Effects of flowering mode and pollinator sharing on reproductive success in natural hybrid of two Epimedium (Berberidaceae) species

Lan-ying Chen
China West Normal University

Qiumei Quan (✉ 284626164@qq.com)
China West Normal University

Yunxiang Li
China West Normal University

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Abstract

Natural hybridization mediated by pollen flow has been considered as an important factor to drive speciation in flowering plants. The diversity of *Epimedium* species is centered in southwestern China, yet the potential role of interspecific pollination in natural hybridization has not been empirically examined. The flowering period and floral visitors, and conducted a series of pollination experiments to examine breeding systems and hybrid compatibility within three *Epimedium* which are commonly sympatric in the Jinchengshan National Forest Park, Nanchong of Sichuan Province. Epimedium pubescens and *Epimedium wushanense* are morphologically clearly distinct and easily recognized by flower morphology, the putative hybrids (not been named) are morphologically between them. We found that *E. pubescens* and *E. wushanense* had 15 days of overlapping anthesis during consecutive three years of observation. Both species in research region are self-incompatible and need pollinators service for successful reproduction. Their pollinators are different in quantity and species, but *Lasioglossum* spp. (Hymenoptera: Halictidae) visited all of them. No breeding barrier between any of the three species were detected by artificial pollination, and the hybrids seeds were fertile. The overlapping time of flowering, common floral visitors and compatible of hybrid may be important factors to make interspecific pollination and speciation successfully for natural hybrids species of *E. pubescens* and *E. wushanense*. The role of potential interspecific pollination is essential to hybrids speciation and the evolution of Epimedium species.

Introduction

Natural hybridization likely influences evolution in a myriad of ways, repeatedly contributing to diversity and speciation in flowering plants (Rieseberg and Ellstrand 1993, Rieseberg 1998, Yakimowski 2016). Even the most stringent self-pollinating plants (such as soybean and barley) have a certain frequency of hybridization events. An estimation of interspecific hybridization is relatively low, approximately 10% in animals, but up to 25% or over 50% in plants (Mallet 2005). In flowering plants, where many lineages are dependent on biotic vectors for pollen transfer, pollinator behaviour may influence the origin and dynamics of plant hybrid zones. Consequently, the movement of pollen influences hereditary combinations and adaptation (Huang and Guo 2000, Huang 2004, Wang et al. 2018, Rita et al. 2018). However, given the lack of research on the formation, maintenance and adaptation of hybrid, the role of hybridization in evolution remains poorly characterized. The mechanisms that how flowering mode and pollination contribution affect reproductive success among different species are still poorly understood (Wesselingh and Arnold 2010, Emmms and Arnold 2000).

The stability of plant hybrid breeding systems is an essential component in the process of natural hybrid species formation (Wendt et al. 2001). In recent years, a wide variety of research techniques have been used to study natural hybrid. Morphological and pollination ecology analyses were historically the main methods for detecting natural hybrid until molecular markers or biochemical markers became easy to use (Edward and Tracey 2012, Choi et al. 2001, Li et al. 2015), alongside artificial hybridization (Motley and Carr, 1998), and molecular biology (Saitou and Nei 1987, Stankiewicz et al. 2019).

*Epimedium* (Berberidaceae) includes well-known medicinal species, as well as potential ground cover and ornamental herbs with more than 60 species globally distributed. Natural variations and interspecific transition types of the original species have been observed in western Sichuan Province (Stein 1995, Quan and Li 2012, Fan et al. 2016, Chen et al., 2019). The differences in morphology of *Epimedium* species have been used as a basis for interspecific classification (Suzuki 1978, Stearn 2002, Wang et al., 2018). Stearn (1938) classified *Epimedium* into two series *Brachycerae* and *Dolichocerae* based on their petal size. Suzuki (1987) yielded hybrid between *Epimedium* species under artificial pollination, for instance between *E. diphyllum* and *E. grandiflorum* in the large-flowered section. However, the natural hybrid of between the two series have not been reported. On the basis of RAPD (Random Amplified Polymorphic DNA) and PCR-RFLP (Polymerase Chain Reaction–RestrictionFragment Length Polymorphisms), the RAPD fingerprints and the phylogenic map of seventeen *Epimedium* species were established by Du et al. (2012). The Chinese species of *Epimedium* can be grouped into the large-flower taxa and the small-flower taxa, *E. pubescens* is the small-flowered taxa, *E. wushanense* is large-flowered taxa (Du et al. 2012, Chen et al. 2019, Sheng et al. 2021).

It is known that *Epimedium* species easily hybridize by hand manipulation (Suzuki 1987, Du and Chen 2013, Li et al. 2015). However, little is known about pollination ecology of natural hybrid within *Epimedium* species (Suzuki 1987, Quan and Li 2012, Chen et al. 2019). *E. pubescens* and *E. wushanense* are frequently allopatric distributed on forests or forest edges of Sichuan Province, but they occur sympatrically at Jinchengshan National Forest Park, Nanchong of Sichuan Province (Quan and Li 2012, Chen et al. 2019, Zhang et al. 2022), where morphological intermediary between these two species has been discovered. To explore the possibility of hybrid between *E. wushanense* and *E. pubescens*, we investigated the floral biology, breeding system, and pollination ecology of these *Epimedium* species and conducted various artificial pollination experiments. We aim to (i) understand the breeding system in *E. pubescens* and *E. wushanense*, and elucidate how the flowering mode affects the reproductive success and interspecific hybridization; (ii) investigate pollinator diversity and sharing in sympatric *Epimedium* species; (iii) examine whether interspecific hybridization is compatible, and if hybrid backcrossing with parental species.

Materials and methods

Study sites

Wild populations of *E. pubescens*, *E. wushanense* and putative hybrid were investigated from March 2014 to May 2017 in the Jinchengshan National Forest Park, Nanchong of Sichuan Province, China (1128 m a.s.l., 30°45′N, 106°28′E), the forefront of the ecological barrier on the upper reaches of the Yangzte River (Bai et al. 2022). The mean annual temperature is 18.6 °C, ranging from an average of 5.4 °C in January to 21.7 °C in July. The mean annual precipitation and evaporation are 1063 mm and 705.8 mm, respectively. The soil in this area is red loam soil (0–30 cm depth). The tree layer and shrub layer of the plots were similar, with the secondary conifer and broad-leaf mixed forest being dominant, belonging to one succession to the evergreen broad-leaf forest (Qian et al. 2017). The habitats of *E. pubescens*, *E. wushanense* and the putative hybrid populations were slightly different (Table 1).

Study species
Epimedium species usually flower from March to May. All species produced terminal inflorescences, per panicle usually bears 10–50 flowers. Each flower consists of two inner and outer layers with four sepals per layer, four exterior conical spurs and four stamens around the interior persistent pistil. The outer sepals are pale green, purple or yellow and fall off when the buds expand and the inner sepals are white. The four petals are pale yellow, spur-shaped. Nectar produced by nectaries (located at the tip of spur) is stored in spurs. Each anther has two chambers, and green or yellow pollen is present after the chamber dehisces. The floral parameters of the study sites Epimedium are shown in Table 2.

Flowering phenology monitoring

From March to May in 2014 and 2016, the flowering phenology (first flowering date, peak flowering date, last flowering date, flowering amplitudes curves, flowering intensity) of each Epimedium species was observed and recorded in three subplots at population and individual levels. The first flowering date, peak flowering date and last flowering date were calculated according to Dafni (1992) statistical methods. Before the Epimedium plants anthesis, 30 buds were randomly selected from three populations in each Epimedium subplot, the total number buds of each inflorescence were labeled with notice plates. The number of open flowers of each inflorescence buds were recorded every two days until the end of the flowering period. First flowering date, approximately 25% of the plants flowered. Peak flowering, over 50% of the plants flowered. Last flowering date, over 75% of the plants have faded at the rest of the end of flowering time. The flowering amplitudes curve and flowering intensity were calculated according to Herrera’s (1986) statistical methods. The flowering amplitudes curve is represented the number of opening flowers per day and per unit time (from 4:30 – 18:30 h ) at per plant individual level. Flowering intensity is the ratio of the maximum number of opening flowers per plant produced on the peak flowering date.

Measurements of plant size and floral morphology

To measure plant height, leaf morphology, and floral morphology, more than 30 anthes plants were randomly selected from three populations in each Epimedium subplot. The floral diameter, the length and height of the spur, the height and width of the entrance of the spur, the length of the stamen, pistil, and style, and the length and width of the anther were measured with Vernier calipers (Guanglu, Guilin, China) at the peak blooming period in each species (n = 30). At least 40 flower buds from 10 plants in each population were randomly selected to determine the volume of secreted nectar. Ten flowers were removed from the different plants at 9:00 h on the first, second, third and fourth days of flowering to immediately measure the nectar volume. The nectar volume was measured using calibrated 5 μL capillary tubes (Hirschmann Laborgerate, Germany).

Floral visitors

Pollination observations were conducted from 5 days in the three populations of each species at the peak blooming period. Pollinator type, pollination behavior, visit frequency, pollination time were recorded, and images of the pollinators were captured (Nikon DSLR, D7000, 16.2 megapixels). All flower visitors were captured for subsequent identification and measurements in the laboratory. To compare pollinator effectiveness in each species, we examined the pollen removal and deposition by effective a single floral visitors per plant. Sixty similar flower buds from 10 plants (6 flowers per plant) at the peak blooming period in each population were randomly selected, labeled and bagged. These bagged flowers were randomly divided into two groups (30 flowers per group), the flowers of one group were opened and emasculated using tweezers before the anthers dehisced, afterward, the bags were covered again. The other group did not do any treatment. On the first day of flowering, when observe floral visitors the bags were opened. Once the flower was visited by effective floral visitors the first time, the flower was removed immediately and fixed with a formalin-acetic acid-alcohol (FAA) solution to determine the pollen grains remaining in anthers (pollen removal) and deposited on stigmas (pollen deposition) (Russell et al. 2021). To determine the total number of pollen grains produced at the population level, at least 20 immature flowers of each species were randomly chosen and fixed with FAA. Afterward, the number of pollen grains were counted using a binocular microscope (Leica DM500, Heerbrugg, Switzerland) in the laboratory.

Manipulated pollination experiments

At the peak blooming period of the three Epimedium species, we conducted five pollination treatments from 2016 to 2017. (1) Natural control: 30 buds belonging to different panicles were labeled and open-pollinated without any treatment until the fruit ripened. (2) Automatically self-pollination: 30 buds belonging to different panicles were labeled and bagged in fine-mesh nylon bags to prevent animal visitors, and no other manipulations were performed. (3) Artificial self-pollination: 30 buds belonging to different panicles were labeled and bagged in fine-mesh nylon bags, and the stigmas were pollinated with pollen from the same flower after the anthers dehisced. Afterward, the bags were used to cover the panicles. (4) Conspecific cross-pollination: 30 buds belonging to different panicles were labeled and bagged in fine-mesh nylon bags, when the flowers opened used tweezers emasculating the anthers before dehisced. Then, the stigmas were pollinated with pollen from other individuals of the same species plants located at least 200 m away. These panicles were bagged after pollination. (5) Interspecific artificial pollination, 60 buds belonging to of different each Epimedium species panicles were labeled and bagged in fine-mesh nylon bags, and the flowers were opened and emasculated using tweezers before the anthers dehisced. Then, (a) the stigmas of E. pubescens were pollinated with pollen from E. wushanense and putative hybrid after the anthers dehisced, (b) the stigmas of E. wushanense were pollinated with pollen from E. pubescens and putative hybrid after the anthers dehisced, (c) the stigmas of putative hybrid were pollinated with pollen from E. pubescens and E. wushanense after the anthers dehisced. These panicles were bagged after pollination. We conducted the seed germination experiment on the seeds obtained from the artificial pollination experiment. Epimedium seeds harvested that year were sown directly in the seedbed of the study sites rich in humus soil, covered with about 1 cm of soil, and kept the soil surface moist, and by the beginning of the second spring, the germination rate was counted.

Statistical analysis

All the data (including the flowering phenology, floral parameters, volume of secreted nectar and concentration, fruit set, germination set, the effective pollinators efficiency) were analyzed using the statistical software SPSS v22.0 (SPSS Inc., Chicago, Illinois, USA). The normality and homoscedasticity of variables were assessed using the Shapiro–Wilk and Levene tests, respectively. Normal and normalizable data were compared using one-way analysis of variance (ANOVA) (with Duncan's multiple range test).
Results

Flowering phenology

Phenological investigations of the sites *Epimedium* indicated that the flowering seasons lasts for 20–30 days from early March to mid-May every year. However, the first flowering date, peak flowering date and last flowering date of each species are obviously different, depending on the plant growing environment and climate (Fig. 1). The flowering amplitude curves of *E. pubescens* and *E. wushanense* displayed a bell curve that peaked on the 15th and 26th days of flowering, respectively (Fig. 1a). At the population level, the flowering period of *E. pubescens* and *E. wushanense* overlapped approximately 15 days. *E. pubescens* and *E. wushanense* were largely characterized by a single peak of flowering amplitude curves, while putative hybrid had a tendency to bloom gradually in batches. And the relative flowering intensities were reasonably over 50%, suggesting that the flowering period of these *Epimedium* species were short and centralized (Fig. 1b).

The longevity of single flower of each species is 5–7 days, bisexual, zygomorphic, inodorous and inverted growing. Intuitively, the floral features of putative hybrid have intermediate forms between *E. pubescens* and *E. wushanense* (Fig. 2). The leaves of *E. pubescens* are acuminate oval, with fine spines toothed edge, foliar with brown patches, thinly leathery, whereas *E. wushanense* are acutely pointed, toothed edges with fine spines, dark green and glossy. The putative hybrid produce acutely pointed, toothed edges with fine spines, and light green with brown patches leaves, with or without hairs on stem. The flowers of all species have inner and outer sepalas, of which each layer contains 4, and grow symmetrically. The outer sepalas are pale purple or green, and fall off gradually at the bud stage. The inner white sepalas unfold petals and male and female organs together with the outer sepalas after the large-bud stage and extend gradually throughout the blooming process (Fig. 2a–i). Four pale yellow spurred petals grow symmetrically, the petals of *E. pubescens* are small and vertical (Fig. 2c), *E. wushanense* (Fig. 2f) and putative hybrid (Fig. 2i) are straight or curve, and the nectaries are located on the tip of the spur. The pistil is slightly longer than the stamen from the large-bud stage to the first day of flowering (Fig. 2b, f, g). The anthers dehisce lengthwise and enclose the stigma on the first day of flowering. After successful pollination, the milky white stigma turns pale yellow and gradually elongates. There are 4–5 ovules in one ovary of *E. pubescens*, but 10–12 in *E. wushanense* and putative hybrid.

Plant size and floral features

The morphological parameters differences among the *Epimedium* species were plant size, leaf shapes (leaf length and width) and floral features are summarized in Table 1. The plant height decreased significantly in *E. wushanense* (47.358 ± 1.121 cm), putative hybrid (31.698 ± 1.815 cm ) and *E. pubescens* (23.422 ± 1.129 cm) (Table 2, p < 0.05), the leave length and width increased significantly in *E. wushanense*, putative hybrid and *E. pubescens* (Table 2, p < 0.05). The diameter of *E. wushanense* flowers (37.215 ± 0.418 mm) were significantly greater than *E. pubescens* (19.252 ± 1.101 mm) and putative hybrid (18.124 ± 0.323 mm ) (Table 2, p < 0.05), but the difference was not significant increased in *E. pubescens* and putative hybrid (Table 2, p > 0.05). The spur of length and width were significantly increased in *E. wushanense*, putative hybrid and *E. pubescens*, respectively (Table 2, p > 0.05). The length and width of the anthers were *E. wushanense* and putative hybrid significantly greater than *E. pubescens* (Table 2, p > 0.05), the difference was not significant increased in *E. wushanense* and putative hybrid, respectively. Evaluations of the plant height, the leaf length and leaf width, the length of out sepal, the length of filament, the length of style, the other floral characters demonstrated that *E. wushanense* was significantly larger than *E. pubescens* and putative hybrid. The morphology of putative hybrid showed one type of apparent transition state between *E. pubescens* and *E. wushanense*. Nectar secretion of three species occurred every day from the large-bud stage until the flowers withered, the nectar volume of the bagged flowers increased over time, but on the second day the increase speed decreased (Fig. 3). The mean nectar volume per flower in the three species were significantly increased in *E. pubescens* (0.35 ± 0.01 µL), putative hybrid (2.28 ± 0.2 µL), and *E. wushanense* (13.51 ± 0.29 µL) (Fig. 3, p < 0.05).

Observations of pollination characteristics

The visiting insects of the three species were more within 2–3 days after flowering of a single flower. Few insects visit flowers in rainy weather. For different species of *Epimedium*, at least sixteen different species of insects were recorded during the field observation period, mainly Hymenoptera and Diptera. By observing the pollen removal of the captured insects specimens' bodies using electron microscopy, various bees carry more pollen and were effective pollinators (Table 3). The effective pollinators of *E. pubescens* were *Lasioglossum* spp. (Hymenoptera:Halictidae), which visited flowers for nectar or pollen (Fig. 4a-b). The pollen grains on the stamens of *E. pubescens* were packed into the pollen baskets by their legs and carried away. Meanwhile, pollen grains on the pollen baskets were deposited on the stigma in the process of visiting flowers. *Apis* sp. (Hymenoptera:Apidae) were opportunists with a low visit frequency and were occasionally observed in *E. pubescens* (Fig. 4c). The nectar-feeding *Bombus trifasciatus* (Hymenoptera:Apidae) and *Bombus grahami* (Hymenoptera:Apidae) were common flower visitors to the *E. wushanense*, but differences in visiting behaviors were observed between the two species of bumblebees. The effective pollinator of *E. wushanense* was *B. trifasciatus* (Fig. 4d), when the bumblebees landed on the flower center, tightly grasped a spur with their front legs, and dipped their proboscis into the spur to suck the nectar, the pollen grains adhered to the neck hair of *B. trifasciatus* and were carried away when bumblebees turned their bodies in the process of visiting flowers. Meanwhile, pollen grains on the neck hair of *B. trifasciatus* were deposited on the stigma. *B. grahami* robbed nectar from the flower (Fig. 4e), drilled a hole in the middle of the spur using their mouthparts and inserted their proboscis into the spur to consume the nectar, no pollen was found on the captured specimens' bodies when performing microscopic examination. *Lasioglossum* spp. also the effective pollinators of *E. wushanense* and paid for the pollen (Fig. 4f). The effective pollinators of putative hybrid were *Lasioglossum* spp. (Fig. 4g) and *Anthophora* spp. (Hymenoptera:Anthophoridae, Fig. 4i). *Anthophora* spp. was observed only in putative hybrid, flower visiting behavior was similar to bumblebees and paid for nectar. *B. grahami* were opportunists with a low visit frequency and were occasionally observed in putative hybrid for nectar, no pollen was found on bodies when performing microscopic examination. We quantified pollen grains remaining in anthers (pollen removal) and deposited on stigmas (pollen deposition) of effective pollinators. The pollen removal and pollen deposition of *Lasioglossum* spp. were significantly greater than *B. trifasciatus* and *Anthophora* spp. (Table 3, p < 0.05).

Manipulated pollination experiments
The results of the artificial pollination combination and the obtained fruit setting rate and germination setting rate results are shown in Table 4. The three *Epimedium* species belonged to outcrossing breeding system. Treatments involving cross-pollination and open pollination resulted in fruit production, and the fruit set and germination setting rate surpassed 50%, 70%, respectively. Whereas treatments involving autonomous and artificial self-pollination resulted in no fruit production. Interspecies artificial pollination of these three species of *Epimedium* resulted in fruit production. The fruit set obtained by using *E. pubescens* as female parent () and *E. wushanense* putative hybrid as the male parent () were 18.92%, 24.11%, respectively, and the seeds obtained by these hybrid also had certain germination setting rate of 30.04%, 25.85%, respectively. When *E. wushanense* was treated as female parent () and *E. pubescens* and putative hybrid as the male parent (), the fruit set were 75.22%, 72.97%, and the germination set were 80.72%, 88.04%, respectively. Putative hybrid as female parent () and *E. pubescens* and *E. wushanense* as the male parent (), the fruit set were 83.33%, 90.63%, and the germination set were up to 87.73%, 85.31%, respectively.

**Discussion**

**Conditions for the formation of natural hybrid**

The most critical and principal factors in the occurrence of hybridization among species are their geographical distributions (Aizawa and Iwaizumi 2020). Gene introgression usually results from spontaneous hybridization occurring among closely related species in sympatric populations, and thus significant impacts the genetic structure of natural populations (Efrain and Oyama 2004). Natural hybridization is most likely to occur in populations where the number of one parent is far less than that of another, because interspecific pollen competition prevents the production of hybrid when there are a large number of both parents (Carney et al. 1994, Rieseberg and Carney 1998). Previous studies showed that *Epimedium* species belong to a typical Old World temperate distribution type, widely and intermittently distributed in the narrow strip between Japan in Asia and Algeria in North Africa (25° ~ 48°N, 5° ~ 143°E), each species is usually narrowly distributed, no widespread species (Ying 2002, Ying et al. 2011). Both *E. pubescens* and *E. wushanense* are small population size, fragmented distributed in the Jincheng Mountain National Forest Park in Sichuan, the population of *E. wushanense* was far less than that *E. pubescens* (Quan and Li 2012, Fan et al. 2016). The coexistence of *Epimedium* species distributed in Jinchengshan National Forest Park, and *E. wushanense* has the relatively narrow ecological niche provided conditions for possibility of hybridization. Pollen movement provided conditions for possibility of hybridization of different species (Patel and Datta 1960, Kulbaba and Worley 2014). Flowering synchrony influences gene flow within species and patterns of interaction among plants and with other trophic levels, including pollinators (Nagahama and Tetsukazu 2019, Fisogni et al. 2022). Relative flowering intensity is an indicator of spatial distribution of plant flower resources, which may affect the movement patterns of pollen gene flow in plants (Wyatt 1982, Burd 2004). The first flowering dates, peak flowering dates and last flowering dates of *E. pubescens* and *E. wushanense* are different, but both of them showed short and centralized flowering period, overlapped approximately 15 days at the population level (Fig. 1), indicating the possibility to hybridize.

Hybridization facilitated ecological divergence, as hybrid expressed a mixture and transition of parental traits, and that new gene combinations generated are speculated to have contributed to ecological divergence (Rieseberg 2006). Suzuki (1986) found that once hybrid were formed, the maintained distinctive traits between the genetic parents and inherited the dominant genes. It was easy to determine by breeding system methods which was the parent of the hybrid, although only for the sexual reproduction of *Epimedium* plants, as clonal plants will not form hybrid (Sheng et al. 2011). The obvious differences between parents were revealed, the inflorescence height, inflorescence number and ovule number of the hybrid represent the middle state between the parents, the characteristics of the flower are the most obviously different between the hybrid and the parents (Du et al. 2012, Sheng et al. 2021). To counted the morphology of *E. wushanense* and *E. pubescens*, as mentioned above (Table 2), and the analysis of the apparent and quantitative traits showed that putative hybrid explicit characteristics were the mixture of the *E. pubescens* and *E. wushanense*.

Most *Epimedium* species have been reported to be self-incompatible, plants avoid selfing through dichogamy, herkogamy and self-incompatibility (Suzuki 1983, 1984, 1987, Sheng et al. 2011, Chen et al., 2019), and the results of the pollination experiment showed that the three *Epimedium* species (Table 4) belonged to outcrossing breeding system, self-impollination, rely on insect pollination. Pollination services are important for maintaining population continuity, as globally two-thirds of crops depend, at least partially, on insect-pollination (Klein et al. 2007, Aizen et al. 2008), with wild pollinators generally contributing to crop yields (Montoya et al. 2020). Asexual reproduction introduces a higher risk of extinction than with sexual reproduction, especially when global pollinator populations are in decline (Vamosi and Knight 2006, Ashman 2008, Buchanan 2015, Panique and Caruso 2019). Insects and plants have evolved a symbiotic relationship, plants attract insects for pollination by color, size, pollen or nectar reward, not only influencing pollinator pollination activity and efficiency, but also affecting the reproductive efficiency of flowers (Földesi et al. 2016, Noman et al. 2019). Through the observation of pollinators, *E. pubescens* and *E. wushanense* share the pollinator *Lasioglossum* spp., indicating the possibility of natural hybridization between them. At the same time, *Anthophora* spp. was observed only in putative hybrid, not found on their parents, which created conditions for the isolation of the hybrid and their parents. The effective pollinator species and their visiting behaviors are crucial for the successful reproduction of plants (Wang et al. 2013, Chakraborty et al. 2021). In plants, the number of conspecific pollen grains per stigma often predicts seed set and is used to quantify pollinator effectiveness (Page et al. 2019). In this study, the pollen removal and pollen deposition of effective pollinators varied among taxa. *Lasioglossum* spp. (Hymenoptera:Halictidae) was the most effective pollinator than others, in the reproductive fitness of putative hybrid in fragmented habitat may result, in part, from pollinator effectiveness of this species. The flower visiting behaviors were different, *Lasioglossum* spp. mainly took pollen as the reward, while *B. trifasciatus* and *Anthophora* spp. rewarded with nectar was due to the feedback mechanism of the sender on the change of flower characteristics. Gene flow caused by pollinator in *Epimedium* species indeed plays an important role in the species diversity.

**Pollination biology and adaptability of natural Epimedium hybridization**

The experimental results of artificial pollination combinations illustrate that different species of *Epimedium* can successfully pollinate each other, and obtain fertile seeds, but the fruit setting rates and seed germination rate of different combinations were different (Table 3). Due to the various isolation mechanisms between species, hybrid incompatibility and hybrid sterility (reproductive isolation after pollination) can prevent hybridization (Wendt et al. 2001). Previous
studies showed that the genetic relationship among *E. pubescens* and *E. wushanense* is relatively close according to ISSR (Inter-simple Sequence Repeat) and RAPD marker clustering, hybridization is not restricted (Du et al. 2012, Chen et al. 2012). Suzuki (1987) and Du et al. (2013) yielded hybrid between large-flower taxa and the small-flower taxa *Epimedium* species under positive and reverse hybridization artificial pollination, showing that large-flower taxa *Epimedium* species is widely affinitive pollen receptor, fit for female parent on interspecific hybridization, small-flower type *Epimedium* species is good pollen affinity donor and is suitable as a male parent in crossbreeding. The seed germination rate of *E. pubescens* as female parent ( ) and *E. wushanense* and putative hybrid as the male parent ( ) were only 30.04%, 25.85%, respectively, the other hybrid combinations was above 70.00%, with the highest rate of 90.63%, indicating that there was a possibility of natural hybridization between them to form species. It also further proves that *E. wushanense* grouped into large-flower taxa is suitable for female parent on interspecific hybridization, *E. pubescens* grouped into small-flower taxa is suitable for male parent. Moreover, it is possible that putative hybrid can backcross with their parents, which also provides a continuation of the formation of putative hybrid species (Motley and Carr 1998, Tastard et al. 2008).

Floral morphology and pollination biology are major topics in studies of floral evolution and plant reproduction. The pollination system is a model to understand the interaction between natural selection and evolution (Husband and Schemske 1996, José María Gómez et al. 2015, Pansarin and Caruso 2019). Since pollination is the necessary stage of fertilization among Spermatophyta, the movement of pollen restricted the individual gene floating and mating modes between colonies (Huang and Guo 2000, Ashman et al. 2004, Alonso et al. 2010). However, recent studies have found that some hybrid can result in novel genotypes through intraspecific hybridization potentially increasing the ability to adapt to the environment (Arnold and Hodges 1995, George et al. 2020, Erickson et al. 2020). Given that hybrid change in the quantity (mating frequency) and quality (efficiency of pollinators) of their pollination services, understanding the effects of pollination on their reproductive success is essential for their conservation and recovery. Evidence of pollination biology has shown that there is no reproductive isolation between *E. pubescens* and *E. wushanense*, which not only provides formation conditions for the natural hybrid of *Epimedium* species, but also provides further research content for taxonomy and phylogeny. Our next work is mainly to investigate the population as a unit, and do in-depth research in morphology and molecular biology, in order to obtain sufficient evidence to prove the formation of natural hybrid species of *E. pubescens* and *E. wushanense*. Therefore, this study presents new theoretical data for *Epimedium* in China, and has important theoretical and practical significance for the effective protection, and utilization of *Epimedium* resources, and the improvement of artificial hybrid cultivation resources, and increases our understanding of *Epimedium* taxonomy and system evolution.

**Declarations**

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**Declaration of Competing Interest**

No conflict of interest exists with the submission of this manuscript, and it is approved by all authors for publication.

**Author Contributions**

Qiumei Quan and Yunxiang Li contributed to the study conception and design. Data collection and experiments were performed by Lanying Chen. Qiumei Quan and Lanying Chen analyzed the data. The first draft of the manuscript was written by Lanying Chen, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 The habitats environmental factors (mean±SE) of Epimedium species in three populations. Different lowercase letters indicate significant differences between species (ANOVA, \( p < 0.05 \)).

<table>
<thead>
<tr>
<th>Species</th>
<th>Coordinates</th>
<th>Altitude/m</th>
<th>Temperature/°C</th>
<th>Relative light intensity%</th>
<th>Relative humidity%</th>
<th>Soil moisture content%</th>
<th>Soil organic content%</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. pubescens</td>
<td>30°45.94′ N, 106°27.83′ E</td>
<td>668</td>
<td>18.62±1.32</td>
<td>52.03±1.48</td>
<td>60.54±4.73</td>
<td>26.22±2.11</td>
<td>4.85±3.11</td>
</tr>
<tr>
<td>E. wushanense</td>
<td>30°45.08′ N, 106°28.01′ E</td>
<td>720</td>
<td>18.84±1.22</td>
<td>54.56±2.90</td>
<td>52.54±3.81</td>
<td>18.17±2.29</td>
<td>3.27±2.15</td>
</tr>
<tr>
<td>Putative hybrid</td>
<td>30°45.09′ N, 106°28.03′ E</td>
<td>725</td>
<td>19.42±1.20</td>
<td>59.28±5.05</td>
<td>54.76±4.66</td>
<td>19.55±2.22</td>
<td>3.12±1.83</td>
</tr>
</tbody>
</table>

Table 2 Comparisons of plant size and oral traits (mean±SE) among three Epimedium species. Different lowercase letters indicate significant differences between species (ANOVA, \( p < 0.05 \)).

<table>
<thead>
<tr>
<th>Traits</th>
<th>E. pubescens</th>
<th>Putative hybrid</th>
<th>E. wushanense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height/cm</td>
<td>23.42±1.129</td>
<td>31.69±1.815</td>
<td>47.35±1.121</td>
</tr>
<tr>
<td>Leaf length/cm</td>
<td>4.53±0.528</td>
<td>6.82±0.611</td>
<td>10.61±0.846</td>
</tr>
<tr>
<td>Leaf width/cm</td>
<td>2.57±0.241</td>
<td>2.84±0.317</td>
<td>3.21±0.666</td>
</tr>
<tr>
<td>Diameter of flower/mm</td>
<td>19.25±1.101</td>
<td>18.12±0.323</td>
<td>37.21±0.418</td>
</tr>
<tr>
<td>Length of outer sepal/mm</td>
<td>3.02±0.065</td>
<td>2.94±0.045</td>
<td>3.04±0.056</td>
</tr>
<tr>
<td>Width of outer sepal/mm</td>
<td>1.20±0.098</td>
<td>1.25±0.063</td>
<td>1.83±0.081</td>
</tr>
<tr>
<td>Length of inner sepal/mm</td>
<td>5.64±0.123</td>
<td>8.59±0.041</td>
<td>8.25±0.131</td>
</tr>
<tr>
<td>Width of inner sepal/mm</td>
<td>2.16±0.042</td>
<td>2.85±0.061</td>
<td>3.22±0.059</td>
</tr>
<tr>
<td>Length of spur/mm</td>
<td>2.20±0.049</td>
<td>9.67±0.109</td>
<td>26.32±0.206</td>
</tr>
<tr>
<td>Width of spur/mm</td>
<td>0.90±0.041</td>
<td>1.28±0.049</td>
<td>1.43±0.014</td>
</tr>
<tr>
<td>Length of anther/mm</td>
<td>2.57±0.047</td>
<td>2.67±0.038</td>
<td>2.74±0.048</td>
</tr>
<tr>
<td>Width of anther/mm</td>
<td>0.93±0.025</td>
<td>1.31±0.019</td>
<td>1.26±0.041</td>
</tr>
<tr>
<td>Length of filament/mm</td>
<td>3.26±0.087</td>
<td>2.83±0.086</td>
<td>2.82±0.101</td>
</tr>
<tr>
<td>Length of style/mm</td>
<td>5.94±0.107</td>
<td>5.68±0.080</td>
<td>5.72±0.071</td>
</tr>
<tr>
<td>Length of pistil/mm</td>
<td>2.56±0.096</td>
<td>2.85±0.049</td>
<td>2.94±0.063</td>
</tr>
<tr>
<td>Width of pistil/mm</td>
<td>0.49±0.019</td>
<td>0.81±0.019</td>
<td>0.84±0.011</td>
</tr>
</tbody>
</table>

Table 3 Floral visitors, pollen removal ratio(%) (mean±SE) and pollen deposition (mean±SE) of three Epimedium species. Different lowercase letters indicate significant differences between species (ANOVA, \( p < 0.05 \)).

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**Note:** The tables display data that are essential for understanding the ecological and evolutionary aspects of Epimedium species, highlighting the environmental factors, plant size, and floral traits that influence their growth and pollination processes. These studies are crucial for conservation efforts and the understanding of biodiversity and hybridization patterns in these species.
<table>
<thead>
<tr>
<th>Visited plants</th>
<th>Order</th>
<th>Family</th>
<th>Floral visitors</th>
<th>Effective pollinator (Y/N)</th>
<th>Pollinator of pollen removal %</th>
<th>Pollinator of deposition on stigmas</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. pubescens</em></td>
<td>Diptera</td>
<td>Syriphae</td>
<td><em>Medianostoma scalare</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Episyrphus balteatus</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Ringia sp</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>Apidae</td>
<td></td>
<td><em>Nomada sp.</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Tetralonia sp.</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Apis sp.</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Colletidae</td>
<td></td>
<td></td>
<td><em>Colletes sp.</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Halictidae</td>
<td></td>
<td></td>
<td><em>Lasioglossum spp.</em></td>
<td>Y</td>
<td>30.378±1.973c</td>
<td>209.378±9.486a</td>
</tr>
<tr>
<td><em>E. wushanense</em></td>
<td>Diptera</td>
<td>Syriphae</td>
<td><em>Sericomyia sp.</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Episyrphus balteatus</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Ringia sp</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>Apidae</td>
<td></td>
<td><em>Bombus trifasciatus</em></td>
<td>Y</td>
<td>20.935±3.098b</td>
<td>91.394±5.191b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Bombus grahami</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Apis cerana</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Andrena sp.</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Halictidae</td>
<td></td>
<td></td>
<td><em>Lasioglossum spp.</em></td>
<td>Y</td>
<td>31.359±2.437a</td>
<td>224.734±4.262a</td>
</tr>
<tr>
<td>Putative hybrid</td>
<td>Diptera</td>
<td>Syriphae</td>
<td><em>Baccha maculata</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Episyrphus balteatus</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Ringia sp</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Meliscaeva cinctila</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>Halictidae</td>
<td></td>
<td><em>Lasioglossum spp.</em></td>
<td>Y</td>
<td>33.688±1.321a</td>
<td>227.034±68.453a</td>
</tr>
<tr>
<td>Anthophoridae</td>
<td></td>
<td></td>
<td><em>Anthophora spp.</em></td>
<td>Y</td>
<td>5.333±0.688c</td>
<td>27.363±5.948c</td>
</tr>
<tr>
<td>Apidae</td>
<td></td>
<td></td>
<td><em>Bombus grahami</em></td>
<td>N</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

**Table 4** Fruit set and germination set of the three *Epimedium* species under different pollination treatments.

<table>
<thead>
<tr>
<th>Pollination treatment</th>
<th><em>E. pubescens</em> ( )</th>
<th><em>E. wushanense</em> ( )</th>
<th>The putative hybrid ( )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inflorescence/flowers</td>
<td>Fruit set (n, %)</td>
<td>Gremination set (%)</td>
</tr>
<tr>
<td>Natural control</td>
<td>14/331</td>
<td>n=219, 66.34%</td>
<td>89.42</td>
</tr>
<tr>
<td>Automatically self-pollination</td>
<td>12/213</td>
<td>0, 0</td>
<td>0</td>
</tr>
<tr>
<td>Artificial self-pollination</td>
<td>10/68</td>
<td>0, 0</td>
<td>0</td>
</tr>
<tr>
<td>Conspecific cross-pollination</td>
<td>9/62</td>
<td>n=54, 87.47%</td>
<td>87.98</td>
</tr>
<tr>
<td>× <em>E. pubescens</em></td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>× hybrid</td>
<td>10/60</td>
<td>n=11, 18.92%</td>
<td>30.04</td>
</tr>
<tr>
<td>× <em>E. wushanense</em></td>
<td>12/60</td>
<td>n=14, 24.11%</td>
<td>25.85</td>
</tr>
</tbody>
</table>
Figure 1

Flowering phenology of *Epimedium* species. (a) presents flowering amplitudes curves, and (b) frequency distributions of the flowering intensity (mean±SE). Different lowercase letters indicate significant differences between species (ANOVA, *p* < 0.05).
Figure 2

Plant morphology (A is *E. pubescens*, B is *E. wushanense*, C is putative hybrid) and the blossom dynamic of three *Epimedium* (a–c is *E. pubescens*, d–f is *E. wushanense*, g–i is putative hybrid).

![Bar chart showing nectar volume for different flowering days and species.](chart)

Figure 3

The single flower presents nectar volume (mean±SE) of three *Epimedium* species. Different lowercase letters indicate significant differences between species (ANOVA, *p* < 0.05).

![Bar chart showing nectar volume for different flowering days and species.](chart)
The pollinators of *Epimedium* species. (a) and (b) are *Lasioglossum* spp. of *E. pubescens*, (c) is *Apis* sp. of *E. pubescens*, (d) is *B. trifasciatus* of *E. wushanense*, (e) is *Lasioglossum* spp. of *E. wushanense*, (f) is *B. grahami* of *E. wushanense*, (g) is *Lasioglossum* spp., of the putative hybrid, (i) is *Anthophora* spp. of putative hybrid, (j) is *B. grahami* of putative hybrid.