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Full Length Article

Effect of Morphs on Reproductive Biology of *Epimedium pubescens* (Berberidaceae): A Species Endemic to China

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Abstract

Differences in floral morphologies can strongly affect pollination behavior and efficient propagation. In this paper, the population of *Epimedium pubescens,* a species endemic to China, was observed to reveal its floral morphs effect on reproduction. In *E. pubescens*, three different floral morphs were known: (i) Styles of 25.33% flowers grew longer than stamens (LS). (ii) Pistil equal to or shorter than stamens was also observed in 7.33% flowers (HS). (iii) Styles of 67.33% flowers grew shorter than stamens, these styles grew abaxially away from stamens or stamens of those flowers would spread out for about half hour to depart from style (CS). The quantitative characters of in leaf number, leaf length, sepal length and width, filament length, style length, pollen number, P/O ratio, the length and width of inner sepal and the length of filament and style were significant differences in three floral morphs. This may be due to the differences in environmental factors, especial the relative humidity. The fruit set and seed set were no significantly different in three floral morphs, but the fruit set and seed set of HS are relatively lower than other two morphs. This may be related to pollinator visiting behavior, pollinators of HS were easily to contact with mixed pollen. The results suggested that floral morphs and pollinator behavior could effect on reproductive biology of *E. pubescens.* © 2018 Friends Science Publishers

Keywords: *Epimedium pubescens*; Reproductive biology; Floral morphs; Pollination

Introduction

Floral morph is very important factor, which can affect pollen removal and deposition by foraging behavior of the pollinators and influence the reproduction (Wolfe and Barrett, 1987; Syafaruddin *et al.*, 2006; Li *et al.*, 2009; Tsai *et al.*, 2017). Among features of floral morph, especially the herkogamy could strongly influence pollination efficiency, which is spatial separation of sexual organs (Webb and Lloyd, 1986; Sakai and Toquenaga, 2004). Through these mechanisms, the pollen–stigma interference is reduced, and self-fertilization could be avoided, and outcross-pollination is promoted (Guo *et al.*, 2013). So it is very important to understand the variation in floral morphs and to study their correlation with the sexual reproductive system.

Epimedium, is known as Chinese traditional medicine herb (Stearn, 2002; Ward, 2004; China Pharmacopoeia, 2005; Xu *et al.*, 2008). It has been widely used for treating cardiovascular diseases and other chronic illness (Wu *et al.*, 2003; Meng *et al.*, 2005; Chiu *et al.*, 2006). Because of over-harvesting and habitats destruction, the wild resources of *Epimedium* species become endangered recently (Ward, 2004; Xu *et al.*, 2007, 2008). Understanding the floral morphs and pollination biology of *Epimedium* is essential for conservation, development and utilization of medicinal materials. The reproductive biology of *Epimedium* such as Japanese *Epimedium* had already been well studied (Suzuki, 1983, 1984, 1987), but only one study on pollination biology of Chinese *Epimedium* has been reported (Li *et al.*, 2009).

Epimedium pubescens, a herkogamic species endemic to China, is considered as one of the most important *Epimedium* species. From the perspective of floral morphology, *E. pubescens* is very different from other species of Japanese *Epimedium* such as *E. diphyllum, E. sempervirens* and *E. trifoliatobinatum* (Suzuki, 1983, 1984). The differences in flowers indicated that each species may have its own pollination system in *Epimedium* spp. (Macior, 1971; Faegri and Pijl, 1979). Furthermore, the habitats of *Epimedium* in Japan and China are also quite different. Fewer studies paid attention to the association between floral morphs and reproductive biology of *E. pubescens.* So we sought to answer the following specific questions (1) What are the details of floral morphs and breeding system? (2) How does floral morphs and the pollinator affect reproductive biology of *E. pubescens*?

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Materials and Methods

Study Site

Field studies were was conducted in a protected area of Jinchengshan Forest Park (106°28′E 30°45′N, at altitudes of 568–790 m) northeastern Sichuan, China from February to April in 2014 and 2015. During experimental period, the climate was rainy and cloudy, the average temperature was about 10.28~17℃ and the humidity 81%~92%. The population of *E. pubescens* covered the size of 400 m^2. The species growing with this species were *Artemisia argyi*, *Anemone hupehensis*, *Capilipedium prviflorum*, *Cayratia japonica*, *Debergeasia edulis*, *Duchesnea indica*, *Mahonia fortunei*, *Pilea notata*, *Iris tectorom* and *Reimeckea camea*.

The Species

E. pubescens is a perennial herb, 15~60 cm high, grows in moist and rocky habitats. Inflorescence is panicle, with 50 to 80 small flowers. The flowers are hermaphrodite, the floral structures from outside to inside are as follow: Four ovate-triangular outer sepals are dark green, four lanceolate inner sepals are white color, four conical spurs are pale yellow, four stamens are green, extended, valvular, rounded with anthers. Inflorescence emerges from February to April and the fruit stage is from April to May (Ying and Chen, 2001).

To see whether floral morphs is associated with various environmental conditions, the air humidity, temperature, relative light intensity were measured using the thermometer and hygrometer (KIMO HD100) and digital lux meter, respectively. (FieldScout Foot-candle Meter, Spectrum Technologies, Inc.).

Vegetative and Floral Traits

To see whether the vegetative and reproductive traits were different among long, homo- and curve-style morphs, we randomly selected 25 plants per morph and measured and calculate four vegetative traits: plant height, leaf number, leaf length and width, fourteen reproductive traits: inflorescence height, inflorescence number, flower number, sepal length, sepal width, spur length, spur width, filament length, style length, pollen grain number, ovule number, pollen/ovule ratio (P/O), fruit set and seed set. Three floral morphs developmental events, including the flowering phase, stamen, style elongation and anther dehiscence was recorded.

Breeding Systems

To study the reproductive system of *E. pubescens*, we randomly chose 50 plants, and four pollination treatments were conducted. (1) Open pollination: the flowers were left for open pollination. (2) Completely enclosed in bags: all flowers were completely bagged before anthesis. (3) Artificial autogamy: flowers from plants were bagged just before flowers started to open. Manual pollination was applied with mature pollen from the same flower until stigmas of all flowers were fully pollinated. (4) Artificial xenogamy: the flowers were pollinated with pollen collected from another population. The fruit sets were recorded during fruiting period.

Pollinator Preference and Efficiency

To determine the visitor species and compare the visit rates among long style, homostyles and curve style, flower visitors were observed and collected in the field and in each population, three patches (patch size: 1 m^2) were established. Observations were performed from 8:00 am to 18:00 pm, and lasted for 4–5 days, for total 90 h under different weather conditions. The bees usually came to the patch to forage for both nectar and pollen. We recorded the visitor species and the visits. If their bodies contacted anthers, we recorded the visits as pollinator visits. Finally in the afternoon, we counted the total number of each morph. The visiting insects were captured and preserved in bottle with cotton containing ethyl acetate for identifying and examining the presence/absence of pollen by dissecting microscope.

To compare of pollinator efficiency to long style, homostyle and curvestyle morphs, the pollen deposition and removal of pollinators were conducted. Thirty plants of each morph were randomly chosen and virgin male-phase inflorescences were bagged until anther dehiscence. Each inflorescence was allowed a single visit by a bee. To estimate pollen removal, we collected about 30 visited flowers and 30 unvisited flowers as controls from these inflorescences, and each flower was stored in a centrifuge tube with 75% alcohol. Pollen removal per flower was calculated from the mean number of pollen grains in unvisited flowers minus the mean number of pollen grains remaining after one visit. To estimate pollen receipt per visit, we removed undehisced anthers from 30 male-phase flowers of each morph and bagged the flowers with cotton mesh until they developed into the female phase. These female-phase inflorescences were uncovered and allowed one visit by the bee. Stigmas of the 30 visited emasculated flowers were collected and stored in a centrifuge tube with alcohol. In the laboratory, we counted pollen grains from the anthers and on the stigmas under a light microscope (Gong and Huang, 2014).

Statistical Analyses

All data were analyzed using GLM analysis as a posterior test with SPSS 19.0 software (IBM Inc., New York, USA). Chi square test ($\chi 2$) was applied to find differences among the treatments.

Results

Effect of Environmental Factors on Three Morphs Distribution

The temperature and relative light intensities did not differ

Fig. 1: The floral characteristics of *E. pubescens.* A. Styles of flowers grew longer than stamens (LS). B. Pistil equal or shorter than stamens was also observed in a few flowers of *E. pubescens* (HS). C. The styles grew abaxially away from stamens (CS). A1 Anther dehiscence of SLS. B1 Anther dehiscence of HS. C1-C2 Anther dehiscence of CS

significantly among longstyle, homostyle and curvestyle morphs, but the relative humidity of homestyle was significantly higher than other flora morphs, while there was no significant difference between long styles and curve styles (Table 1).

Vegetative and Floral Traits

There was no significant difference among three floral morphs in the plant height, leaf width, inflorescence height, inflorescence number, spur length and width, ovule number (Table 2). The leaf numbers of long styles and homestyle were significantly higher than curve styles ($P<0.01$). The leaf lengths of long styles were remarkably longer than the other morphs, while the curve styles were shortest among three morphs ($P<0.01$). The sepal length of long styles was significantly higher than curve styles, which were significantly higher than the homestyle ($P<0.01$). There was no remarkably difference between long styles and curve styles in sepal width, which was significantly higher than homestyle ($P<0.01$). The filament length of curve styles was much longer than other floral morphs, while the style length of long styles was longer than other morphs. There was no remarkably difference between long styles and homestyle in pollen grain number, which were significantly higher than long styles ($P<0.01$). The P/O ratio in curve styles was higher than long styles, while there was no significantly between curve styles and homestyle or between long styles and homestyle ($P<0.05$). The flower is scentless, nectariferous and grows in a downward orientation. Each flower has four erect anthers. The shedding sequence is out sepal, stamen, spur and inter sepal sequentially.

Three floral morphs in terms of the relative position between pistil and stamens were observed during flowering (Fig. 1A–C). 1) Styles of 25.33% flowers grew longer than stamens (LS), the split anther could hardly contact to stigmas when pollen were shed in this scenario (Fig. 1A–A1). 2) Pistil equal to or shorter than stamens was also observed in 7.33% flowers (HS) and no obvious changes of distance between them were observed during flowering (Fig. 1B–B1). 3) About 67.33% flowers showed equal length of pistil and stamens or the pistil shorter than stamens (Fig. 1C), and the style would grow abaxially away from stamens (Fig. 1C1) or stamens of those flowers would spread out for about half hour to depart from style, then close slightly to pistil (Fig. 1C2). The fruit sets indicated that there are no significant difference in fruit sets ($P>0.05$), but the fruit sets of HS are relatively lower than other two morphs (Table 2).

Pollinators Behavior and Visitation Frequency

Nine insect species were found visiting flowers of *E. pubescens* in plots (Table 3). Generally, floral visitors of *E. pubescens* did not visit flowers until the anthers dehisced. *E. pubescens* was visited by both pollen-collecting and nectar-foraging bees. Detection by light microscopy showed that pollen was only found on the abdomen and legs of *Andrena emeishanica*, which implied that *A. emeishanica* was the only effective pollinators. *A. emeishanica* hovered around the racemose base for choosing a flower with dehisced anthers, and hung reversely on the flower by grasping the filaments with its front legs, then held the anthers firmly with its middle and hind legs. After staying on a flower, the slightly tilted tail of *A. emeishanica* could cover nearly the whole anther, which kept turning its body to forage nectar from four spurs. While foraging nectar, the *A. emeishanica* scraped continuously pollen grains with its middle and back legs. During this process, pollen grains on the abdomen of *A. emeishanica* gathered from other flowers were easily loaded on the stigma of long styles (Fig. 2A). In homostyle flowers, the pollen could be spread on the abdomen of *A. emeishanica*, which could hardly contact to stigmas until the filament loosed by pollinators, then the stigma received mixed pollens (pollen in this flowers and other pollen) (Fig. 2B). In curve style flowers, the stigma can easily touch the extraneous pollen carried by *A. emeishanica* (Fig. 2C).

Because of relatively high temperature and low humidity, the peak period of visiting activity by insects in a day occurred from 13:00 to 14:00 pm. No visitor was observed during rainy days. The visitation frequency was not significant different among the three morphs (Wald $\chi 2=0.71$, $P=0.70$, N=35, 33, 26) (Fig. 3).

Pollen Deposition and Pollen Removal

In emasculated treatment, there was no significantly

Table 1: Comparison of environmental factors (mean ± SE) between the three morphs in *E. pubescens* tested by GLM analysis

Weather condition	Long styles	Homestyles	Curve styles	Wald χ2	Sig.
Temperature (°C)	18.37±0.32[a]	16.67±0.55[a]	18.03±1.77[a]	1.37	0.50
Relative humidity (%)	59.28±5.05[b]	67.44±0.32[a]	57.59±1.44[b]	74.40	0.00
Relative light intensity (%)	62.26±8.49[a]	42.73±1.39[a]	55.73±6.92[a]	4.86	0.08

Table 2: comparisons of vegetative and reproductive traits (mean ± SE) among three floral morphs in *E. pubescens*

Plant characters	Long styles	Homestyles	Curve styles	Wald χ2	*p*
Plant height (cm)	27.41±2.12[a]	23.34±2.32[a]	30.40±2.32[a]	4.64	0.09
Leaf number	39.67±4.23[a]	43.40±4.63[a]	14.40±4.63[b]	23.59	0.00
Leaf length (mm)	14.17±1.23[a]	8.40±1.34[b]	5.00±1.34[c]	26.20	0.00
Leaf width (mm)	10.40±0.62[a]	10.23±0.68[a]	8.72±0.68[a]	3.80	0.14
Inflorescence height (cm)	37.41±2.12[a]	33.34±2.32[a]	40.40±2.32[a]	4.64	0.09
Inflorescence number	2.00±0.50[a]	1.20±0.54[a]	2.20±0.54[a]	1.88	0.39
Flower number	54.09±5.37[a]	42.00±7.82[a]	37.82±5.37[a]	4.81	0.09
Sepal length (mm)	12.68±0.30[a]	10.01±0.176[c]	11.48±0.42[b]	22.72	0.00
Sepal width (mm)	2.56±0.09[a]	2.24±0.06[b]	2.51±0.06[a]	6.10	0.00
Spur length (mm)	2.64±0.06[a]	2.51±0.08[a]	2.66±0.07[a]	1.05	0.35
Spur width (mm)	1.17±0.07[a]	1.06±0.02[a]	1.15±0.04[a]	1.93	0.15
Filament length (mm)	5.06±0.09[b]	5.09±0.08[b]	5.16±0.05[a]	3.14	0.04
Style length (mm)	3.51±0.09[a]	2.87±0.06[c]	3.17±0.05[b]	16.82	0.00
Pollen grain number	7357.40±27.12[b]	7785.80±27.90[a]	7760.50±27.85[a]	154.12	0.00
Ovule number	6.10±0.78[a]	5.20±0.72[a]	4.800±0.69[a]	1.58	0.45
Pollen/ovule ratio	1221.40±119.39[b]	1573.24±119.39[ab]	1925.24±119.39[a]	8.92	0.01
Fruit set (%)	33.08± 5.27[a]	20.75±5.55[a]	24.65± 5.16[a]	2.98	0.22
Seed set (%)	82.95± 1.86[a]	72.01± 4.43[a]	81.00± 2.57[a]	3.57	0.17

different among three morphs in pollen deposition. In non-emasculated treatment, the pollen removal of long and curve styles had no significant difference, which were significantly higher than the homestyle. While the pollen deposition of the pollen removal long and curve styles were significantly lower than the homestyle (Table 4).

Breeding Systems

Thirteen panicles were bagged to test the self-incompatibility and no fruits were formed in those inflorescences (Table 5). Other 22 blooming inflorescences in the same population, which were subjected to open pollination gave high fruit-set. The fruit setting percentages were 46.86 – 56.57%. Fruits could not be set in the flowers only with stamens or pistil, or in the flowers being artificial self-pollinated. Artificial cross pollination among genets improved fruit setting with 54.55–58.00% setting rate. Variation among years for all data was insignificant (P＞0.05).

Discussion

Our survey indicated that the *E. pubescens* has three floral morphs widespread in natural populations. An investigation of environmental factors suggested that the homostyled morph tended to occur in higher relative humidity.

The P/O ratio and bagging experiment indicated that breeding systems of *E. pubescens* is self-incompatible and outcrossing. This is similar to that of *E. grandiflorum, E. sempervirens* and *E. sagittatum*, but is diffrent from *E. diphyllum* and *E. trifoliatobinatum*, which are not completely self-incompatible in Japanese *Epimedium* species (Suzuki, 1983, 1984).

Table 3: Floral visitors of *E. pubescens*

Order	Family	Species	Reward
Diptera	Syrphidae	*Melanostoma scalare*	Pollen
		Episyrphus balteatus	Pollen
		Ringia sp	Pollen
Hymenoptera	Apidae	*Nomada* sp1	Pollen
		Nomada sp2	Pollen
		Tetralonia sp	Pollen
		Apis sp	Pollen
	Colletidae	*Colletes* sp	Pollen
	Andrenidae	*Andrena emeishanica*	Nectar and pollen

Fig. 2: *Andrena emeishanica* visiting behavior in long styles (A), homestyle (B) and curve styles (C) of *E. pubescens.* Red arrow showed the position that the stigma contacted the pollinator' body

Table 4: The deposition and pollen removal of three floral morphs

Item		Long styles	Homostyled	Curve styles	Wald χ2	Sig.
Emasculated (15, 16, 16)	Deposition on stigmas	28.86±1.38[a]	30.500±1.38[a]	22.56±1.18[b]	22.13	0.00
Unemasculated (26, 30, 26)	Pollen removal (%)	38.28±4.17[a]	33.03±3.88[a]	34.79±4.17[a]	0.86	0.64
	Deposition on stigmas	91.62±1.87[c]	228.33±2.75[a]	99.58±1.951[b]	1878.47	0.00

Means with same letter in a column different non-significantly (P>0.05)

Table 5: The capsule set of *E. pubescens* under different treatments

Treatment	Type	plants	flowers	Fruit	Fruit set (%)
Open pollinated	LS	11	595	190	33.08
	HS	11	522	102	20.75
	CS	11	416	86	24.65
Completely enclosed in bags	LS	4	188	0	0
	HS	4	260	0	0
	CS	4	291	0	0
Artificial autogamy	LS	4	101	0	0
	HS	4	83	0	0
	CS	4	77	0	0
Artificial xenogamy	LS	10	36	23	63.88
	HS	10	24	12	50.00
	CS	10	30	12	40.00

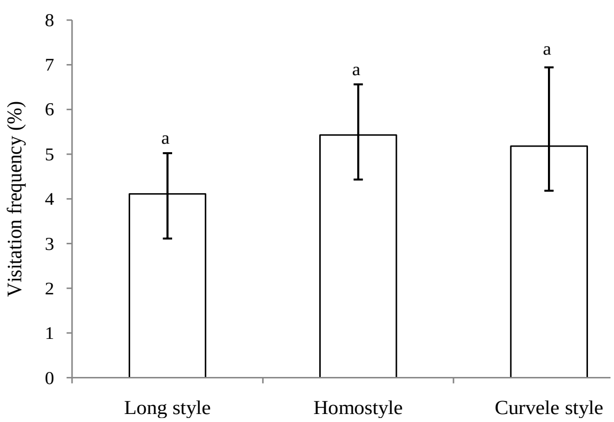

Fig. 3: The visitation frequency of three floral morphs

Floral morph is usually considered to be very important factors to affect the pollination efficiency during visitation by pollinators (Syafaruddin *et al.*, 2006; Pang *et al.*, 2012; Freitas *et al.*, 2017) and these traits promote pollinator-mediated crosspollination and to reduce the wastage of pollen grains, which can lead to affect the fruit set and seed set (Tsai *et al.*, 2017). Among the flower structures, especially pollen-stigma interference strongly influences pollination efficiency (Elle and Hare, 2002; Guo *et al.*, 2013; Freitas *et al.*, 2017) The SLS and SAS could increase the distance between stigma and anthers, which avoided interference between the sexes in *E. pubescens*. When pollinators come, the pollen grains carried by visitors could be easily make contact with the extended or curved stigma, which might enhance the possibility of outcrossing. Previous studies demonstrate that extended distance between pistil and stamen or longer styles could enhance outcrossing (Van kleunen and Ritland, 2004; Syafaruddin *et al.*, 2006). If the lengths of style and stamen are equal, the dehisced anthers would be higher than stigma and the pollen might be very easy to contact pollinators, which might lead to contact stigmas, interfere with the growth of outcrossed pollen tubes, and reduce fecundity (Palmer *et al.*, 1989). This may explain why flowers of LS and CS got high fruit set. The movement of HS in *E. pubescens* is an effective mechanism to reduce interference between male and female sexual functions, encourage outcrossing and avoid selfing. That might be the reason that number of fruits in HS is obviously no significantly different than that in LS and CS. Thus, we can conclude that the herkogamy, which would make the anther–stigma be separated are favorable to outcrossing. This is analogous to the floral characteristics of *E. sagittatum* (Li *et al.*, 2009), while in *Brassica rapa* the position of the pistil had less effect on F1 (Syafaruddin *et al.*, 2006). Because of the foraging behavior of pollinators, the herkogamy of *Narcissus longispathus* did not enhance outcrossing pollination (Medrano *et al.*, 2005).

Our results indicated that the *A. emeishanica* was an the effective pollinators of *E. pubescens*, as their bodies carrying and transporting pollen grains. The nectar-sucking and pollen were the important food for *A. emeishanica*, which plays an important role for outcrossing pollination in *E. pubescens*. In Japanese *Epimedium* and *Tetralonia* sp. are the effective pollinators in *E. sempervirens* and hybrid derivatives of *Epimedium* sp. *Tetralonia* sp. was observed on *E. pubescens*, but it was not an effective pollinators of *E. pubescens* (Suzuki, 1984). The *Andrena* sp. was commonly observed in populations of all the species of Japanese *Epimedium*. *E. pubescens* has short spurs compared with other *Epimedium* species, the visiting behavior of *Andrena* sp. was similar to the pollinators in *E. grandiflorum*. The spurs and pollen are the attractants to pollinators in *E. pubescens*, and the rewards for pollinators are pollen and nectar.

Conclusion

According to the relative position between pistil and stamens, the *E. pubescens* have three floral morphs, which are LS, HS and CS. The fruit set and seed set of HS are relatively lower than other two morphs. The *A. emeishanica* is the pollinator of *E. pubescens*, and the rewards are pollen and nectar. They have different behaviors in three floral morphs in *E. pubescens*. The floral morphs and pollinator behavior could remarkably effect on reproductive biology of *E. pubescens*.

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