

Reproductive success of non-rewarding *Cypripedium japonicum* benefits from low spatial dispersion pattern and asynchronous flowering

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- **Background and Aims** Outcrossing animal-pollinated plants, particularly non-rewarding species, often experience pollinator limitation to reproduction. Pollinator visitation is affected by various factors, and it is hypothesized that reproduction in non-rewarding plants would benefit from low spatial flower abundance and asynchronous flowering. In order to test this hypothesis, the influence of spatial pattern and flowering phenology on male and female reproductive success (RS) was investigated in a non-rewarding orchid, *Cypripedium japonicum*, in central China over two flowering seasons.
- **Methods** The probabilities of intrafloral self-pollination and geitonogamy caused by pollinator behaviours were estimated from field observations. Pollinator limitation was evaluated by hand-pollination experiments. RS was surveyed in different spatial flower dispersal patterns and local flower densities. The effects of flowering phenological traits on RS were assessed by univariate and multivariate regression analyses.
- **Key Results** Hand-pollination experiments revealed that fruit production was strongly pollen limited throughout the entire reproductive season – over two seasons, 74.3 % of individuals set fruit following hand pollination, but only 5.2–7.7 % did so under natural conditions. Intrafloral self-pollination and geitonogamy within the potential clones might be rare. Both male and female fitness were substantially lower in clustered plants than in those growing singly. An increase in local conspecific flower density significantly and negatively influenced male RS, but had no effect on female RS. Phenotypic selection analysis indicated that individuals flowering earlier have the greatest probability of RS. Over 85 % of sampled flowering individuals had a flowering synchrony value >0.7 ; however, highly synchronous flowering was not advantageous for RS, as indicated by the negative directional selection differentials and gradients, and by the positive quadratic selection gradients.
- **Conclusions** These results support the hypothesis that, as a consequence of density-dependent selection, low spatio-temporal flower abundance is advantageous for attracting pollinators and for reproduction in natural populations of non-rewarding *C. japonicum*.

Key words: *Cypripedium japonicum*, pollen limitation, spatial dispersal pattern, flowering synchrony, reproductive success, selection differential, selection gradient, deceptive flower.

INTRODUCTION

Outcrossing species of angiosperms generally experience low reproductive success (RS), which is often ascribed to pollinator limitation at the pollination stage (Zimmerman and Aide, 1989; Calvo and Horvitz, 1990) and resource constraints at the post-pollination stage (Stephenson, 1981). Pollinator availability and visitation rate are influenced by various factors, including floral display, local floral density and flowering phenology (Kunin, 1992; Sabat and Ackerman, 1996; Mustajärvi *et al.*, 2001; Kirchner *et al.*, 2005). Natural selection is expected to favour phenotypes or attributes that increase pollinator visits and pollen exchange among conspecifics (Rathcke, 1983; Johnston, 1991). For instance, large floral displays generally have higher pollinator visitation (Broyles and Wyatt, 1990; Harder and Barrett, 1995) and a greater likelihood of male and/or female RS (Schemske, 1980; Firmage and Cole, 1988; Trapnell and Hamrick, 2006). Mass-flowering plants generally attract more pollinators and facilitate a higher RS through synchronous flowering within

inflorescences or plant patches, leading to large floral displays (Augspurger, 1980; Melampy, 1987). However, simultaneous opening of flowers within the same flowering cluster or inflorescence may also increase the probability of geitonogamy (de Jong *et al.*, 1993; Back *et al.*, 1996; Eckert, 2000; Harder and Johnson, 2005) because the pollinators might visit more than one flower within flowering clusters or inflorescences during individual foraging bouts.

Pollinator visitation and the RS of flowering plants also depend on the availability and quantity of rewards that the plants offer foraging pollinators. Some plants, namely deceptive or non-rewarding plants, provide no harvestable reward to their pollinators. Such plants are surprisingly common (Renner, 2006), and include approximately one-third of the species of Orchidaceae, which is considered to be the largest angiosperm family (Dafni, 1984; Ackerman, 1986). In order to attract more pollinators and increase RS, some non-rewarding plants rely on mimicry of co-flowering rewarding plants or even female insects (Ackerman, 1986; Nilsson, 1992). The large majority, however, simply exploit the instinctive food-seeking behaviours of naïve pollinators. Inexperienced pollinators explore their environment and

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sample non-rewarding flowers in their exploratory trips (Nilsson, 1992). However, pollinators might learn to discriminate between non-rewarding and rewarding flowers (Smithson and Macnair, 1997), avoiding the former and switching to more profitable food sources to optimize their foraging efficiency (Ollason and Ren, 2002). Consequently, non-rewarding plants generally experience a relatively lower RS than their rewarding counterparts (Nilsson, 1992; Neiland and Wilcock, 1998). Therefore, any factor that is likely to modify the rates at which inexperienced pollinators learn to discriminate between rewarding and non-rewarding flowers could affect the RS of non-rewarding plants.

One condition that probably impedes discrimination by inexperienced pollinators and maximizes the RS of non-rewarding plants is low density and widely dispersed populations (Ackerman, 1986; Sabat and Ackerman, 1996; Jersáková *et al.*, 2006), which may reduce the frequency with which pollinators encounter non-rewarding flowers during their exploratory foraging visits (Ferdy *et al.*, 1999; Castillo *et al.*, 2002). However, populations of some non-rewarding plants are often found growing in highly dense patches or in aggregated patterns of dispersion (Nilsson, 1979; Brzosko *et al.*, 2002; Bänziger *et al.*, 2005; Trapnell and Hamrick, 2006). The effect of local density or aggregation level on RS in both natural and experimental plant populations is revealed to be much less consistent. In natural populations of some non-rewarding species, the flowers on those plants growing in clusters comprising an intermediate number of conspecifics have a greater probability of setting fruit than either solitary plants or clusters comprising a large number of flowers (Firmage and Cole, 1988; Sabat and Ackerman, 1996). A positive density effect on pollinator visitation and RS has been found in some non-rewarding species (Johnson *et al.*, 2003; H.-Q. Sun *et al.*, unpubl. res.); however, a negative effect has been detected in other cases (Gumbert and Kunze, 2001). In manipulated populations of *Dactylorhiza sambucina*, increased aggregation of non-rewarding plants had a significant negative influence on female RS and a marginally negative influence on male RS (Internicola *et al.*, 2006).

Another strategy for reducing the frequency with which inexperienced pollinators avoid non-rewarding flowers and enhancing RS is asynchronous flowering; by temporally reducing pollinator encounters with non-rewarding flowers, this strategy might reduce the frequency with which pollinators recognize the deceit (Ferdy *et al.*, 1998; Castillo *et al.*, 2002; Parra-Tabla and Vargas, 2004; but see Gentry, 1974). To date, however, few studies have examined this possibility. Recently, Parra-Tabla and Vargas (2007) found a negative effect of flowering synchrony on male and female pollination success in the non-rewarding *Myrmecophila christinae*. In non-rewarding *Changnienia amoena*, however, it was found that synchronous flowering individuals have a greater RS than those flowering asynchronously (H.-Q. Sun *et al.*, unpubl. res.). In summary, knowledge of the potential advantages or disadvantages of flower density or dispersion pattern and flowering synchrony in non-rewarding plants remains limited.

Cypripedium japonicum (Orchidaceae), a non-rewarding species bearing a single flower that reproduces both sexually and vegetatively, is a particularly well suited species for this

type of investigation. Each growing season, the horizontal underground rhizome of *C. japonicum* produces a single new ramet or, more often, a cluster of shoots. Variation in cluster size and simultaneous blooming of flowers within the same cluster provide a good opportunity to explore cluster (aggregation) and synchrony effects. Moreover, in common with most orchids and asclepiads, *C. japonicum* is particularly suitable for measuring both male and female RS at the pollination stage. This is because its pollinium, which comprises a mass of sticky pollen, is removed as a unit by pollinators (Fig. 1B), and pollen removal (male function) and deposition (female function) are easily scored in the field. Although male RS is ultimately the number of seeds sired, a positive correlation between pollinia removal and deposition or seeds sired has been found in many pollinia-bearing species; therefore, pollinia removal can serve as an estimate of male RS (Broyles and Wyatt, 1990; Nilsson, 1992; O'Connell and Johnston, 1998; O'Connell and Eckert, 1999).

Non-rewarding plants are expected to promote cross-pollination between plants and reduce geitonogamous pollen transfer (Dafni and Ivri, 1979; Dressler, 1981; Johnson and Nilsson, 1999; Johnson *et al.*, 2004; Jersáková *et al.*, 2006; but see Salguero-Farías and Ackerman, 1999) because pollinators that do not find a reward spend less time and probe fewer flowers on non-rewarding inflorescences. In the present study, the composition of the pollinator assemblage of *C. japonicum* was initially investigated and observations were made to determine whether pollinator visiting behaviour results in self-pollination within a flower or geitonogamy within a cluster. The following questions were then addressed. (a) Is RS in *C. japonicum* limited by pollinator visitations? (b) Do plants growing in a dispersed pattern have higher male and/or female RS than those growing in large clusters? (c) Is asynchronous flowering beneficial in terms of male and/or female functions?

MATERIALS AND METHODS

Study system and study sites

Cypripedium japonicum Thunb. is a hermaphroditic orchid that occurs in the understorey of deciduous forests and bamboo groves at altitudes ranging from 1000 to 1800 m in East Asia (Cribb, 1997; Chen *et al.*, 1999). Most plants have two leaves and a 25–39 cm tall stalk bearing a single flower (Fig. 1A). In the present study, a few plants that produced two flower stalks were found. The flower has a large, pendent, sac-like labellum (mean \pm s.d.; 5.2 ± 0.54 cm long, 2.8 ± 0.4 cm wide, 2.8 ± 0.4 cm high, $n = 50$) with a crimson mouth (1.8 ± 0.48 cm long, 1.9 ± 0.3 cm wide, $n = 55$) on its upper surface (Fig. 1A). This species produces no nectar despite the large floral display and the conspicuous 'nectar guides' (the crimson mouth). There are two anthers, each situated above an exit from the labellum. Each anther bears a pollinium, a mass of sticky pollen that is usually removed as a unit when a pollinator squeezes through the exit (Fig. 1B). In the Shennongjia Mountains of Hubei Province, central China, *C. japonicum* blooms from mid-April to early May. Individual flowers remain open for approx. 3 weeks, but senesce ≤ 5 d after pollination (H.-Q. Sun, unpubl. res.).

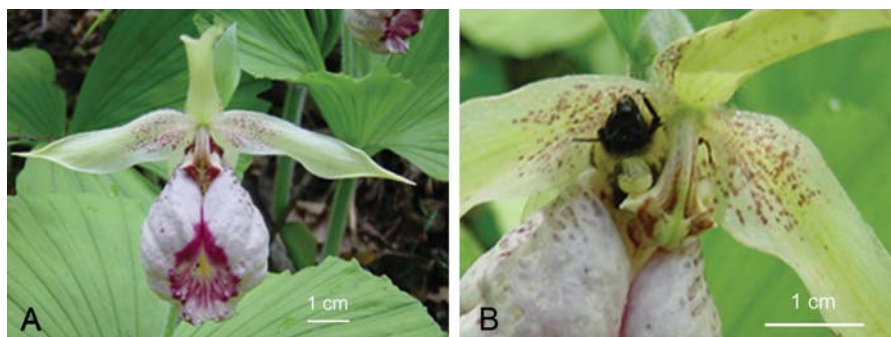


FIG. 1. *Cypripedium japonicum* and its pollinator. (A) Close-up view of a pendent orchid flower, showing the crimson mouth, through which pollinators get into the labellum. (B) A bumble-bee squeezing under the anther and removing an intact pollinium by the mesothorax.

Three populations (>200 flower-producing individuals) were studied in 2003 and six populations (>500 flower-producing individuals) were studied in 2004 during the early spring at the Shennongjia National Nature Reserve and Shennongjia Biodiversity Research Station of the Chinese Academy of Sciences, Hubei Province (31°21'N, 110°03'E). The populations were separated by a distance of 1.5–2.5 km. The vegetation in which these populations grow is similar – secondary deciduous woodland mixed with a few evergreen trees and shrubs. During the flowering season of *C. japonicum*, the understorey is well lit because at this time of year the leaves of the deciduous trees and shrubs have yet to flush fully. During the study period, there were few concurrently blooming entomophilous species within the communities. Therefore, it was assumed that the effect of co-flowering rewarding species on the RS of the orchid would be minimal. A sympatric, non-rewarding orchid, *Changnienia amoena* Chien, although present in the study area, flowers before *C. japonicum*.

Pollinator observations

In order to assess the composition of the pollinator assemblage associated with *C. japonicum*, during the initial and peak flowering (from 29 April to 7 May) in 2003, all insects visiting solitary and clustered *C. japonicum* flowers in the three populations were observed for >40 h. Observations were made during the period of highest pollinator activity (1000 h–1600 h) for 3–6 h d⁻¹. Pollinators were defined as those species that not only visited *C. japonicum* but also carried its pollen. Some of these insect visitors were captured for identification.

To explore whether pollinators cause intrafloral self-pollination and geitonogamy within a potential clonal cluster, the following two questions were addressed by conducting investigations of pollinator behaviour while assessing the pollinator pool. (1) Can visitors trapped within the labellum remove the pollinium and then smear it onto the stigma (transferring a pollinium within flowers)? (2) Do pollinators forage the nearest neighbours or do they move away after a non-rewarding visit (potentially leading to the transfer of pollinium within clones, or geitonogamy)? Five potential clonal clusters ranging in size from 20 to >80 flowering plants were chosen to address the latter question. A potential clonal

cluster was defined as an aggregative group of flowers that grew in very close proximity, the furthest distance between neighbouring flowering shoots being not more than the mean length of the rhizome (1.38 m, $n = 7$), regardless of the area they occupied.

Estimation of pollinator limitation

In 2004, hand-pollination experiments were performed to estimate pollinator limitation. For each of the following treatments, flower buds from different plants were randomly chosen and marked. In order to investigate whether current-year fruit set was limited by pollen receipt, each experimental flower was bagged using fine mesh nets after it was assigned to one of four treatments early in the flowering season: self-pollination ($n = 20$), cross-pollination ($n = 20$), non-pollination to test for autonomous self-fertilization ($n = 8$) and open without treatment as the control ($n = 51$). Pollen donors for cross-pollination were selected from plants growing at least 10 m apart. In order to investigate whether pollinator limitation varied over the course of the flowering season, randomly selected flowers ($n = 20$ for each phase) were marked and cross-pollinated in three flowering phenological phases. These phases were determined by creating a scale using the proportion of individuals in flower among the plants in the population: the early phase, 0–60% of the surface of the flowers in bloom; the middle phase, >60% of the surface in flower through the peak flowering; and the late phase, 60–0% in flower. The number of hand-pollinated flowering individuals successfully setting fruit was compared with the fruit set of controls that initiated flowering on the same dates as the manipulated plants.

Spatial distribution pattern and reproductive success

In the field, the distances between shoots within aggregations are very small and it is not possible to identify individuals (genets) in a genetic sense. However, flowering clusters and isolated flowering individuals can easily be distinguished in the field. The distribution pattern of *C. japonicum* was classified into two types: the isolated pattern, in which flowering individuals grew singly and had no neighbours within 1 m, and the clustered pattern in which flowering individuals occurred within clusters of shoots regardless of the area they

occupied. In the present study, cluster size varied considerably in each population, from several to >80 flowering individuals within a cluster. In each reproductive season, the number of flowering individuals in two distribution patterns was surveyed in all populations. In order to evaluate whether male and female RS varied with local densities of conspecific flowering plants, a focal flower or a focal cluster was randomly selected, around which was set up a quadrat of 1 m radius. The quadrats encompassed a wide density range, from several to >50 flowering individuals. A total of 14 and 31 quadrats were set up in 2003 and 2004, respectively. Flowering individuals of the two distribution types, as well as every quadrat in each population, were visited every 2 or 3 d during the flowering season, and the numbers of plants with pollen removed (male RS) and deposited (female RS) were counted. An analysis of variance (ANOVA) was conducted to evaluate variation in RS between the isolated and clustered patterns. A linear regression analysis was also performed for each reproductive season in order to determine whether there was an effect of local density on male and female RS.

Flowering phenology, reproductive success and phenotypic selection

In order to detect phenotypic selection on flowering phenology, a number of phenological traits and fitness measures were censused on randomly selected flowers from all populations in both years (209 and 346 flowering individuals in 2003 and 2004, respectively). One to five flowers were randomly selected from the different clusters since clusters may consist of more than one genet (Brzosko *et al.*, 2002). The following three variables were used to describe the flowering phenology: (1) opening date; (2) flowering duration; and (3) flowering synchrony index. Opening date was defined as the first day on which the dorsal petal lifted sufficiently to allow a pollinator to enter the labellum. Flowering duration was calculated as the days elapsed between the flower opening and wilting. A flowering synchrony index, measuring the overlap between the flowering of a given individual and the flowering of every other individual in the sample, was calculated using the methods of Augspurger (1983):

$$X_i = [1/(n-1)](1/f_i)\sum e_{j \neq i}$$

where n is the number of flowering plants in the sample, f_i is the number of days individual i is in flower, and $e_{j \neq i}$ is the days on which both individual i and j flower simultaneously. This index ranges from 0, when there is no overlap, to 1 when the flowering time of an individual overlaps completely with that of all other individuals in the sample. Fitness was evaluated as RS. For each flower, male absolute RS was measured as the number of pollinia removed (0, 1 or 2), whereas female absolute RS was measured as the number of pollinia received (0 or 1). The relative male (or female) RS of each flowering plant was calculated as absolute male (or female) RS divided by the mean absolute male (or female) RS of the study individuals. For the phenological census, each selected flowering individual was monitored every 2 or 3 d throughout the flowering season, and pollinium removal (male RS) and deposition (female RS) were examined.

Univariate and multiple regression analyses (Lande and Arnold, 1983; O'Connell and Johnston, 1998; Conner, 2007) were used to estimate the magnitude and direction of selection acting on the quantitative phenological traits (opening date, flowering synchrony and flowering duration) of *C. japonicum*. Because the relationships between the phenological traits and RS did not vary among populations within a flowering season (H.-Q. Sun, unpubl. res.), the data for each season were pooled. Separate analyses were conducted for each year. Flowering duration was highly correlated with opening date (2003: Pearson $r = -0.240$, $P \leq 0.05$, 2004: $r = -0.312$, $P \leq 0.01$) and flowering synchrony (2003: Pearson $r = -0.373$, $P \leq 0.01$, 2004: $r = -0.535$, $P \leq 0.01$), and was therefore excluded from the multiple regression analyses because selection gradients will not accurately represent selection on a trait if the traits are highly intercorrelated (Neter *et al.*, 1983; Mitchell-Olds and Shaw, 1987). Before analysis, all phenological traits were standardized to unit variance by dividing each character by the standard deviation; this allows expression of any change in the fitness value due to a change in the character under selection in terms of standard deviation units (Lande and Arnold, 1983). The standardized linear (directional) selection differentials were estimated as the univariate (single-character) regression coefficient between relative fitness and each of the standardized traits. Univariate regression coefficients estimate the total strength and sign (positive or negative) of selection acting on a phenological trait both directly and indirectly through correlated phenological traits. The standardized linear selection gradients were estimated as the vector of the regression coefficients from the multivariate (multiple-character) regression of relative fitness on the set of standardized traits measured. The standardized non-linear selection gradients were calculated from the multivariate regression, including linear and quadratic terms of all standardized traits. Standardized selection gradients describe the direct selection on a trait after accounting for selection on the other potentially correlated traits included in the analysis. A significant positive regression coefficient of a quadratic term indicates either non-linear or disruptive selection on that trait or correlated traits included in the analysis, whereas a negative value indicates non-linear or stabilizing selection on that trait or correlated traits included in the analysis (Lande and Arnold, 1983; Mitchell-Olds and Shaw, 1987). Univariate and multiple regression analyses were performed using SPSS (2002). Finally, a sequential Bonferroni correction (Rice, 1989) was used to evaluate the table-wide significance ($P < 0.05$) of regression coefficients (standardized selection differentials and standardized linear and quadratic selection gradients) for each year and sex function (male and female), which reduces the probability of obtaining significant results by chance alone.

RESULTS

Pollinator observation and pollinator limitation

In 45 h of observations, it was found that *C. japonicum* was visited by 19 anthophilous insect species. However, the workers of only two species of bumble-bee, *Bombus (Thoracobombus) remotus* (Tkalců) and *B. (Tricornibombus)*

imitator Pittioni, were effective primary pollinators, and *B. (Pyrobombus) picipes* Richards workers were occasional pollinators. Pollen removal occurred when a bumble-bee squeezed past of one of the lateral anthers (Fig. 1B), while pollinium deposition occurred when a bumble-bee carrying a pollinium crept under the stigma exiting past an anther. *Bombus imitator* is also the primary pollinator of *C. amoena*, a sympatric but distantly related orchid. During the course of the study, a waxy pollinium of *C. amoena* was found on the stigma of a *C. japonicum* plant. However, no potential hybrids were found in the field, indicating that these two plant species are not crossable.

On the basis of 21 observations, it is suspected that the behaviour of bumble-bees during their escape from the labellum might not lead to intrafloral self-pollination. No bumble-bees were observed to manoeuvre to the bottom of the labellum after removing the pollinium from the anthers. Although transfer of a pollinium within a cluster (potential geitonogamy) is conceivable, this event would appear to be rare. Deceived bumble-bees were found to leave non-rewarding clusters immediately after escaping from the labellum.

Population surveys of *C. japonicum* revealed two clear patterns in male and female fitness. First, among open-pollinated flowers, pollination frequency was only 5.2% in 2003 and 7.7% in 2004 (Fig. 2). Secondly, the number of plants with pollen removed was considerably higher than those with pollen received. In 2003, 13.4% of the flowers had one pollinium removed and 3.3% had two removed. The corresponding percentages in 2004 were 22.9 and 4.1% (Fig. 2). Pollinium removal and pollination were highly correlated in both years (Pearson correlation: 2003, $r = 0.33$, $P < 0.05$; 2004, $r = 0.49$, $P < 0.05$).

All plants hand pollinated during the early flowering phase set fruit. Bagged flowers (pollinator exclusion) failed to develop fruits, indicating that autogamy does not occur in this species. Pollinator limitation was prevalent in *C. japonicum*. Over the entire season, the mean fruit set of the control flowers was significantly lower than that of

outcrossing flowers hand pollinated during three flowering phases (control = 11.1%, $n = 185$; supplemented = 74.3%, $n = 51$; $\chi^2 = 10.75$, d.f. = 1, $P < 0.001$, G -test of independence), with higher fruit set in the control flowers in the middle flowering phase than that in the early and late phases.

Spatial distribution pattern and reproductive success

In both reproductive seasons, the number of flowering individuals growing in two dispersion patterns was different, with considerably fewer flowering individuals of the isolated type than the clustered type. In these populations, 84.7% (2003) and 82.7% (2004) of the shoots with flowers occurred in the clustered pattern. However, the trends in RS associated with two dispersion patterns were clearly different. In both seasons, male RS of the flowering individuals growing in clusters was substantially lower than that of plants growing in an isolated pattern (Fig. 3; 2003, $t = 2.3$, d.f. = 9, $P = 0.05$; 2004, $t = 2.64$, d.f. = 17, $P = 0.03$). Female RS in clustered plants was substantially lower than that in isolated plants in 2004 (Fig. 3; $t = 3.19$, d.f. = 30, $P = 0.01$), although in 2003 the difference between the two patterns was not significant (Fig. 3, $t = 1.3$, d.f. = 13, $P = 0.14$). Furthermore, the percentage of flowering individuals having two pollinia removed was higher in the isolated pattern (mean \pm s.d., $2.4 \pm 0.35\%$ in 2003; $3.1 \pm 0.42\%$ in 2004) than in the clustered pattern ($0.9 \pm 0.15\%$ in 2003; $1.1 \pm 1.85\%$ in 2004). Regression analysis revealed that in both reproductive seasons local floral density had a significantly negative influence on male RS, but that there was no effect of density on female RS (Fig. 4).

Flowering phenology, reproductive success and phenotypic selection

Of the phenological traits measured, significant differences between years were observed only in flower opening date (Table 1). Average values for flowering synchrony for most flowers were high – more than 85% of marked flowers had

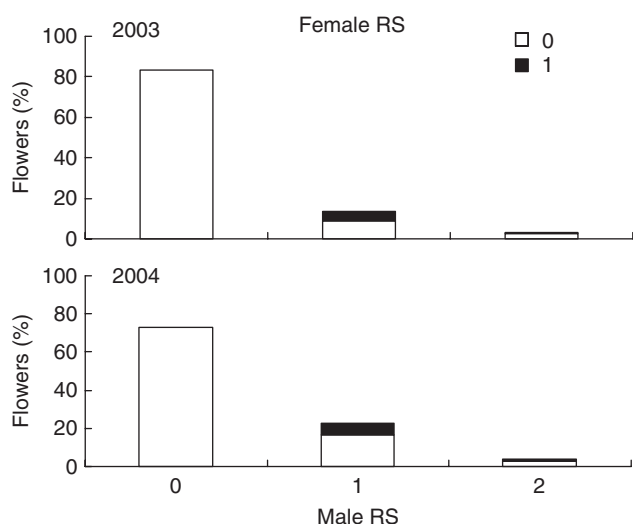


FIG. 2. Distribution of male and female reproductive success in *C. japonicum* in two years, expressed as a percentage of flowers (209 individuals in 2003 and 757 individuals in 2004). Plants may receive 0 or 1 pollinium and may disperse 0, 1 or 2 pollinia.

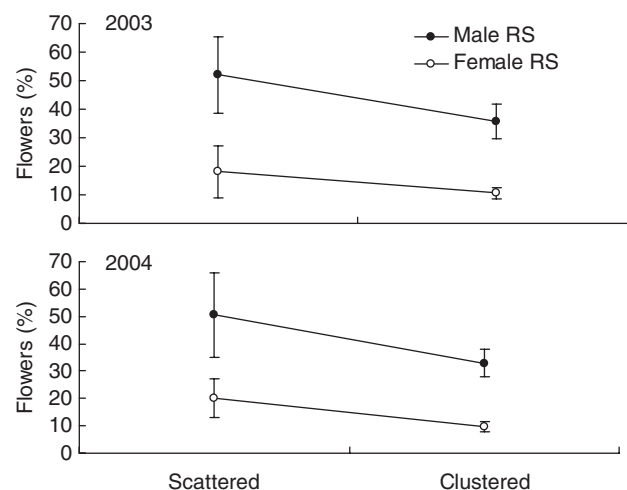


FIG. 3. Male and female reproductive success in different dispersion patterns of *C. japonicum* at Shennongjia Mountains in two seasons. The data are the means \pm s.e.

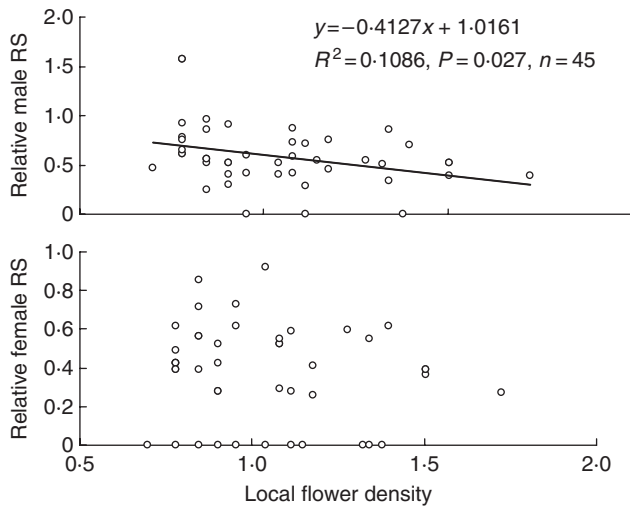


FIG. 4. Relationship between local floral density and relative male and female reproductive success of *C. japonicum*. Data are pooled over two seasons (2003–2004). n = number of quadrats of 1-m radius (local floral density). Local floral density was log-transformed and the ratio variables were arcsine square-root-transformed to meet the assumptions for parametric tests. Regression lines are only shown if they are statistically significant.

a synchrony value >0.7 , although the values ranged from 0.42 to 0.95 (Table 1). In both seasons, the distribution of flowering synchrony values was leptokurtic and skewed to the left (skewness and kurtosis coefficients were -1.390 and 5.004 , respectively, in 2003; and -0.97 and 4.026 , respectively, in 2004).

In both seasons, there were linear selection differentials in the same direction (negative) for opening date and synchrony through male and female RS, indicating that early and asynchronous flowering were favoured. In contrast, the selection differentials were significantly positive for flowering duration through male RS in both seasons and through female RS in 2004, indicating that a long flowering duration was advantageous (Table 2).

Multivariate regression analysis detected a significant direct linear selection favouring early and asynchronous flowering through both male and female RS in two reproductive seasons, as indicated by the significantly negative selection gradients (Table 3). In addition to the linear selection gradients on flower opening date, this trait was also exposed to direct non-linear (disruptive) selection. That is, early or late flowering was more advantageous through both male and female fitness, as indicated by the positive selection gradients for this trait. Multivariate selection regression also detected a significant non-linear selection on flowering synchrony in two

reproductive seasons (Table 3). Asynchronous flowering was advantageous through both male and female functions, as indicated by the positive gradients (disruptive selection) of this trait. The correlational selection gradients through male fitness were weak in both seasons, and only in 2003 was there significantly disruptive selection on covariance of opening date and synchrony through female fitness, as indicated by the positive gradient of this trait (Table 3), i.e. a combination of earlier and asynchronously flowering individuals have an advantage through female fitness.

DISCUSSION

Pollinator limitation to reproductive success

Over two reproductive seasons, Shennongjia Mountain populations of *C. japonicum* exhibited low natural female relative RS and a high male relative RS (Fig. 2). Low RS in non-rewarding plants is often attributed to pollinator limitation (Zimmerman and Aide, 1989; Calvo and Horvitz, 1990; Neiland and Wilcock, 1998; Tremblay *et al.*, 2005). Such pollinator limitation appears to be pronounced in *C. japonicum* since supplementary self- and cross-handed pollinations of this species significantly increased fruit set compared with that in control plants. The extent of pollinator limitation was, however, not constant over the entire flowering season, being least severe in the first half of the season. One explanation for such temporal variation in the extent of pollinator limitation is the inherent variation in the abundance and foraging experience (density and activity) of the pollinators, i.e. pollinators are likely to be more numerous and more naïve earlier in the season. Flowering seasons with high pollinator visitation (male RS) are less pollen limited for RS than those with low visitation (Ramsey, 1995). In *C. japonicum*, visitations by bumble-bee workers occurred mainly in the early half of the flowering season (H.-Q. Sun, unpubl. res.). Early flowering advantage has also been demonstrated in the deceptive orchid, *Tolumnia variegata* (Sabat and Ackerman, 1996). In *Calypso bulbosa*, a species in a genus closely related to *Changnienia*, an early flowering advantage was not so obvious since it was obscured by pulses of newly emerged naïve queen bumble-bees (Ackerman, 1981). After a few non-profitable visits, naïve pollinators are likely to switch their foraging preference to species providing a high reward, even though the non-rewarding flowers are still numerous in the populations. The temporal variation in pollen limitation within a season observed in *C. japonicum* is not unique. A certain degree of pollen limitation has also been found in the non-pollen-limited dioecious plant *Aucuba japonica*

TABLE 1. Measurements for flowering phenological traits of *C. japonicum* in two years

	2002 ($n = 209$)		2003 ($n = 346$)	
	Mean \pm s.d.	Range	Mean \pm s.d.	Range
Opening date**	25 April \pm 1.80 d	19 April–5 May	16 April \pm 3.45 d	11 April–25 April
Duration	12.59 \pm 2.89 d	3–21 d	12.67 \pm 2.34 d	7–19 d
Synchrony	0.81 \pm 0.10	0.42–0.95	0.79 \pm 0.08	0.45–0.92

**Significant difference between years, $P < 0.01$ (ANOVA).

TABLE 2. Standardized selection differentials (S') of three traits through male and female reproductive success in *C. japonicum* during two flowering seasons

Trait i	Male $S' (\beta') \pm$ s.e.	Female $S' (\beta') \pm$ s.e.
2003 ($n = 209$)		
Opening date	$-0.727 \pm 0.151^{***}$	$-0.841 \pm 0.241^{***}$
Duration	$0.912 \pm 0.162^{**}$	$0.998 \pm 0.256^{**}$
Synchrony	$-0.553 \pm 0.102^{**}$	$-0.651 \pm 0.287^{**}$
2004 ($n = 346$)		
Opening date	$-0.830 \pm 0.603^{***}$	$-0.479 \pm 0.159^{**}$
Duration	$0.814 \pm 0.656^{**}$	0.311 ± 0.231
Synchrony	$-0.773 \pm 0.156^{**}$	$-0.489 \pm 0.292^*$

Directional selection differentials (S' or β') \pm s.e. are the total effect of traits on relative fitness. Selection differentials are significant after Bonferroni correction as follows: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

during the part of the flowering season when male flowers were scarce (Abe, 2001).

Although non-rewarding plants have a low RS compared with rewarding plants, an overall fitness advantage might be accrued to the former from increased outcrossing and reduced selfing or geitonogamy (Dressler, 1981; Smithson, 2002; Johnson *et al.*, 2004). Hand-pollination experiments demonstrated that *C. japonicum* is self-compatible, although self-pollination in nature might be rare. As in most other members of the subfamily Cyripedioideae, the mechanics of pollination in *C. japonicum* effectively prevent pollinator-mediated self-pollination like other cogenics (Davis, 1986; Bänziger *et al.*, 2005; Li *et al.*, 2006). It is also assumed

that revisits to the same non-rewarding flower are rare since bumble-bee workers should learn to avoid such flowers after a few exploratory visits. Indeed, bumble-bees were observed to fly away quickly from the non-rewarding clonal clusters after a single visit, suggesting that geitonogamy within a clonal cluster could also be a rare event. Given this behaviour of the bumble-bee pollinators, it is expected that inbreeding would be minimal. Nevertheless, the extent of selfing or geitonogamy within a clone still requires further investigation.

Effect of spatial distribution on RS

In each of the two study seasons, both the male and female RS of isolated flowering individuals was higher than that of the individuals growing in clusters (Fig. 3). A negative correlation was found between local floral density and male RS, whereas female RS was independent of local floral density (Fig. 4). These results strongly suggest that the isolated pattern of conspecific flowering individuals is more advantageous compared with the clustered pattern. In *C. japonicum*, selection may have focused on improving fitness through higher male RS rather than through increased female RS by decreasing local floral densities. This strategy might be related to the optimal foraging behaviour of pollinators. Pollinators will sample a variety of rewarding as well as non-rewarding flowers in the initial stage of their exploratory trips (Heinrich, 1976). However, they may learn to discriminate between non-rewarding and rewarding flowers (Smithson and Macnair, 1997), subsequently avoiding the former in order to minimize energy expenditure (Ollason and Ren, 2002). Therefore, a pollinator attracted to a cluster of flowers will visit only a few

TABLE 3. Analysis of phenotypic selection through male and female reproductive success on three phenological traits in *C. japonicum* during two flowering seasons

(A) Male				
Traits	$\beta' \pm$ s.e.	95 % CI	$\gamma' \pm$ s.e.	95 % CI
2003 ($n = 209$)				
Opening date	$-0.690 \pm 0.287^{**}$	-1.255 to -0.125	$1.417 \pm 0.309^{***}$	0.908 to 2.126
Synchrony	$-0.934 \pm 0.188^{**}$	-1.306 to -0.561	$0.438 \pm 0.162^*$	0.118 to 0.758
Opening date \times synchrony			-0.252 ± 0.183	-0.613 to 0.108
2004 ($n = 346$)				
Opening date	$-0.561 \pm 0.155^*$	-0.866 to -0.255	$1.043 \pm 0.202^{***}$	0.645 to 1.440
Synchrony	$-0.906 \pm 0.170^{**}$	-1.240 to -0.572	$0.551 \pm 0.230^*$	0.099 to 1.003
Opening date \times synchrony			-0.173 ± 0.117	-0.403 to 0.057
(B) Female				
Traits	$\beta' \pm$ s.e.	95 % CI	$\gamma' \pm$ s.e.	95 % CI
2003 ($n = 209$)				
Opening date	$-1.068 \pm 0.453^{***}$	-1.962 to -0.174	$1.342 \pm 0.488^{***}$	0.795 to 1.705
Synchrony	$-0.642 \pm 0.250^*$	-1.135 to -0.149	$1.186 \pm 0.406^{***}$	-1.990 to -0.381
Opening date \times synchrony			$0.662 \pm 0.257^{**}$	0.156 to 1.167
2004 ($n = 346$)				
Opening date	$-0.844 \pm 0.234^{**}$	-1.304 to -0.384	$1.404 \pm 0.304^{**}$	0.806 to 2.002
Synchrony	$-0.980 \pm 0.257^{**}$	-1.487 to -0.474	$1.204 \pm 0.348^{**}$	0.518 to 1.889
Opening date \times synchrony			-0.286 ± 0.176	-0.632 to 0.060

Directional (β'), and concave/convex selection gradients (γ') \pm s.e. are the direct effect of traits on relative fitness. Selection gradients are significant after Bonferroni correction as follows: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

flowers before learning that they contain no reward (Heinrich, 1976; Johnson *et al.*, 2004). As a consequence, a large number of flowers within a cluster might act as a negative reinforcement, thereby decreasing the probability that an individual flower will be visited (Firmage and Cole, 1988; Smithson and Gigord, 2003). In the present study, it was found that the percentage of flowering individuals with two pollinia removed was higher in the isolated pattern (2.4–3.1 %) than in the clustered pattern (0.9–1.1 %), implying that pollinators might have a preference for small overall floral displays rather than for the larger conspecific clusters, even though they may initially prefer large floral displays. This finding appears to be consistent with the results of a study by Schemske (1980), who demonstrated that pollinators focus on individual inflorescences as the unit of attraction rather than on clusters of flowers. It does, nevertheless, run counter to the observation of Sabat and Ackerman (1996), who demonstrated that rewardless *T. variegata* flowers with an intermediate number of conspecific flowers exhibited a greater probability of RS than those with fewer or more flowers. In addition, higher rates of pollinium removal from solitary individuals and small clusters may enhance heterozygosity within a population (Brzosko *et al.*, 2002).

On the basis of these results, it is contended that the larger clusters of *C. japonicum* with large floral displays may be maladaptive in terms of reproduction under current ecological conditions. The low probability of male and female RS in plants growing in such clusters, compared with that of isolated flowering individuals and those growing in small clusters, suggests that this is the case. However, the overwhelming dominance of the clustered flowering individuals (>80 %) in our study populations does not support the notion that there is a strong selection against larger clusters of flowers. This then raises the question of what is the adaptive significance of large clusters or clonal growth in *C. japonicum*. One explanation might be the low sexual RS – less than 10 % of flowering individuals produced a fruit within a reproductive season due to pollinator limitation. Larger clusters formed by the clonal growth of genets could function as a compensatory strategy for low sexual reproduction rather than to increase pollinator visitations through an enlarged overall floral display. The combination of and balance between sexual reproduction and clonal propagation would be advantageous for the population demography and evolutionary potential of populations of *C. japonicum* as well as other clonal plants (Dorken and Eckert, 2001; Thompson and Eckert, 2004). Another potential explanation for the significance of clonal growth in *C. japonicum* is that orchid seeds have no endosperm and the germination and growth of seedlings is highly dependent on the presence of mycorrhizal fungi (Rasmussen, 1995; Shefferson *et al.*, 2005). Soil microenvironments around large clusters might be richer in the specific fungi and more favourable for seed germination and the growth of seedlings, since new juvenile individuals usually appear within and in the vicinity of such clusters (Brzosko *et al.*, 2002; H.-Q. Sun, unpubl. res.). On the other hand, the seeds of most temperate terrestrial orchid species may spend a considerable time, undetected, underground (Gill, 1996; Primack and Stacy, 1998; Willems and Melser, 1998; Kindlmann *et al.*, 2002; Shefferson *et al.*, 2003; Shefferson and Tali, 2007),

and seedling recruitment is very low in populations of some clonal species, including *C. japonicum*. The low rate of recruitment might, nevertheless, be sufficient to maintain or even increase local genetic variability (Soane and Watkinson, 1979; Stehlik and Holderegger, 2000; Brzosko *et al.*, 2002). Finally, clonal propagation may be very advantageous under certain ecological conditions, since it minimizes the risk of genet extinction by spreading the risk of mortality among the ramets of some clonal plants (Cook, 1985; Eriksson and Jerling, 1990; Piqueras, 1999).

Phenotypic selection on flowering phenology

It is often suggested that male RS is limited by success in mating whereas female RS is limited by the amount of resources available to produce progeny; thus, selection for floral characteristics will be more intense through the effects on male function than on that of females (Bateman, 1948). Selection intensities in some hermaphrodite plants are, however, similar through male and female functions (Campbell, 1989; O'Connell and Johnston, 1998). Likewise, in *C. japonicum*, similar overall selection intensities were found for male and female functions for two phenological traits. In both flowering seasons, variances in the mean absolute values of standardized selection differentials and gradients were similar, despite slightly different magnitudes of selection on different traits in different years (Tables 2 and 3). This result does not support the hypothesis of greater selection intensity on male function. Equal variances in male and female success may be more common in animal-pollinated hermaphroditic plants than in those with separate sexes (Bateman, 1948), since a single pollinator visit is likely to increase both male and female RS simultaneously. In *C. japonicum* and many other single-flowered species with two pollen packets, relative RS is expressed at only two levels in terms of female function (zero and high), and only three levels with respect to the male function (zero, medium and high). At the pollination stage, a visit by a pollinator will almost certainly lead to the removal of one pollinium. Therefore, one visit can potentially saturate the female fitness gain curve, and two visits can saturate the male curve (O'Connell and Johnston, 1998). In the case of *C. japonicum*, male success and female success were positively correlated, though relative donation of pollen was slightly higher than pollen receipt. Those flowers receiving any pollinator visits will therefore gain relative female and male RS simultaneously. Thus, there was no less opportunity for selection through pollen receipt than through pollen donation.

There is some evidence that selection favours earlier blooming in both rewarding and non-rewarding plants (Campbell, 1991; Sabat and Ackerman, 1996; O'Connell and Johnston, 1998; Kelly and Levin, 2000; Maad, 2000; Parra-Tabla and Vargas, 2004). Likewise, in *C. japonicum*, earlier flowering individuals have the greatest probability of achieving male and female RS, as indicated by the significant negative directional selection differentials and gradients, and the positive quadratic selection gradients (Tables 2 and 3). One possible adaptive advantage of earlier flowering is that such plants have a greater opportunity of encountering inexperienced

foraging bumble-bees (Little, 1983; Nilsson, 1992; Sabat and Ackerman, 1996; O'Connell and Johnston, 1998). Later flowering might not be as advantageous, because by the end of May deciduous trees and shrubs in *C. japonicum* communities have a full canopy of leaves that reduces the apparency of orchid flowers. Moreover, bumble-bee abundance also gradually declines within the forest as the season progresses due to their preference for foraging in more exposed sites where food is available (H.-Q. Sun, pers. obs.). If there is selection for early flowering, why then has *C. japonicum* not evolved to bloom earlier? A probable explanation lies in the avoidance of competition for pollinators between *C. japonicum* and *Changnienia amoena*, the latter of which is a non-rewarding species that co-occurs with *C. japonicum* but flowers earlier in the season. *Changnienia amoena* and *C. japonicum* have slightly overlapping flowering phenologies and both are pollinated exclusively by bumble bees, one species of which, *B. imitator*, is a shared pollinator. During the course of this study, a pollinium of *C. amoena* was discovered on the stigma of a *C. japonicum* flower, indicating that bumble-bees switched their temporal foraging preference to later flowering *C. japonicum*. If *C. japonicum* were to flower earlier in the season, such that its flowering time coincided exactly with the reproductive season of *C. amoena*, competition between these two deceptive species would probably be inevitable.

It is predicted that in non-rewarding species natural selection will act against synchronous flowering because this trait may accelerate the rate at which inexperienced pollinators distinguish and subsequently avoid non-rewarding flowers (Ferdy *et al.*, 1999; Castillo *et al.*, 2002; Parra-Tabla and Vargas, 2004). However, to date, there have been only a few field studies that have sought to test this prediction. In the case of *C. japonicum*, flowering synchrony for most flowers was high – over 85% of sampled flowers had a flowering synchrony value >0.7. However, these plants did not have a higher pollinator visitation rate and RS than asynchronously flowering plants. Quite the contrary; asynchronous flowering appeared to be more advantageous. This result is consistent with the analysis of RS according to dispersion pattern. Flowering individuals growing in clusters generally have a higher degree of flowering synchrony due to the genetic similarity among shoots (probably ramets of the same genet), and they received fewer visitations from bumble-bees than those growing singly. This observation is not unique; a significant negative effect of flowering synchrony on male success has also been found in the multiflowered inflorescences of the non-rewarding *Myrmecophila christinae* (Parra-Tabla and Vargas, 2007). A contrasting trend was, nevertheless, found in our recent investigation of the non-rewarding *C. amoena*. The individuals of *C. amoena* with high flowering synchrony have a greater probability of attracting pollinators and a higher fitness than those flowering asynchronously (H.-Q. Sun *et al.*, unpubl. res.). The effect of flowering synchronization appears to be related to the abundance of flowering conspecifics, because the population densities in *C. amoena* (not more than 20 flowers per 10 m²) were far lower than those of the *C. japonicum* investigated in the present study (>50 flowers within a 1 m radius). High flowering synchrony under the conditions of low floral density might be a facilitative factor attracting a greater number of pollinators, thereby increasing

the RS of non-rewarding plants. However, as demonstrated in the present study, under conditions of high floral density, the same high flowering synchrony may become disadvantageous.

CONCLUSIONS

Although rewardlessness is surprisingly common in angiosperms (Johnson *et al.*, 2003; Renner, 2006) and its evolutionary significance has been widely discussed (Dressler, 1981; Ackerman, 1986; Nilsson, 1992; Smithson, 2002; Johnson *et al.*, 2004), there have been few investigations that have sought to examine the effects of spatial and temporal variations in conspecific flower abundance on reproduction in non-rewarding plants (Tremblay *et al.*, 2005). The findings of the present study support the hypothesis that non-rewarding plants that are sparsely distributed, grow at low conspecific density and flower asynchronously have a selective advantage in terms of attracting pollinators and reproduction. These results provide insights into the strategies of increasing RS in non-rewarding species.

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