant species in different samples are *Q. phillyraeoides* (sample FP), *T. vernicifluum* (samples AK, BJ, and MX), and *Celastrus orbiculatus* Thunb. (sample TC), respectively (Fig 3). *T. vernicifluum* is the dominant species visited by *A. cerana*. The distribution characteristics of *A. cerana* and its preferred nectar and pollen plant, *T. vernicifluum,* based on the data from GBIF (GBIF Secretariat, 2021) are shown in Fig 4. The main distribution areas of the two species are overlapping in eastern Asia.

Table 3. Areal-types of genera of nectar and pollen plants around the Qinling area.

Types	Number of genera	Percentage
1. Cosmopolitan	3	5.7%
2. Pantropic	7	13.2%
3. Tropical Asia and Tropical America disjunct	2	3.8%
4. Old World Tropic	1	1.9%
5. Tropical Asia and Tropical Australasia	2	3.8%
6. Tropical Asia to Topical Africa	3	5.7%
7. Tropical Asia	3	5.7%
8. North Temperate	16	30.2%
9. East Asia and North America disjunct	8	15.1%
10. Old World Temperate	2	3.8%
11. Temperate Asia	0	0.0%
12. Mediterranean, West Asia to Central Asia	0	0.0%
13. Central Asia	0	0.0%
14. East Asia	5	9.4%
15. Endemic to China	1	1.9%
Total	53	100%

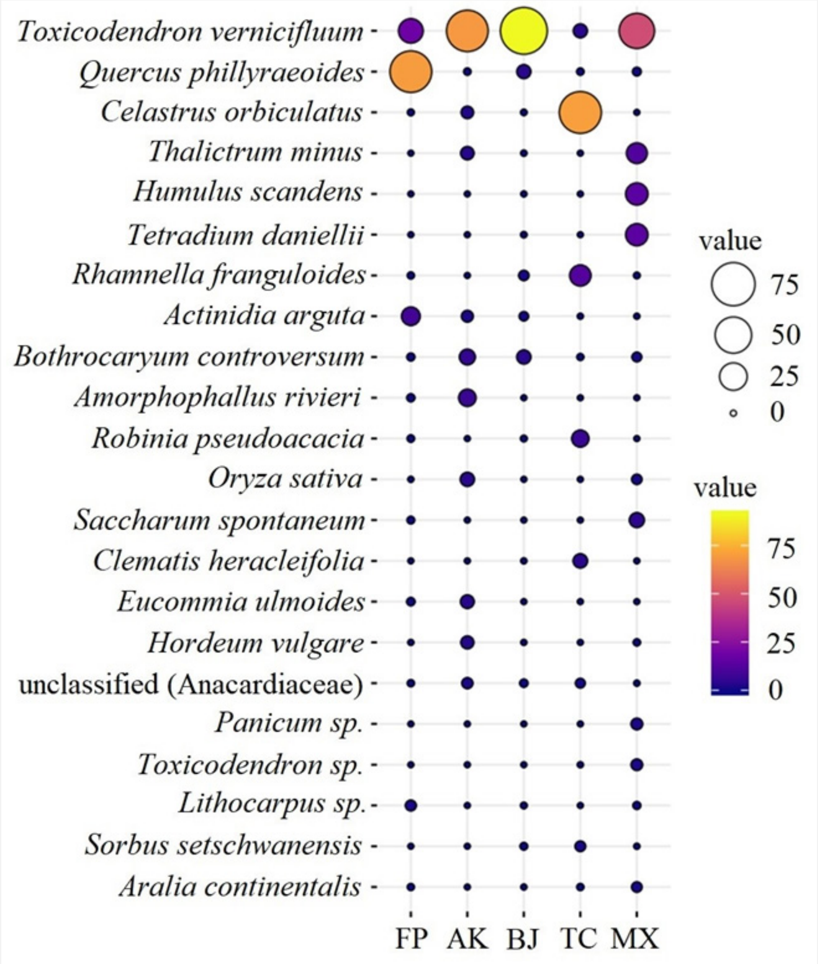

Fig 3. Bubble chart showing dominant species and their abundance in different samples. The bubble size and color indicate the value of species abundance.

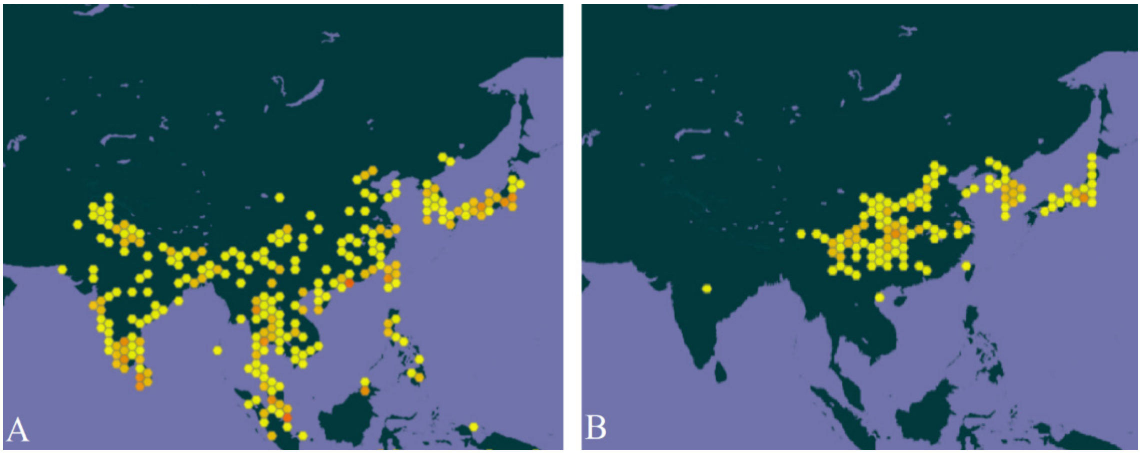

Fig 4. Distribution regions of *A. cerana* (A) and *T. vernicifluum* (B). The dots represent areas where species have been recorded, with darker colors indicating more records.

Evolutionary relationships among species of nectar and pollen plants around the Qinling area

The phylogenetic relationships among nectar and pollen plants around the Qinling area are shown in Fig 5. Different groups, such as rosids and asterids or eudicots and monocots, can be clearly distinguished. For the plants found in three or more samples, they are concentrated in rosids, the major clade of core eudicots, such as species of Rosales or Sapindales. *A. cerana* has some preference in selecting these plants, suggesting close coevolutionary relationships between these plants and *A. cerana.*

Discussion

The Qinling Mountains are the dividing line of China's North-South climate, with significant plant biodiversity differences between the north and the south. The correlation between the samples reflects the difference in the composition of nectar and pollen plants between the northern (samples BJ and TC) and southern (samples FP, AK, and MX) Qinling Mountains.

There are abundant nectar and pollen plants around the Qinling area, and the native bee, *A. cerana,* is an essential pollinator in this area. Studying pollen composition in the honey of *A. cerana* would help explain the diversity of nectar and pollen plants visited by *A. cerana* and then help us understand the role of *A. cerana* in maintaining biodiversity. According to the traditional beekeeping practices in China, the honey of *A. cerana* is generally collected from the hive only once a year. Hence, its honey has a long accumulation time and can contain pollen from plants that bloom in different seasons. The plants identified in the honey samples in this experiment have different flowering periods as early as March and as late as November, suggesting that *A. cerana* has a long-lasting foraging period and the ability to adapt to lower temperatures.

The studies on the pollen composition in honey from this area have shown a relatively high species diversity of honeybee foraging plants. Using palynology, Sang and Xu (2021) studied nectar woody plants in Qinling Mountains. They identified 20 species of nectar woody plants foraged by *A. cerana*, among which *T. vernicifluum*, *C. orbiculatus*, *A. arguta*, etc., were also found in our results. Our results show that the composition of nectar and pollen plants is very complex, including wild plants or cultivated crops, herbs or woody plants, and dicotyledons or monocotyledons.

Most of these plants can provide honey and pollen, but some are pure pollen plants, such as the corn *Zea mays* L. Most of the plants visited by *A. cerana* are sporadic nectar and pollen plants, except for a few species, such as *Robinia pseudoacacia* L., cultivated in large areas. *A. cerana* is excellent at collecting sporadic nectar and pollen plants, even in colder seasons or environments (Tan et al., 2012), so it is especially suitable for beekeeping in mountainous areas with scattered nectar sources. This study found no obvious preference for *A. cerana* in selecting plant characters and flowering dates. The effect of flower characteristics and pollen morphology on the selection of nectar and pollen plants for *A. cerana* is not involved in this paper and needs further investigation.

The construction of a phylogenetic tree based on the nucleic acid sequences of nectar and pollen plants not only provides a certain basis for the determination of some species or genera according to the sequencing results but also provides some information for understanding the evolutionary relationships of nectar and pollen plants and the selection preference of *A. cerana*. Based on the results, we assume a close coevolutionary relationship between *A. cerana* and plants of rosids.

DNA barcoding and high-throughput sequencing can quickly identify nectar and pollen plants in honey. Using these technologies, researchers analyzed the diversity of plants visited by pollinating bees, such as *Nomia chalybeate* Smith, *Megachile strupigera* Cockerell, and *A. mellifera* in different regions (He et al., 2020; Jones et al., 2021; Zhang et al., 2022), providing new ideas for the identification of nectar and pollen plants.

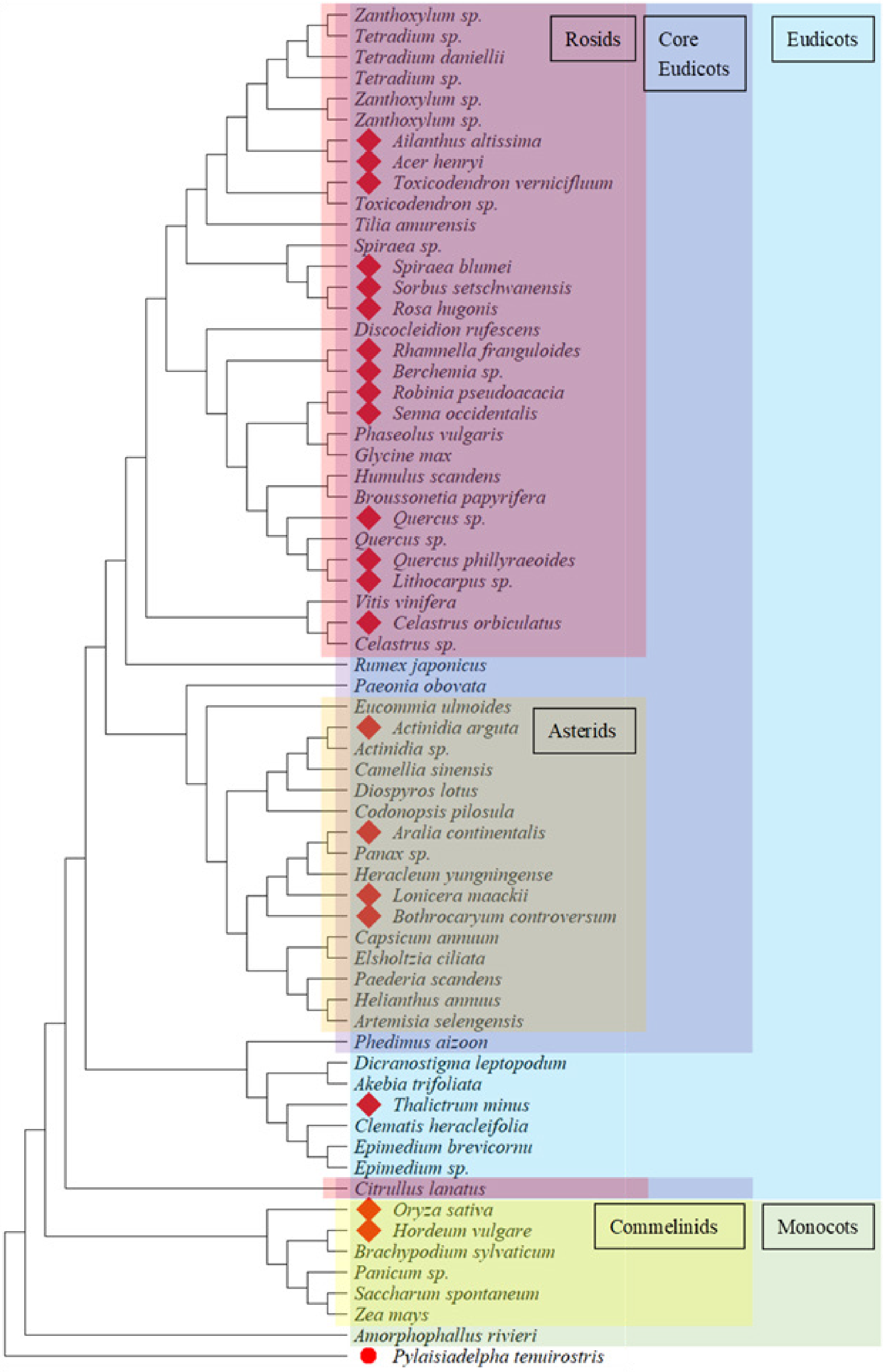

Fig 5. Evolutionary relationships among species of nectar and pollen plants based on partial *rbcL* sequences. ◆ means species found in 3 or more samples, and ● means outgroup.

The *rbcL* gene is a commonly used barcode DNA for identifying and confirming plant species (von Cräutlein et al., 2011; Erickson et al., 2017; Liu et al., 2018b; Basak et al., 2019). However, high-throughput sequencing currently requires relatively short nucleic acid fragments, making it somewhat difficult to identify species based on these short fragments. The identification process would be more efficient, and the results would be more accurate if a longer sequence could be used in high-throughput sequencing in this study. At the same time, the identification results are considerably affected by the data in the searched sequence database. During the BLAST search, it was discovered that some query sequences might match sequences from various plants, some of which are even divided into different genera in the database. The reason for this may be that the species information of the relevant sequences recorded in the database is wrong, or the traditional classification of these plants is flawed, with the same species being identified as different, or the selected target fragment is inappropriate and does not provide sufficient nucleic acid information to distinguish closely related species. Combining the distribution regions of the corresponding species and the evolutionary relationships obtained based on the sequences will help to identify related species. Some sequences that could not be matched to appropriate species or genera in the database were not further analyzed. On this basis, the direct identification of pollen by palynological methods would help to confirm the species of nectar and pollen plants. Still, more expertise in pollen morphological classification is needed.

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Authors' Contribution

Xinjun Guo: conceptualization, methodology, writing-original draft, writing-review and editing.
Saiting Wang: investigation, formal analysis, writing-review & editing.
Jing Feng: investigation, formal analysis, writing-review & editing.

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