Determination of an effective pollinator for the rare and endangered *Urophysa rochkii* and the effects of its floral organs on flower visiting by insects

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Pollination is an important part of fertilization and reproduction in seed plants. Pollen movement largely restricts gene flow among individual plants and the manner of reproduction in a population, thereby affecting the genetic traits and fitness of plant offspring. For insect-pollinator-based plants, the flower-visiting characteristics, frequency and timing of the pollinating insects have decisive effects on the successful pollination of plants, whereas the colour and morphology of various floral components are important factors affecting the pollinating insects. Based on the pollination biology of plants, we studied the pollination mechanisms and links of *Urophysa rochkii* to understand its endangered status. Using *U. rochkii* at the full flowering period for the study, and using methods to observe, record, and perform experimental analysis, we recorded the species, frequency and flower-visiting time of these insects every 15 min and observed the effects of weather and temperature on the insects. Additionally, we captured the flower-visiting insects, brought them to the laboratory, and took photographs under a dissecting electron microscope for observation and identification. *U. rochkii* plants were grouped under different treatments that included removal of sepals, stamens and or pistils, while untreated plants were included as control group. In summary, the effects of floral components on the pollination process were examined. The experimental results showed that the effective pollinator of *U. rochkii* was *Apis cerana* Fabricius, and that sky-blue sepal was the most important factor affecting the flower-visiting frequency of the pollinators.

Keywords: Pollination biology, flower-visiting insects, floral organs, *Urophysa rochkii*.

POLLINATION is a fundamental and important process in plant breeding systems and ecosystem functioning[1]. It also plays an important role in plant diversity[2]. Most plants have bisexual flowers, with the peculiarities of flower types increasing the output of pollen grains (stamens) and its reception (stigma)[3]. Approximately 90% of flowering plants are insect pollinator-based[4]. In addition to achieving pollination by taking advantage of animals, plants can also use water and wind for pollen transport during pollination[5–7]. For insect pollinator-based plants, the behaviour of the pollinators and the plant breeding system are influenced by multiple plant characteristics[8,9], including flower morphological characteristics and biological phenological characteristics[10], self-incompatibility and inflorescence structure[11]. The floral features not only promote pollination efficiency but also limit the flower-visiting frequency of ineffective flower-visiting insects[12], thereby avoiding 'wastage' of pollen grains[13]. For example, in *Syringa oblata* Lindl, the white sepals and blue corolla form a strong contrast, which plays a role in attracting pollinators[14].

The flowering biological characteristics, plant flower number and flower arrangement pattern affect breeding systems[8,11]. For example, while an increase in flower number in plants would enhance the attraction of pollinators, it may also result in more self-pollination in the same plant[10,15]. The establishment of a new environment for a plant population may lead to significant changes in its floral characteristics and mating system, especially if pollinators are scarce[16]. For endangered plants in fragmented habitats, a small number of individuals within a population is not conducive to effectively attract pollinators, and the fragmented habitat may also affect the survival of pollinators per se. Therefore, the interaction between plants and pollinators may be blocked[17,18], and the resultant pollen limitation may further affect the reproductive success of the plants[19]. With changes in human habitat and climate, the stability of pollination systems is being threatened[20,21], the number of plant populations and pollinators is decreasing[22], and the seasonal mismatch between plants and pollinators is increasing[23–25]. Under natural conditions, due to the effects of climatic and environmental factors, pollinating insects for some plants have low flower-visiting frequency or carry insufficient pollen, but these insects could increase their

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pollination efficiency through other mechanisms. For example, in a field study, Herrera *et al.*[26] observed that although insects visited *Helleborus foetidus* L. (Ranunculaceae) at a very low frequency, its seed-setting rate was not affected by prolonging its flowering period to increase pollination efficiency.

To date, studies on *Urophysa rochkii* have been limited to its genetic diversity[27,28], seed distribution[29], soil physico-chemical properties and biological characteristics of communities as well as to biological and ecological aspects, such as the community characteristics of its habitat[30]. However, no studies have been made on its productive ecology, such as that observed with the pollination ecology of *U. rochkii*. In this study, we explored whether the *U. rochkii* flower can effectively attract enough pollinating insects and the effect of each floral organ on attracting flower visitors. To develop reasonable protective measures and provide adequate and reasonable theoretical bases, we addressed the following two issues: (1) Which insects are the main pollinators in the flowering period of *U. rochkii* and which of them are most effective? (2) What are the main factors that attract insects in the pollination process?

Materials and methods

Research background

Urophysa rochkii Ulbr. is a plant species belonging to the genus *Urophysa* family Ranunculaceae. It is an endemic species, with only a small distribution in the upper reaches of the Fujiang River in Jiangyou City, Sichuan Province, China. In 1925, an American plant collector, J. F. Rock, found this species in Jiangyou, for the first time and collected a sample. In 1929, Ulbrich[31] published a paper on this species (Figure 1).

Here, Xiangjiagou, Yongsheng Township was selected as the study site (Figure 2). The geographical location of the study site is 31°59′14.6″N, 104°51′01.8″E; it has a slope of 85°, a northwest aspect of 20° and an elevation of 910–970 m. This area belongs to the subtropical monsoon climate zone and has the climatic characteristics of warm winter and early spring, long summer and short winter, and a long frost-free period, as well as abundant rainfall. It has four distinct seasons, with summer and rainy seasons co-occurring and exhibiting obvious geographical changes. *U. rochkii* grows in relatively humid places on limestone cliffs in the middle of the mountains, accompanied mainly by *Adiantum capillus-veneris* L., *Gelsemium elegans* (Gardner and Champ.) Benth., *Primula malacoides* Franch, and *Epimedium davidii* Franch. In the study location, *U. rochkii* has a clustered distribution[32].

The petals pacing characteristics in *U. rochkii* flowers have important scientific value for establishing phylogeny within the *Aquilegia* taxa of family Ranunculaceae. *U. rochkii* plays an important role in plant taxonomy and in the genetic protection of rare plants. This species is rich in aromatic lipids, and the colours of the petals and leaves change over time. It has a long flowering period (usually from December to April) and is thus a winter-flowering plant, providing the species with potential medicinal and ornamental value[33].

Flowering process and floral characteristics of U. rochkii

During the bud stage in December 2011 and December 2012, we randomly selected 50 *U. rochkii* plants from different populations for labelling, with flower shape, size, colour and spatio-temporal dynamics being recorded (while noting to the positions of stigma and anther). After the full blossom stage, 50 flowers were randomly selected and the major floral morphological indices, such as

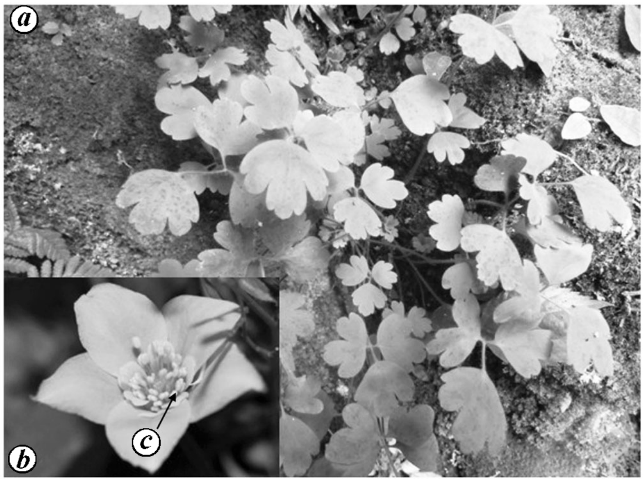

Figure 1. ***a***, *Urophysa rochkii* Plant, ***b***, Flower; ***c***, Anther. Arrow denotes groove in the ventral surface of the anthers.

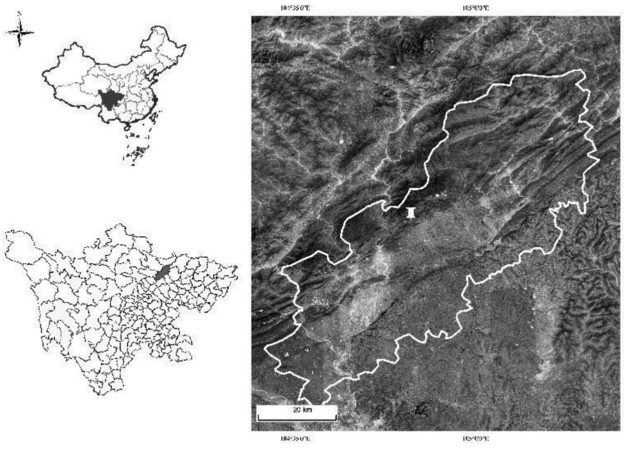

Figure 2. Location of study site and *U. rochkii* morphology.

sepal length, petal length, anther length and style length were measured with a Vernier caliper, and the floral were recorded.

In addition, according to the criteria of Dafni[34], the flowering process of *U. rochkii* populations at the observation site was described, and initiation times were recorded for (i) beginning of the flowering stage; (ii) when less than 25% of the plants were in the flowering stage; (iii) when more than 50% of the plants were in flowering stage; (4) when less than 25% of the plants were in the flowering stage while the remaining flowers were withering, and (5) late flowering (when less than 10% of the plants were still in the flowering stage).

Observation and identification of flower-visiting insects

During the flowering period, 50 flowers with robust growth were selected for observation. With every 15 min considered as a statistical unit, the species of flower-visiting insects were recorded (and photographs taken), and the visiting time and temperature when the insects visited were recorded. Flower-visiting insects were captured, and individuals used for species identification were fixed in 50% alcohol and photographed for observation and identification under a dissecting electron microscope.

Effects of weather on flower-visiting insects

Temperature and weather conditions are factors that affect insect visitation. At the full flowering stage, for 30 labelled flowers, we collected statistics on the flower visiting behaviour of insects at the same time under different weather conditions. Table 1 shows the observed weather conditions and temperature.

Effects of floral organs on insect pollination

Flowers are important in plant breeding: To determine the effects of sepals, stamens and pistils on the pollinator insects and on the pollination process, four groups of treatment were included in the field: with sepals removed (Group A, 50 flowers), with stamens removed (Group B, 50 flowers), with pistils removed (Group C, 50 flowers), and control group (Group D, 50 flowers). Each individual was labelled with a tag. In the full flowering period, 15 flowers were selected from the treated flowers in each group for observation. For five consecutive days, the flower-visiting behaviour of the insects was observed each day from 08 : 00 to 18 : 00 h, with every hour serving as a statistical unit. The insect visiting number was recorded by separate groups, and all data from the five days were averaged and considered as the flower-visiting frequency of the insects for each treatment. Next, each group/treatment was maintained until the flowers withered and seeds were set, and data on seed-setting rate for each group were recorded.

Table 1. Temperature and weather conditions during the *Urophysa rochkii* flowering period

Year	Date (month, day)	Weather	Temperature (°C)
2012	3, 7	Sunny	6–12
	3, 8	Sunny	6–12
	3, 9	Cloudy	5–10
	3, 1	Rain	3–10
	3, 11	Sunny	6–12
	3, 12	Sunny	6–13
	3, 13	Sunny	8–15
	3, 14	Cloudy	10–17
	3, 15	Cloudy	10–18
	3, 16	Sunny	11–18

Results

U. rochkii flowering process and floral organ statistics

Four to five days after the emergence of *U. rochkii* buds, the sepals opened one after another. The opening of the first sepal of each flower marked the beginning of the flowering period for that flower. In the first 2–3 days of the flowering period, the stigmas were at a higher position than the anthers for all 50 flowers under observation. With the progression of observation time, filaments of *U. rochkii* continued to elongate. In the 4th–6th day of the flowering period, 21 flowers had elongated filaments, and their anthers were significantly higher than the stigmas, whereas the remaining 29 flowers still had stigmas higher than the anthers. In the following days, the filaments of the 29 flowers gradually elongated. On the 10th day of the flowering period, the anthers were higher than the stigmas for all 50 flowers, and half of the flowers began to wither. Field measurements showed the diameter of *U. rochkii* flowers was 19.26 ± 2.80 mm (mean ± SD). Table 2 shows the statistical results for the number and length of floral organs for the 50 *U. rochkii* flowers.

According to field experiments in 2011 and 2012, *U. rochkii* withered in summer, began to revive and grow in early August, and formed flower buds in early October. The flower buds emerged and gradually enlarged in early December, and the duration of this process was relatively long, usually approximately one month. Plants then underwent the stages of flowering and withering, until most of the flowers had withered, which marked the end of the flowering phenology. The flowering phenology of *U. rochkii* extended from early December of the first year to middle or late April of the second year. Fruiting started in late March; in early June, the fruits ripened, the pericarp split and the seeds were released.

Table 2. Maximum and minimum length, and average (SD) length of floral organs, as well as maximum and minimum number and average number of flower organs in each flower of *U. rochkii*

Origin	*N*	Maximum length	Minimum length	Length (mean ± SD)	Maximum number in a single flower	Minimum number in a single flower	Average number in a single flower
Calyx	252	30.99	16.04	22.89 ± 3.39	6	5	5.04
Petal	255	9.95	5.85	7.71 ± 0.79	7	5	5.1
Anther	250	2.33	0.81	1.57 ± 0.27	50	31	38.82
Style	244	11.21	4.35	7.56 ± 1.24	6	4	4.9

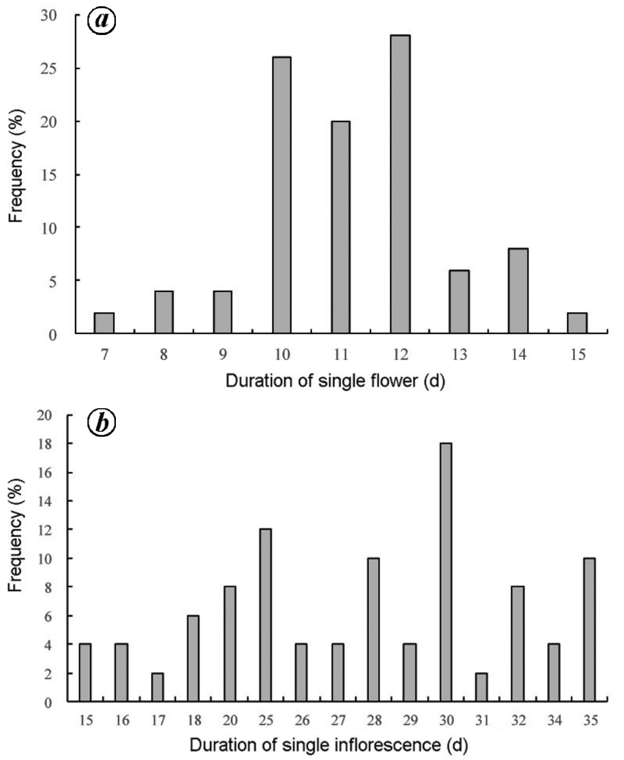

Figure 3. Frequency of flowering duration of a single flower and inflorescence of *U. rochkii*. (***a***) Frequency distribution of single flower longevity. (***b***) Frequency distribution of inflorescence longevity.

Our phenological observations on *U. rochkii* showed that the flowering of single flowers did not have a fixed time. The flowering duration of single flowers had a range of 7–15 d, with 12 d showing the highest frequency (Figure 3), and the average flowering time of a single flower was 11.18 ± 1.64 d (ref. 32). In the bud stage, the sepals were often light green or light purple, and flowers on the inflorescence occurred in an alternating manner. When the flowers had opened, the sepals expanded one after the other, and their colour changed from light green to white or sky blue (light purple); generally, flowers at the top of the same inflorescence were the first to open. On sunny days, the buds opened mostly around noon, which may be related to air temperature on the day of flowering.

Bisexual flowers are borne on cymose inflorescences. There are usually 4–22 inflorescences in each plant, with 2–4 flower buds on each inflorescence in an alternating manner. Inflorescence phenology is related to the spatial location of the on the flower branch. The flowering period of a single-flower inflorescence was approximately 15–35, with the highest frequency being 30 d (Figure 3) and the average being 26.78 ± 5.93 d.

When the flowers had opened, the stigmas were at a significantly higher position than the anthers. The styles did not elongate significantly during the flowering period, whereas the filaments began to elongate on the flowering day or on the second day until the anthers began to crack to release the pollen. Among all filaments, those in the inner whorls elongated first, and the anthers on these filaments shed their pollen.

Flower-visiting insects and their behaviour

Observe 50 flowers between 11 : 00–14 : 00 every day, observe and statistics 4 times, 15 min each time, record the number of visits to a flower, observe 5 days continuously, take average as an insect visiting frequency. The flower-visiting insects of *U. rochkii* included three orders: Diptera (*Muscidae*; *Culicidae*), Hymenoptera (*Apoidea*; *Formicidae*), and Homoptera (*Aphidae*; *Psyllidae*), which totaled five species. Among them, only *Mitroplatia* sp. of the order Diptera, *Apis cerana* Fabricius of the order Hymenoptera, and *Myzus persicae* of the order Homoptera were accurately identified, whereas other insects could not be identified at the species level due to research limitations (Figure 4). The flower-visiting insects of *U. rochkii* mainly had two body types: one was medium-sized, such as *Myzus* sp. and *A. cerana* Fabricius, while the other was small-sized, such as *M. persicae*.

The flower-visiting frequency varied greatly among the different insect species. The flower-visiting frequency of *A. cerana* Fabricius was significantly higher than that of other insects, while the flower-visiting frequency of *Myzus* sp. was almost the same as *M. persicae*. Within 15 min, a flower was visited 1.65 ± 0.33, 0.72 ± 0.024, and 0.73 ± 0.024 times on average by *A. cerana* Fabricius, *Mitroplatia* sp. and *M. persicae* respectively. In contrast, the visiting frequencies of Culicidae, Formicidae and Psyllidae insects were low, and less than 0.15 times. We observed that the active time of flower-visitors of *U. rochkii* was during 11 : 00–14 : 00 h (Figure 5).

The effective pollinator of *U. rochkii* was *A. cerana* Fabricius. The insects of this species stayed on the flowers for approximately 5–10 sec, mainly to feed and collect pollen or nectar. They hovered around the inflorescences to look for targets, mostly flowers with cracking anthers. They firmly captured the filaments with their forelegs and held the stamens with their middle and hind legs; in this position, their tails were slightly raised so that the stamens of *U. rochkii* were close to the abdomens of the insects. In the pollen feeding process, they constantly turned their bodies around the stamens to ensure high pollen collection. They scraped the pollen with their middle and hind legs, with the scraped pollen grains falling close to their abdomens (Figure 6 *a–d*).

The species of flower-visiting insects of *U. rochkii* were relatively few, and the number was relatively small. When an electronic microscope was used to detect flower-visiting insects, only the head, thorax, abdomen and feet of *A. cerana* Fabricius carried a large number of pollen grains, indicating that this species can play a key role in pollination, and is an effective pollinator. Observations of several other flower-visiting insects showed that they carried no pollen grains.

Effects of weather on flower-visiting behaviour of insects

Insects visit *U. rochkii* flowers in two ways: flying directly to the flowers on sunny days, and climbing along the pedicel to the flower surface and resting on the sepals on cloudy or rainy days. Flower-visiting insects feed on nectar by moving among the flowers, and in the process, pollen particles stick to their head, tentacles and feet, thus increasing pollination efficiency. Field observations showed that the flower-visiting times of insects changed significantly with change in temperature. The time insects spent visiting flowers increased with increase in the ambient temperature. No flying insects were observed when the air temperature was below 8°C; at these times, the insects usually slowly crawled upward along the pedicels and hid themselves under the sepals or stayed on the leaves. Therefore, the behaviour of stationary insects directly affects pollination efficiency, which is lower than that of volitant insects.

Weather condition is another factor that affects flower visiting by insects. The time that insects spent visiting flowers on sunny days was more than that on rainy days. Between 12 : 30–13 : 00 in from 7–16 March 2012, a continuous observation of 30 *U. rochkii* flowers for 10 days, Statistics of the number of Volitant insects and Quiescent insects, 6 days are sunny, 3 days are cloudy, and the 1 day is rainy. The frequency of flower visits by insects on sunny days was significantly higher than that on rainy days (Figure 7). On clear days, *A. cerana* Fabricius, *M. persicae*, and *Myzus* sp. visited the flowers, and moved among them searching for food (Figure 6 *a–g*). On rainy days, *A. cerana* Fabricius was nearly inactive, while *M. persicae* and *Myzus* sp. remained at the back of the leaves of the plant for a long time (Figure 6 *f*).

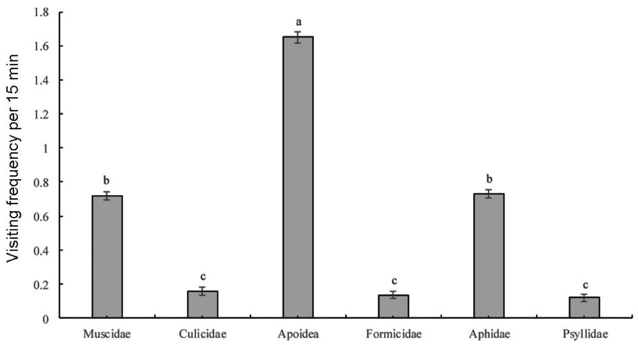

Figure 4. Visiting frequency (mean ± SE) of insects.

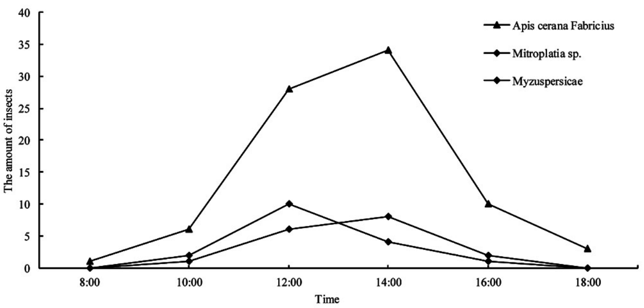

Figure 5. Visiting time of different insects (a – *Apis cerana* Fabricius; b – *Mitroplatia* sp.; c – *Myzus persicae*).

Effects of floral organs on insect pollination

According to experimental observations and statistical data analysis, the flower-visiting frequency of *U. rochkii* showed the following ascending order of groups: A (with sepals removed) < B (with stamens removed) < C (with pistils removed) < D (control) (Figure 8 *a*). The control group had the most species of flower-visiting insects and highest frequency of flower visiting. There was significant difference between the group with sepals removed and that with stamens removed ($P < 0.05$). When the stamens and pistils were removed from the flowers, the flower-visiting insect species of both groups were slightly different from those of the control group. The flower-visiting frequencies of the two groups were reduced compared with that of the control group. Additionally, with the sepals removed, the flower-visiting insect species and the flower-visiting frequency were lowest: the main flower visitor was *A. cerana* Fabricius; it stayed on the stamens only for a short time. These results indicate that the attractiveness of floral organs to visiting insects follows the descending order: sepals > stamens > pistils.

The seed-setting rate of each group also decreased compared with the control group (Figure 8 *b*). The

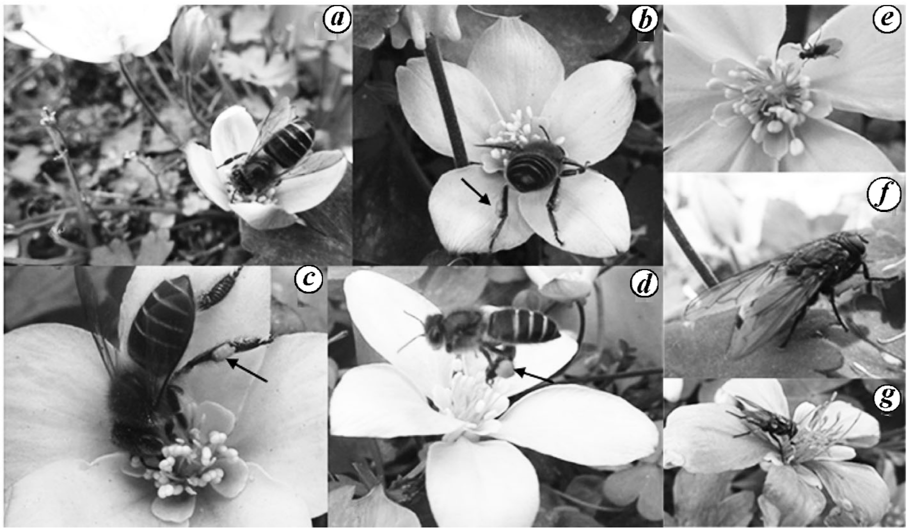

Figure 6. Flower-visiting activities of insects. ***a–d***, *A. cerana* Fabricius visiting *U. rochkii* flowers. The insects move around the stamen (arrow showing a large number of pollen grains adhering to the hind legs of *A. cerana* Fabricius). (***e***) A Formicine insect visiting *U. rochkii* flower. ***f–g***, *Mitroplatia* sp. visiting a *U. rochkii* flower. (***f***) *Myzus* sp. resting on the leaf surface. (***g***) *Myzus* sp. visiting a nearly withered flower.

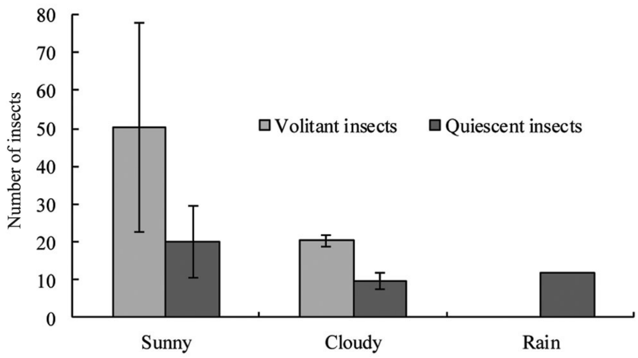

Figure 7. Flower-visiting of insects (mean ± SD) during different weather conditions.

seed-setting rate showed the following ascending order of groups: A (with sepals removed) < B (with stamens removed) < D (control). No seed setting occurred for *U. rochkii* when the pistils were removed.

Discussion

U. rochkii flowering process and integrated pollination characteristics

Observations on the flowering phenology of *U. rochkii* showed that the flowering period was relatively long, generally extending from early December in a particular year to mid-April of the following year. The plant had a long flowering period and exhibited an obvious flowering asynchrony. At the single-flower and single-inflorescence levels, a single flower had a flowering duration ranging from 8 to 15 d, with a single plant having flowering duration lasting approximately two months; a population could have flowering duration lasting approximately four months. The long duration of flowering and asynchronized flowering not only ensures a sufficiently long time for the plants to successfully complete pollination and fertilization, but also reduces the negative effects of an adverse natural environment during the flowering period on the reproductive success of the plant. This is a reproductive strategy that *U. rochkii* has developed in its long-term adaptation to environmental impacts.

Ranunculus is one of the base groups of angiosperms[35,36]. Previous studies have shown that plants offer various forms of reward for pollinating insects, with the most common ones being pollen, nectar, or both[37,38]. The sky-blue sepals and yellow, uncracked anthers of *U. rochkii* display a significant visual contrast in their colour. Anthers crack and expose a large number of pollen grains, which can provide food for some flower-visiting insects. Therefore, sepals, stamens, pistils and the rewards that they provide together constitute the pollination characteristics of *U. rochkii*.

Flower-visiting behaviour of insects to U. rochkii

The frequency and mutual effects of the interaction between flower visitors and the flowers jointly determine the effectiveness of pollination[39–41]. The identification of an effective pollinating insect isan important aspect for understanding the characteristics of reproductive biology; it is also a prerequisite for understanding why a species is rare and endangered[42]. The present study demonstrates

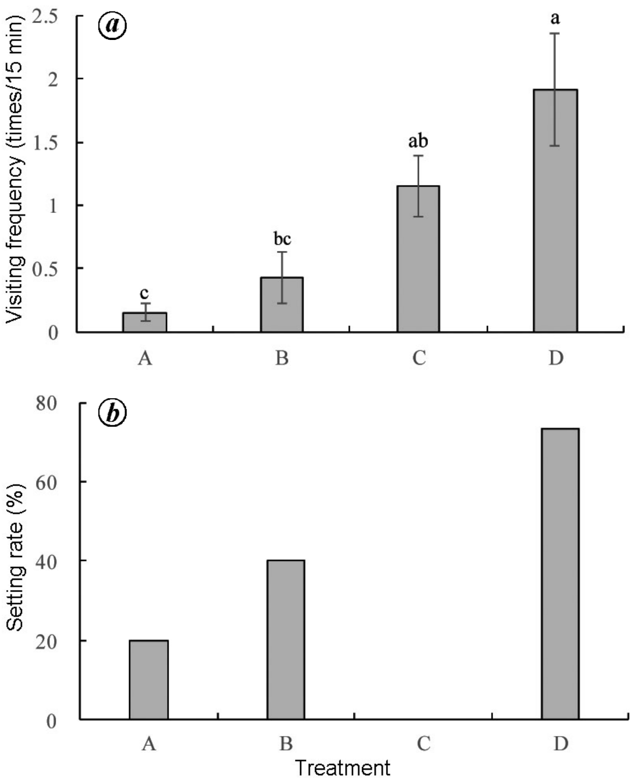

Figure 8. ***a***, Visiting frequency of insects after different treatments. ***b***, Comparison of seed setting after different treatments in *U. rochkii*. A – Sepals removed. B – Stamens removed. C – Pistils removed. D – Control.

that the effective pollinator of *U. rochkii* is *A. cerana* Fabricius, while other insects do not carry pollen grains and thus do not play a role in pollination even though they visit the flowers.

The amount of pollen dispersal and nectar secretion of plants are synergistic with pollinating insects in time. The peak period of pollination visits corresponds to the time of pollen dispersal and nectar secretion[43]. We found that under sunny conditions, the *U. rochkii* anthers cracked mostly during 11 : 00–14 : 00. As the number of cracking anthers increased, the amount of pollen being dispersed increased, with substantial reward being provided to the insects. On the other hand, experimental results show that the active time of flower-visiting insects is also 11 : 00–14 : 00, which is consistent with the time of anther cracking.

Effects of floral organs on pollination by flower-visiting insects

The floral organ that most readily undergoes evolution in Ranunculaceae plants is the petal[44]. The petals of *U. rochkii* show no obvious differentiation and have no obvious attraction to insects, which may be one of the reasons for the endangered status of the plant.

For bio-pollinated plants, the floral characteristics are evolutionarily related to the pollination system and have the most direct effects on attracting pollinators[45]. For insect pollinator-based plants, flower colour, corolla structure, and the existence of odours directly affect the pollination efficiency[46]. *U. rochkii* has multiple stamens and its petals secrete nectar. Therefore, its flower is typical of an insect-pollinated plant. The most important signal in the pollination system is the sense of sight, and bright flower colour can have a visual impact on the pollinators[47]. The sepals of *U. rochkii* account for a large proportion of the entire flower; the colour contrast between the sky-blue sepals and the yellow petals and anthers can visually attract pollinators to the flowers and provide a resting platform for the insects. With sepals removed in *U. rochkii*, the flower-visiting frequency of insects per flower was 0.1553 times/min, which was significantly lower than that of the groups with the stamens and pistils removed as well as the control group. In *U. rochkii*, seed-setting rate for the treatment group with sepals removed was only 20%, which was lower than that for the group with stamens removed (40%) group and significantly lower than that for the control group (70%). The seed-setting rate for the group with pistils removed was 0, indicating that no apomixis occurred. Therefore, we conclude that the sky-blue sepal is the most important factor for attracting insects to the flowers.

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