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FLOWERING PHENOLOGY AND REPRODUCTIVE CHARACTERISTICS OF *CYPRIPEDIUM MACRANTHOS* (ORCHIDACEAE) IN CHINA AND THEIR IMPLICATION IN CONSERVATION

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Abstract

The phenology and reproductive characteristics of *Cypripedium macranthos* were studied in China. This slipper orchid is mainly distributed outside of the diversity center of the genus *Cypripedium* in China and needed urgent protection. The plant favored the weakly acidic soil and the pH of soil of 7 populations in Beijing varied from 5.85-6.92. Moreover, the organic matter differed from 9.66%-22.84%, which belonged to the first grade according to the classification criterion of soil nutrient in China. The process of germination, anthesis, pollination, fructification, flower withering, plant withering and dormancy of *C. macranthos* were recorded. It usually opened around the first ten-days period of June and proceeded until the end of June or the beginning of July at population level. A single flower without pollinium removal or deposition lasted about 9.42±1.81 d (*n*=36), while the mean flowering time of those with pollinium deposition is 6.80 ±1.30 d (*n*=109). The period with low temperature might play an important role in development of plant and bud. *C. macranthos* is self-compatible and pollinator limitation. The number of seeds was 16254±9750 per capsule in hand-self-pollination and 15638±8257 in hand-cross-pollination, which were about 2.5 times more than that (5808±2633 per capsule) in natural pollination. So hand-pollination with more pollen might increase the number of fruits and seeds. Therefore, vegetation protection, suitable temperature of selected nursery place and hand-pollination would be important strategies in conservation of *C. macranthos* and might contribute more or less to resume its population.

Introduction

Slipper orchids such as *Cypripedium* spp. and *Paphiopedilum* spp. are very popular as ornamental in many countries, especially in western countries (Cribb, 1997; Chen & Tsi, 1998). For their high economic value, slipper orchids face much more critical situation to be protected and earned lots of attention. The genus *Cypripedium* consists of approximately 50 species and has a wide distribution from sea level to high altitude and from subtropical zone to temperate zone (Cribb, 1997). Its high diversity can been found in North America and temperate Asia. China is the main distribution and has 36 species, and most of them are peculiar in Southwest China (Li *et al*., 2005). But as an exceptional case, *C. macranthos* cannot be found in Southwest China which is the center of diversity of the genus *Cypripedium*. It is mainly distributed in Northeast China, North China and Taiwan (Chen, 1999).

Until now, few cultivated plant of *Cypripedium macranthos* can be provided to business. The wild plants collected from China are one of the main sources for smuggled goods. At present, *C. macranthos* is sporadic and scattered in the field (Luo *et al.*, 2003; Zhang, 2009). If there is no full-scale conservation, it is forecasted that several populations will soon be disappeared in near future. Nowadays, several studies focus on the phylogeny and biogeography of the genus *Cypripedium* (Cox *et al*., 1997; Li *et al*., 2011; Guo *et al.*, 2012). Moreover, there are some studies on pollination biology of the slipper orchids, especially those in Southwest China (Li *et al.*, 2008a, 2008b; Ren *et al.*, 2011; Zheng *et al.*, 2011).

Flowering phenology, pollination and genetic diversity of *Cypripedium macranthos* var *.rebunense*, a threatened lady's slipper (Orchidaceae) in Japan, have been reported (Sugiura *et al*., 2001, 2002; Izawa *et al*., 2007). However, knowledge about the process of native plant growth and its suitable habitat will help us to formulate professional protection strategy (Qiao *et al*., 2012). In this study, we focused on the key steps in life history of *C. macranthos*, such as growth, flowering and reproduction. And we hoped the basic data would play important role in protection of *C. macranthos*.

Materials and Methods

Study species: *Cypripedium macranthos*, one of the endangered species in Orchidaceae, usually grows on humus-rich soils and well-drained places in forests, forest margins and grassy slopes (Cribb, 1999). As a slipper orchid, its lip is deeply pouched, subglobose or ellipsoid and its pollen is glutinous (Fig. 1A, B) (van der Pijl & Dodson, 1966; Chen, 1999).

Study site: We did resources surveys in the main distribution of *Cypripedium macranthos* in China, especially in Beijing in 2004-2011 and did soil analysis of 7 populations in Beijing. The soil organic matter concentration was determined by ash content test. The total N content was tested by Kjeldahl nitrogen method. The other mineral elements were measured by atomic absorption spectrum (AAS, GBC9932AA).The pH of soil was measured in distilled water with a soil: H_2O ratio of 1:5.

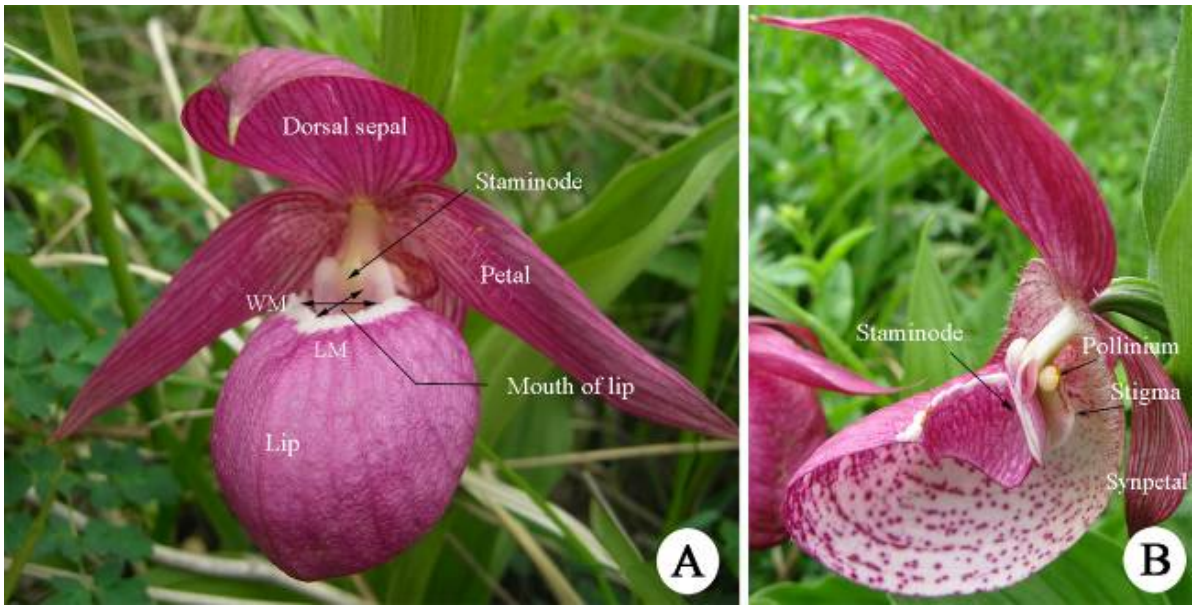

Fig. 1. Flower morphology.
A: Close view of an individual flower of *Cypripedium macranthos*
LM: The length of mouth of lip
WM: The width of mouth of lip
B: Longitudinal section of the flower of *C. macranthos*

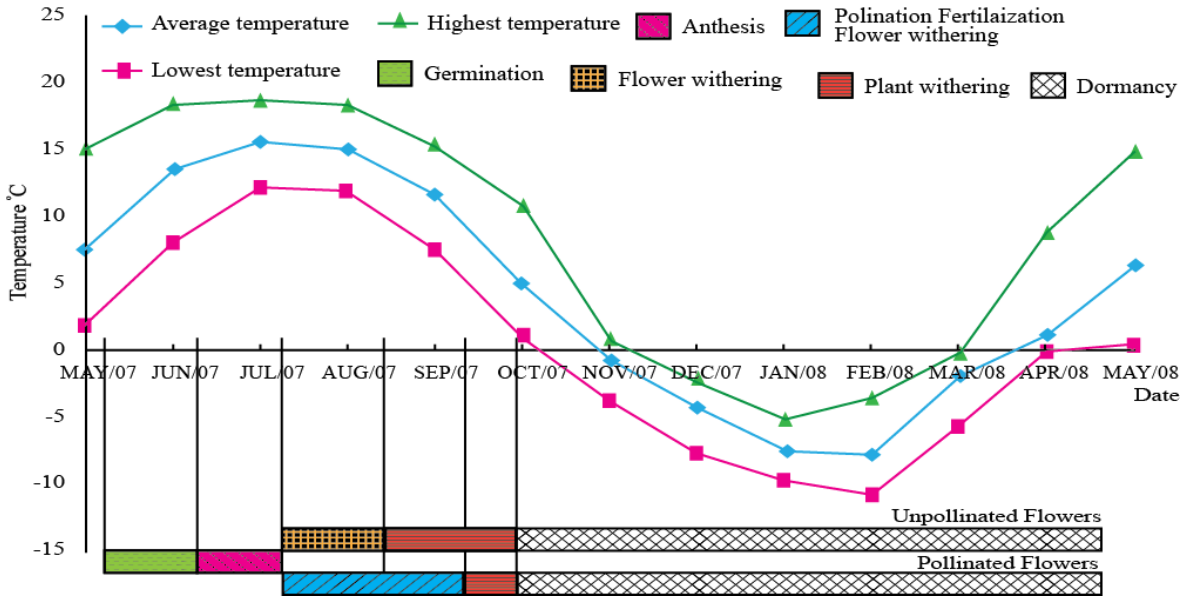

Fig. 2. The phenology and temperature record in life cycle of *Cypripedium macranthos*.

Baihua Mountain Nature Reserve was selected to study plant growth and development. As it is a perennial plants and cannot be located consecutively because their overground parts wither away in autumn every year. In order to fix the plants for constant observation, the sites of plants in a quadrat (10m×10m) were marked with tags in June, 2005. And we recorded phenology of *Cypripedium macranthos* in this quadrat where we did not do any manual intervention.

Flower morphology: 33 opened flowers were randomly chosen for morphological measurement. The height of plant were measured with a ruler to the closet 1 mm while the length and width of dorsal sepal, sysepal, petal, staminode and leaf were measured with digital caliper to the closest 0.01 mm. Moreover, the length (LM) and width (WM) of mouth of lip were also recorded (Fig. 1A).

Phenology of *Cypripedium macranthos*: We recorded the process of germination, anthesis, pollination, fructification, flower withering, plant withering and

dormancy of *Cypripedium macranthos*. If necessarily, we dug out the underground part to study its development. We monitored both the flowering time of the individual flower and the flowering period of the population. A flower was recorded as open when its dorsal sepal separated from the labellum and as withered when the flower colour changed. At population level, the flowering period was recorded from the anthesis of the first flower to the end of the last one.

At the same time, one temperature recorder (HOBO TBI32-20+50) was set in root zone underground to successively record soil temperature one time per half hour during the whole growth season. Then we found the lowest temperature, the highest one and calculated the average temperature every month during the whole life of *Cypripedium macranthos* in 2007 and 2008.

Moreover, as sympatric plants may play important roles in life cycle of *Cypripedium macranthos*, such as pollination stage, we recorded the co-flowering plants.

Manual reproductive experiments and natural pollination success: Buds were randomly selected and bagged in order to study the breeding system. The following manual pollination trials were carried out on the second or third day of anthesis: 1), Unmanipulation. No hand-pollination was attempted to find whether autogamy occurred. 2), Castration. The pollinaria were taken away to find whether apomixes occurred. 3), Hand-manipulated self-pollination. The pollinium was directly transferred to the stigma of the same flower. 4), Hand-manipulated cross-pollination. The stigma of the treated flower was deposited by pollinium of the flower from another clone at least 5 m away. All the flowers were rebagged after management. Fruit set of the plants in those four treatments were recorded at the end of flowering periods when ovaries of the flowers were distinctly expanded. Manual reproductive experiments in treatment 3 and 4 were repeated in 2005-2008, while those in treatment 1 and 2 were only set in 2005. Moreover, the randomly marked plants in the population were noted as natural pollination treatment each year. Because the total number of flowers was limited and different among years, we did not set the same number of each treatment each year.

Number of seeds per capsule in different treatments: Five capsules of self-pollination, cross-pollination and natural pollination were randomly chosen in order to count seeds.

Statistical analysis: All analyses were performed in SPSS 13.0 for Windows. The data of the morphology of the plants were analyzed by SPSS using descriptive statistics. The variations in the rates of fruit set between different treatments were analyzed by on-way ANOVA.

Results

Resource distribution: After years' survey in China, we totally found about 24 populations of *Cypripedium macranthos*. Among these, 7 populations were in Beijing. But all of the populations are considered as extremely small populations and needs urgent conservation according to the criterion of State Forestry Bureau in China.

Soil constituent analysis: The results of soil analysis are given in Table 1. The pH values of soil from 7 populations in Beijing are 5.85-6.92, so *Cypripedium macranthos* favors the weakly acidic condition. The contents of organic matter in the soils where the slipper orchids grow differed from 9.66%-22.84%.

Phenology of *Cypripedium macranthos* and temperature record: The earth in Baihua Mountain Nature Reserve usually thaws in May every year. *Cypripedium macranthos* can always be found in meadow or forest edge. Its co-flowering plants were *Lysimachia stenosepala, Deutzia parviflora, Spiraea pubescens Aquilegia yabeana* and *Thalictrum petaloideum*. The plant of *C. macranthos* began to germinate and grow rapidly at middle of May when most of sympatric species were still at dormancy period. It usually flowered around the first ten-days period of June and proceeded until the end of June or the beginning of July at population level. The flowering period usually lasted almost 20 days. Single flower without pollinium removal or deposition lasted about 9.42±1.81 d (n=36), while the mean flowering time of those with pollinium deposition was 6.80 ±1.30 d (n=109). The ovary of the pollinated flower developed into fruit. The overground parts successively withered from late August to early September, but the whole plant that had capsule withered after fruit maturation and could be found until the end of September. The complete life cycle is shown with the curve of ground temperature supplied by temperature recorder from May, 2007 to May, 2008 (Fig. 2).

Floral morphology: The results of characteristics of floral morphology are shown in Table 2. The coefficients of variation of both length and width of vegetative organs (stem and leaf), were higher than those of reproductive organs (dorsal sepal, synsepal, petal, staminode and lip) except the width of petal.

Table 1. The soil nutrient content in seven populations in Beijing.

Population	Organic matter %	Total nitrogen %	CaO %	K_20 %	MgO %	P_2O_5 %	pH
1	21.35	0.43	2.17	2.28	1.50	0.32	6.37
2	9.66	0.26	1.26	2.46	1.74	0.25	6.28
3	18.17	0.49	1.94	2.26	1.51	0.30	6.00
4	22.84	0.66	2.32	2.21	1.48	0.33	5.99
5	12.00	0.66	1.50	2.37	1.65	0.30	5.85
6	16.85	0.55	1.93	2.28	1.54	0.32	5.90
7	9.84	0.26	3.67	1.93	2.76	0.27	6.92

Table 2. The morphology of *Cypripedium macranthos*.

Plant morphology		Size (mm)	Coefficients of variation
		Mean ± SD	
Stem	Height	218.6 ± 35.9	0.164
Leaf	Length	126.994 ± 26.165	0.206
	Width	57.455 ± 11.748	0.204
Dorsal sepal	Length	47.424 ± 5.272	0.111
	Width	30.816 ± 4.914	0.159
Synsepal	Length	38.751 ± 5.117	0.132
	Width	25.374 ± 4.367	0.172
Petal	Length	52.439 ± 5.799	0.111
	Width	17.574 ± 3.534	0.201
Staminode	Length	14.925 ± 1.829	0.123
	Width	9.767 ± 1.074	0.110
Lip	LM	12.024 ± 2.084	0.173
	WM	11.521 ± 1.426	0.124

Table 3. The fruit set of manual reproductive experiments and natural pollination in 2005-2008.

Treatment	2005	2006	2007	2008
Unmanipulation	0	–	–	–
Castration	0	–	–	–
Hand-manipulated self-pollination	95.00%	95.92%	90.91%	80.00%
Hand-manipulated cross-pollination	90.00%	89.22%	78.49%	84.51%
Natural pollination	24.29%	25.71%	12.62%	10.13%

Table 4. The number of seeds in capsule of different treatments.

Treatment	No. of seeds per capsule
	Mean ± SD
Natural pollination	5808 ± 2633
Hand-manipulated self-pollination	16254 ± 9750
Hand-manipulated cross-pollination	15638 ± 8257

Fruit set of manual reproductive experiments and natural pollination: The results of manual reproductive experiments and natural pollination in 2005-2008 are shown in Table 3. No unmanipulated flowers and castrated ones matured to capsules. Totally, the rate of fruit set in hand-self-pollination was 80.00%-95.92%, while that of hand-cross-pollination was 78.49%-90.00%. The rate of fruit set in natural pollination was 10.13%-25.71%. The difference of the rate of fruit set was not significant between self-pollination and cross-pollination (p=0.319). The rate of fruit set in natural pollination had significant differences with self-pollination (p=0.000) and cross-pollination (p=0.000), respectively.

Number of seed per capsule in different treatments: The number of seeds per capsule was 16254±9750 in the capsules of hand-self-pollination (n=5) and 15638±8257 in hand-cross-pollination (n=5), which were about 2.5 times in both the treatments more than that (5808±2633 per capsule) in natural pollination (n=5) (Table 4).

Discussion

The specific habitat: The soil analysis showed that the organic matter (9.66%-22.84%) of the habitat of *Cypripedium macranthos* was higher than 4%, which is the minimum standard of the first grade according to the classification criterion of soil nutrient in China. The content of organic matter of uncultivated area mostly depended on degree of vegetation conservation (National Soil Survey Office, 1998). The orchids usually grew in the undisturbed site and the vegetation was well protected. The result of soil analysis was consistent with the habitat of orchids.

Low temperature and phenology: It is reported that vernalization has an effect on the growth and flowering of *Cypripedium macranthos* (Olver, 1981). During the life cycle of *C. macranthos*, subzero temperature usually can be found from October to the next April in Baihua mountain. This period might play an important role in the development of plant and bud, although no overground parts could be seen.

Cypripedium macranthos is reported in humus-rich soils and well-drained places in forests, forest margins and grassy slopes in China, Korean Peninsula and Russia.

In China, *Cypripedium macranthos* mainly distributed in Northeast China, North China and Taiwan, and no plant can be found in Southwest China which is the center of diversity of the genus *Cypripedium* (Chen, 1999). The high temperature in summer in southwest China might be one reason.

The effect of flowering in early spring: The soil in Baihua Mountain Nature Reserve does not ice-out until early May. The plant of *Cypripedium macranthos* usually germinated when most of the other plants were still in dormancy period. The flowers of *C. macranthos* were very noticeably for their purplish red color, while there were few other plants around. The fresh color might help to attract the limited pollinators in early spring (Nilsson, 1992; Jersakova *et al.*, 2006). Moreover, considering microenvironment of the subalpine mountaintop, it was usually rainy in our study site. According to the weather record, it was actually rainy in more than half of days in June. However, the sunny day still appeared more frequently in June than that in July and August. And the rain was usually much heavier in July and August. *C. macranthos* mainly bloomed in June when there were relatively larger number of sunny days and pollinators were active, so it could gain much more chance for pollination.

Reproductive success of *Cypripedium macranthos*: Our manual reproductive experiments demonstrated that *Cypripedium macranthos* is self-compatible but incapable of automatic self-pollination. Moreover, no apomixia was employed. Pollinium deposition can observably short the flowering time of *C. macranthos* from 9.42±1.81 (n=36) to 6.80 ±1.30 d (n=109).

The fruit set of natural pollination was much lower than that of hand-pollination and there were significant differences between them in 2005-2008 (p=0.000 between natural pollination and self-pollination, p=0.000 between natural pollination and cross-pollination). The mean number of seeds in the capsule of hand-pollination was much bigger than that of natural pollination. We speculated that it was caused by different amount of pollen onto the stigma. When we did hand-pollination experiment, the whole pollinium was used. But the pollinator could only be touched by a few of pollen in the natural pollination. Moreover, not all the pollen on the body of pollinator could reach the stigma of flower. So hand-pollination increased both the fruit set and the number of seeds in each capsule. Although it is a complicated course from seed to plant, the increase of fruits and seeds might contribute more or less to resume the population of *Cypripedium macranthos*.

The implications of specific habitat, phenology and reproductive characteristics for a conservation policy of *Cypripedium macranthos*: As the high content of organic matter in the habitat of *Cypripedium macranthos*, it is very important to protect the vegetation around and keep away from disturbance and damage during in-situ conservation. When we carry out ex-situ conservation, the weakly acidic and rich environment should be chosen. Moreover, a long period with subzero temperature is needed for plant development and blossom in the selected nursery place. Hand-pollination with enough pollen can be employed during breeding season in order to gain more seeds.

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