



Positive effects of flower abundance and synchronous flowering on pollination success, and pollinia dispersal in rewardless *Changnienia amoena* (Orchidaceae)

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Pollination success and pollen dispersal in natural populations depend on the spatial-temporal variation of flower abundance. For plants that lack rewards for pollinators, pollination success is predicted to be negatively related to flower density and flowering synchrony. We investigated the relationships between pollination success and flower abundance and flowering synchrony, and estimated pollinia dispersal distance in a rewardless species, *Changnienia amoena* (Orchidaceae). The results obtained in the present study revealed that male pollination success was negatively influenced by population size but was positively affected by population density, whereas female pollination success was independent of both population size and density. Phenotypic analysis suggested that highly synchronous flowering was advantageous through total pollination success, which is in contrast to previous studies. These results indicate that pollination facilitation rather than competition for pollinator visits occurs in this rewardless plant. The median distance of pollinia dispersal was 11.5 m (mean distance = 17.5 m), which is comparable to that of other rewardless plants but longer than for rewarding plants. However, pollen transfer occurred mainly within populations; pollen import was a rare event. Restricted gene flow by pollinia and seeds probably explains the previous population genetic reporting a high degree of genetic differentiation between populations. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, **99**, 477–488.

ADDITIONAL KEYWORDS: phenotypic selection – population density – selection coefficient – synchronous flowering.

INTRODUCTION

Pollination success and pollen dispersal are important determinants of the demographic and genetic properties of plant populations, and might influence the persistence of small populations. In animal-pollinated plants, pollination success and patterns of pollen dispersal are affected by various factors, and one of the most important factors is the abundance of flowering conspecifics (population size and density;

Kunin, 1993, 1997; Morris, 1993; Karron *et al.*, 1995; Richards, Church & McCauley, 1999; Ishihama *et al.*, 2006; Spigler & Chang, 2008). A positive relationship between pollination success or fruit/seed production of individuals and the number or density of conspecifics, also known as the Allee effect (Allee, 1931), was revealed in many studies (Byers, 1995; Ågren, 1996; Bosch & Waser, 1999; Kirchner *et al.*, 2005; Spigler & Chang, 2008). However, most empirical studies focused mainly on single factor of either population density or population size (Kirchner *et al.*, 2005) that actually are correlated in natural populations (Ågren, 1996; Kunin, 1997). Therefore, it is necessary to take both population size and population density into account when assessing the effect of flower abundance

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on pollination success in natural populations (Mustajärvi *et al.*, 2001; Wagenius, 2006; Gunton & Kunin, 2007; Spigler & Chang, 2008; Johnson, Torninger & Ågren, 2009).

Although low population densities usually have a negative effect on pollination success or fruit/seed production (Kunin, 1993; Bosch & Waser, 1999; Kirchner *et al.*, 2005), plants growing in sparse populations might have relatively long pollen dispersal distance compared to those growing in denser populations (Morris, 1993; Stacy *et al.*, 1996; Ishihama *et al.*, 2006). This pattern is ascribed to optimal foraging strategy; most pollinators tend to visit adjacent flowering individuals or patches in sequence as a result of energy limitation (Harder, 1988; Chittka, Gumbert & Kunze, 1997). However, this visitation behaviour may reduce pollen dispersal between populations regardless of densities when they are isolated by distance. Isolation by a few hundred metres was found to seriously reduce the import of pollen in some plant populations (Ellstrand & Marshall, 1985; Goodell *et al.*, 1997; Richards *et al.*, 1999).

Another important factor that may have a significant impact on pollination success or fruit/set production is flowering phenology (Rathcke & Lacey, 1985; Elzinga *et al.*, 2007). Many studies indicated that plants flowering early have selective advantages in terms of pollination success and fruit predation avoidance compared to those flowering late (Widén, 1991; Totland, 2001), although stabilizing selection favours intermediate-flowering plants in some cases (Widén, 1991; Ollerton & Diaz, 1999). As a consequence of the highly synchronized flowering of most shoots or individuals, mass-flowering reward-producing shrubs and trees induce high pollinator visitation rates and pollination success (Marquis, 1988; Domínguez & Dirzo, 1995; Méndez & Díaz, 2001; but see also Gómez, 1993) because they provide massive reward for foraging pollinators.

By contrast to reward-producing plants, some plants (i.e. deceptive or rewardless plants) provide no harvestable reward to their pollinators. In such plants, the relationships between pollination success and flower abundance and flowering synchrony, and the patterns of pollen dispersal were considered to be different from that of those rewarding counterparts. Pollination success in terms of proportion of pollen removed and/or fruit set in these species has been demonstrated to be higher in small populations than in large populations (Fritz & Nilsson, 1994; Alexandersson & Ågren, 1996) and higher in sparse populations/patches than in dense populations/patches (Gumbert & Kunze, 2001; Castillo, Cordero & Domínguez, 2002; Sun *et al.*, 2009). Pollinators that do not find a reward spend less time and probe fewer flowers within the rewardless patches/populations

(Peakall & Beattie, 1996). This may facilitate long-distance pollen dispersal relative to their rewarding counterparts (Peakall & Beattie, 1996). However, pollen dispersal distances in these rewardless plants were rarely measured. Some studies have indicated that selection favours earlier blooming in rewardless plants (Sabat & Ackerman, 1996; O'Connell & Johnston, 1998; Parra-Tabla & Vargas, 2007; Sun *et al.*, 2009). Nevertheless, from the studies conducted thus far, there is little evidence for the effect of flowering synchrony on pollination success of rewardless plants (Parra-Tabla & Vargas, 2007), although asynchronous flowering is predicted to be advantageous in these rewardless plants because a high level of aggregative blooming in such plants may cause pollinators to learn to avoid them more quickly (avoidance learning; Ollason & Ren, 2002) and, consequently, reduce their number of visitations and plant's pollination success.

Changnienia amoena Chien is a rewardless orchid and an endangered species endemic to China (Li & Ge, 2006; Sun *et al.*, 2006). Although this species has a relatively wide distribution, its populations have become highly fragmented and varied in size as a consequence of destruction and degradation of its habitats by various human activities during the past decades (Li & Ge, 2006). In the previous study on 11 natural populations of *C. amoena*, Li & Ge (2006) detected a high degree of genetic differentiation between populations, implying a low level of gene flow between populations. In common with most orchids and asclepiads, the pollen of *C. amoena* is packaged in relatively large pollinia that is easily labelled by microtags (Nilsson, Rabakonandrianina & Pettersson, 1992) or histochemical stains (Peakall, 1989), and pollinia removal and deposition is easily determined to estimate pollinia dispersal and pollination success in terms of male and female functions in the field. Therefore, *C. amoena* comprises a good system for investigating the effect of temporal/spatial variation of flower abundance on pollination success, and pollinia dispersal.

In the present study, we specifically addressed three main areas: (1) Is there a negative relationship between pollination success and population size and/or density in rewardless *C. amoena*? (2) Does *C. amoena* benefit from flowering asynchrony? Or, is asynchronous flowering advantageous for male and female pollination success in this species? (3) Are pollinia dispersal distances in this rewardless species longer than those in rewarding counterparts? And is short-distance pollinia dispersal responsible for the high degree of genetic differentiation between populations in this species? Such information will not only enhance our understanding of the adaptive reproductive strategies and patterns of pollinia dispersal in

natural populations, but also has implications for the conservation of this endangered orchid.

MATERIAL AND METHODS

STUDY SPECIES AND SITE

Changnienia amoena Chien is a perennial, self-compatible, terrestrial orchid that is mainly restricted to the mountainous regions in eastern and central China (Li & Ge, 2006; Sun *et al.*, 2006). In the early spring, reproductive individuals produce a single leaf from an underground corm as well as an inflorescence (height 5–12 cm) bearing a solitary purplish–pink flower. The flower has a three-lobed lip and a horn-like spur that does not contain nectar. During the daytime the flower produces a sweet odor under direct irradiation or high temperature (H.-Q. Sun, pers. observ.). *Changnienia amoena* flowers for 3–4 weeks beginning in middle or late March (Sun *et al.*, 2006). The pollen is packaged in four hard, solid pollinia, arranged in two pairs on the sticky viscidium and covered by an anther cap (Sun *et al.*, 2006). In the Shennongjia Mountains, two *Bombus* species, workers of *Bombus (Diversobombus) trifasciatus* Smith and *Bombus (Tricornibombus) imitator* Pittoni, pollinate *C. amoena* (Sun *et al.*, 2006). A bumblebee usually removes the entire pollinarium (the four pollinia and the viscidium) on its mesothorax when retreating from the flower and, at the same times, may deposit pollinia individually, or in groups of two, three or four pollinia together (Sun *et al.*, 2006). *Changnienia amoena* is highly fertile and sets 100% of its fruits after cross-pollination (Sun *et al.*, 2006). However, fruit production in natural populations is low, and less than 15% of individuals set a fruit (Sun *et al.*, 2006), indicating that pollinator limitation is pronounced in this orchid.

The present study was carried out in the early spring of two consecutive years (2002 and 2003) in the Shennongjia Mountains, Hubei Province (31°21'N, 110°03'E; 1275–1290 m a.s.l.) (Sun *et al.*, 2006). The weather system of the studied area is cold and humid during the reproductive season of *C. amoena* (Sun *et al.*, 2006). *Changnienia amoena* grows in relatively nutrient-rich secondary deciduous woodlands mixed with a few evergreen trees and shrubs. During the anthesis of *C. amoena*, the understory of the woodlands is still brightly lit because, at this time of year, the new leaves of the deciduous trees and shrubs within these habitats have yet to fully flush. Therefore, the effect of tree cover on pollinator visitation and reproductive success in *C. amoena* is considered to be minimal. During the study period, within and in the vicinity of some populations, there are a few but not many rewarding co-flowering plants, mainly

including *Viola grypoceras* A. Gray, *Glechoma biondiana* (Diels) C. Y. Wu & C. Chen var. *glabrescens* C. Y. Wu & C. Chen, two species of *Primula* and one species of *Prunus*. In most study populations, however, there are no co-flowering plants. Therefore, we assumed that the effect of rewarding co-flowering species on the pollination success of *C. amoena* would be minimal. A sympatric, rewardless orchid, *Cypripedium japonicum* Thunb., although present in the study area, flowers after *C. amoena* (Sun *et al.*, 2009).

POPULATION SIZE, DENSITY, AND POLLINATION SUCCESS

Data on pollination success were collected from the same 12 populations examined in both 2002 and 2003. All populations chosen for study were located on different hillsides that were separated by at least a distance of 3 km, except for two populations that were approximately 300 m apart. We defined a population as a group of plants separated from other conspecific groups by at least 300 m. For each population, we recorded the number of flowering plants (population size), the percentage of flowering plants that exported pollen (male pollination success), and the percentage of flowering plants that set a fruit (female pollination success). The pollinarium removal of each plant was censused at the end of flowering. Fruit set was recorded 3–7 weeks after flowering (May to June).

Every flowering plant in the study populations was individually marked and mapped in two-dimensional space; therefore, we could generate x and y coordinates for each flowering plant. On the basis of the map, we censused the number of flowering plants per 10 m² (local conspecific density). Population density was determined as the local conspecific density when the population covered an area of less than 10 m². When the population covered an area larger than 10 m², it was divided into patches of 10 m², and the population density was calculated as the mean of the local conspecific densities of these patches. The percentage of flowering plants that exported pollen (male pollination success) and the percentage of flowering plants that set a fruit (female pollination success) were counted on an area of per 10 m² in each season.

We examined the relationship between population size, density, and plant pollination success using analysis of covariance (ANCOVA) model for each season. For this analysis, male and female pollination were used as dependent variables, and population size and density were included as independent variables. Population size was log-transformed and density was square root-transformed, and male and female pollination success were arcsine-square root-transformed prior to the analysis to meet the assumptions for parametric tests. A number of flowers (5.4%

in 2002 and 5.7% in 2003) exhibited signs of predation; these were most likely unable to attract pollinators and were thus excluded from the analysis.

FLOWERING PHENOLOGY, POLLINATION SUCCESS, AND PHENOTYPIC SELECTION

The census of phenological traits and pollination success was undertaken by monitoring random samples of 73 and 122 flowering individuals in 2002 and 2003, respectively, from all study populations that were used to investigate the effect of flower abundance on pollination success. We recorded the opening and wilting dates for every marked flowering individual on every fine day throughout the flowering season. Three variables of flowering phenology were evaluated: (1) flower-opening date; (2) flowering duration; and (3) a flowering synchrony index. Flower-opening date was measured as the first day that the petals lifted high enough to allow a pollinator to enter the flower. In each year, the opening date of the first plant flowered was counted as 1, and the next date of the other plants that flowered was counted as 2. By analogy, the flower-opening date was set up as an ordinal variable. Flowering duration was calculated as the number of days that elapsed between flower opening and wilting. The flowering synchrony index, a measure of the overlap between the flowering of a given flower and the flowering of every other individual in the population, was calculated using the method of Augspurger (1983): $X_i = [1/(n-1)](1/f_i)\sum_{j \neq i} e_{j+i}$, where n is the number of flowering plants in all sampled populations, e_{j+i} is the days on which both individuals i and j flower simultaneously, and f_i is the number of days individuals i is in flower. This index ranges from 0, when no overlap occurs, to 1, when complete overlap occurs.

Plant fitness was recorded as pollination success in terms of pollinium removal (male pollination success) and fruit set (female pollination success). Pollinium removal was censused by examining all flowers at the end of flowering. Fruit set was recorded 3–7 weeks after flowering. For each flowering plant, absolute male pollination success was scored as either 0 or 1, depending, respectively, on whether the pollinarium was removed or not. Similarly, absolute female pollination success was scored as either 0 or 1, depending on whether the plant set a fruit or not. Relative male (or female) pollination success of each flowering plant was calculated as absolute male (or female) pollination success divided the mean absolute male (or female) pollination success of study individuals. To obtain an estimate of the relative ‘total’ pollination success of each flower, we divided the sum of relative male and female pollination success by two (O’Connell & Johnston, 1998).

We used regression analysis (Lande & Arnold, 1983) to estimate the magnitude and direction of selection acting on the quantitative phenological traits in *C. amoena*. Flowering duration was highly correlated with opening date (2002: Pearson correlation coefficient $r = -0.245$, $P < 0.05$; 2003: $r = -0.305$, $P < 0.01$) and flowering synchrony (2002: $r = -0.314$, $P < 0.01$; 2003: $r = -0.397$, $P < 0.01$), and was therefore excluded from the multivariate regression analyses because selection gradients do not accurately represent selection on a trait if the traits are highly inter-correlated (Mitchell-Olds & Shaw, 1987). We conducted separate analyses for each season by pooling the data of all sampled populations because there were no significant differences in the results of selection on flowering phenological traits among different densities (H.-Q. Sun, unpubl. data) and the habitats where the study populations located were similar. All phenological characters were standardized to unit variance prior to analysis by dividing each character by the standard deviation because this allows the expression of any change in fitness value as a result of a change in the character under selection in terms of standard deviation units (Lande & Arnold, 1983). Coefficients from a univariate (single-character) regression estimate the total strength and sign (positive or negative) of selection acting on a phenological trait, both directly and indirectly, through correlated phenological traits. When expressed in units of standard deviation, they are equivalent to linear (directional) selection differentials. Coefficients from multivariate (multiple character) regression estimate the strength of selection acting independently of any potentially correlated phenological traits included in the analysis. When expressed in units of standard deviation, they are equivalent to selection gradients including linear (directional) and quadratic terms. A significant positive regression coefficient of a quadratic term indicates concave upwards or disruptive selection on that trait or correlated traits included in the analysis, whereas a negative value indicates downward concavity or stabilizing selection on that trait or correlated traits included in the analysis (Lande & Arnold, 1983; Mitchell-Olds & Shaw, 1987). The coefficients were estimated by the least squares method (Lande & Arnold, 1983). Finally, we used a sequential Bonferroni correction (Rice, 1989) to evaluate the table-wide significance ($P < 0.05$) of the regression coefficients for each year and sex function, which reduces the probability of obtaining significant results by chance alone (Rice, 1989).

POPULATION DENSITY AND POLLINIA DISPERSAL

The pollen of *C. amoena* is packaged in four pollinia, arranged in two pairs on the sticky viscidium (Sun

et al., 2006). In 2003, the pollinia dispersal distance of *C. amoena* was estimated by labelling all pollinia of every flower of all study populations used to explore the relationship between conspecific flower abundance and pollination success. Before labelling, we removed carefully the anther cap that covered the pollinarium, then marked individually the two front pollinia with microtags, on which coloured letter-plus-number combinations were printed (Nilsson *et al.*, 1992). It was hard to mark the two pollinia hidden behind with microtags without the risk of moving the front pollinia out of their position. The two pollinia hidden behind therefore were individually marked using a dot of water resistant coloured stain using a 10- μ l syringe (Peakall, 1989): methylene blue (1% w/v), brilliant green (1% w/v), rhodamine (0.2% w/v), trypan Red (2% w/v), orange G (2% w/v), and methyl violet (1% w/v). The two pollinia hidden behind were labelled with one randomly-chosen colour on a flower and with different colours for neighbouring flowers around a focal flower within a population. There were the cases which more than one flower was labelled by the same colour within a population because of limited kinds of stain. Each marked flower was visited every fine day throughout the entire season, and checked to determine whether it had been pollinated or whether its pollinarium had been removed. Pollinia found deposited on a stigma were checked with a hand lens ($\times 15$) to determine whether or not they were marked. Because every flower was mapped in two-dimensional space, we were able to calculate the distance between any pair of flowers within a population. Pollinia dispersal distance was measured as the distance between donor and recipient. The donors of flowers that received the pollinium with microtags could be unambiguously determined because 'letter plus number' combinations were printed on them. When locating stain-coloured pollinia on stigmas, we determined the pollinia donor by assessing all flowering plants with matching coloured pollinia within a population. We classified the populations into two categories (i.e. sparse and dense) based on the median of population density. The relationship between pollinia dispersal distance and population density was examined by ANCOVA, in which population size was introduced as the covariate.

RESULTS

POPULATION SIZE, DENSITY, AND POLLINATION SUCCESS

The number of flowering plants in the study populations (population size) of *C. amoena* was small, ranging from fewer than ten to more than 40 over two seasons (mean \pm SD flowering plants: 2002: 12 \pm 5.4, $N = 12$; 2003: 16 \pm 8.5, $N = 12$). The number of flow-

Table 1. Analysis of covariance of the relationships between population size, density, and pollination success over two seasons

Dependable variables	Population size	Population density
2002		
Male pollination success	13.7**	8.75*
Female pollination success	0.93	0.85
2003		
Male pollination success	8.1*	6.58*
Female pollination success	0.69	0.58

Values given are F -ratios, N (the number of populations) = 12. Population size was log-transformed, local conspecific density was square root-transformed, and pollination success was arcsine-square root-transformed prior to analysis. (* $P < 0.05$, ** $P < 0.01$).

ering plants within an area of 10 m² (local conspecific density) ranged from one individual to more than ten over two seasons (mean \pm SD flowering plants per 10 m²: 2002: 2.8 \pm 2.4, $N = 18$; 2003: 2.6 \pm 2.6, $N = 18$). We detected significant relationships between population size, density and male pollination success (Fig. 1, Table 1). Although female pollination success was apparently unaffected by both population size and density, male pollination success was negatively related to population size, but positively related to local conspecific density (Fig. 1, Table 1).

FLOWERING PHENOLOGY, POLLINATION SUCCESS, AND PHENOTYPIC SELECTION

A significant difference was observed between years in the date of flowering initiation, but not in flowering synchrony and duration (Table 2). The average values for flowering synchrony for most flowers were high, and more than 80% of marked flowers had a flowering synchrony value of greater than 0.7 (Fig. 2) despite values in the range 0.44–0.98 (Table 2). In both years, the distributions were leptokurtic and skewed to the left (Fig. 2; skewness coefficient and kurtosis coefficient were -0.884 and 4.299 in 2002, and -0.90 and 3.975 in 2003, respectively).

The selection differentials changed through the three components of pollination success in both seasons (Table 3). There was strong selection for earlier flowering in 2002 via male and female pollination success, and in 2003 via female and total pollination success, that was expressed by the negative selection differentials. For all components of pollination success, there was a tendency for positive selection on flowering synchrony; however, this was consistently weak and nonsignificant in both years.

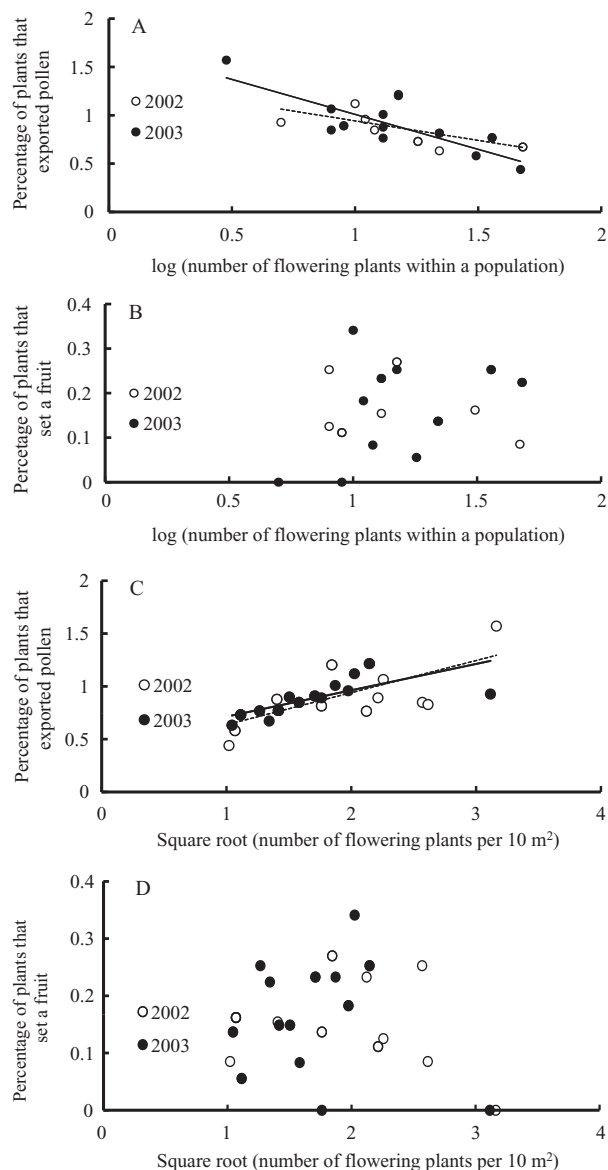


Figure 1. Relationships between the log of population size (number of flowering plants in a population), and male pollination success (percentage of plants that exported pollinarium) (A) and female pollination success (percentage of plants that set a fruit), (B) between the square root of local conspecific density (number of flowering plants per 10 m²) and male (C) and female (D) pollination success of *Changnienia amoena* in two reproductive seasons. Male pollination success and female pollination success were arcsine-square root-transformed prior to the analysis. Only significant regression lines are shown.

Selection differentials for flowering duration were positive and statistically significant for all components of pollination success in 2002 but nonsignificant for male pollination success in 2003.

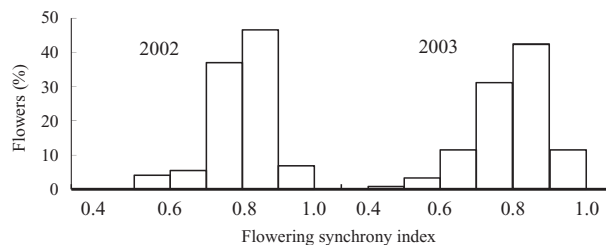


Figure 2. Frequency distribution for values of flowering synchrony ($N = 73$ flowering individuals in 2002; $N = 122$ flowering individuals in 2003).

Similar to the univariate regressions, multivariate analyses showed that directional selection gradients for opening date were significantly negative for all three components of pollination success in both years, with the exception of male pollination success in 2002 (Table 4). Quadratic regression analyses, however, revealed that, in both 2002 and 2003, opening date underwent nonsignificantly convex selection with respect to the three components of fitness. In 2003, flowering synchrony had a significantly positive directional selection coefficient for total pollination success only, indicating that individuals with highly synchronized flowering gained an advantage (Table 4). However, in both seasons, there was a nonsignificant concave selection on flowering synchrony through the three components of pollination success. Correlational selection gradients for the interaction between opening date and synchrony were negative for all fitness functions in the two seasons.

POPULATION DENSITY AND POLLINIA DISPERSAL

Pollinia dispersal distance varied greatly among populations, ranging from 0 to 75 m (median distance, 11.5 m; mean \pm SD distance, 17.5 \pm 19.2 m; $N = 75$). Pollinia dispersal distribution based on the unambiguously determined distances and all possible pairwise distances between recipient and donors with the matched colours revealed that over 50% of marked pollen was dispersed over a distance of less than 10 m, and that less than 7% of pollen was dispersed over a distance greater than 50 m. Distributions were leptokurtic and skewed to the right (skewness coefficient = 2.4; kurtosis coefficient = 10.1; Fig. 3). Pollen transfers occurred mainly within the populations and there were few transfers between populations. One population was found to have received an unlabelled pollinium from another unmarked population, indicating gene flow from a foreign population.

There was a negative correlation between pollinia dispersal distance and population densities ($r = -0.78$, $P = 0.037$, $N = 10$). There was a significant

Table 2. Measurement of flowering phenological traits of *Changnienia amoena* over two seasons

Variable	2002			2003		
	N	Mean ± SD	Range	N	Mean ± SD	Range
Opening date**	73	25 March ± 2.84 days	19 March to 31 March	122	5 April ± 3.04 days	30 March to 2 May
Synchrony	73	0.79 ± 0.09	0.50–0.98	122	0.81 ± 0.1	0.44–0.98
Duration	73	18.1 ± 2.61	10–23	122	17.6 ± 4.5	7–28

Values represent the mean ± SD and range. Asterisk indicates significance between two seasons, based on analysis of variance (**P* < 0.05, ***P* < 0.01).

Table 3. Standardized selection differentials (*S'*) of three phenological traits through relative male, female and total pollination success in *Changnienia amoena* during two seasons

Year	Trait	Male		Female		Total	
		N	<i>S'</i> (β') ± SE	N	<i>S'</i> (β') ± SE	N	<i>S'</i> (β') ± SE
2002	Open date	73	-0.364 ± 0.094**	72	-0.613 ± 0.204**	73	-0.338 ± 0.130*
	Synchrony	70	0.240 ± 0.09*	73	0.115 ± 0.216	71	0.178 ± 0.133
	Duration	71	0.429 ± 0.079**	72	0.972 ± 0.184***	72	0.701 ± 0.107***
2003	Open date	122	-0.290 ± 0.087*	121	-0.646 ± 0.181***	120	-0.418 ± 0.111**
	Synchrony	121	0.252 ± 0.088*	122	0.303 ± 0.108*	120	0.317 ± 0.116*
	Duration	121	0.189 ± 0.087*	120	0.560 ± 0.133***	121	0.374 ± 0.113**

Directional selection differentials (*S'* or β') ± standard errors (SE) are the total effect of traits on relative fitness. Selection differentials shown in bold are significant after Bonferroni correction (**P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001).

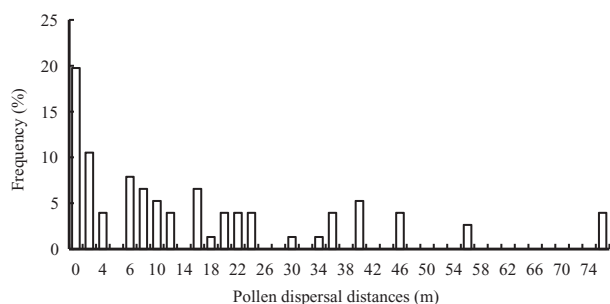


Figure 3. Frequency distribution of labeled pollinia dispersal in *Changnienia amoena* with the unambiguously determined distances and all possible pairwise distances between recipients and donors (*N* = 75).

difference in pollinia dispersal distance of sparse and dense populations. The mean ± SD pollinia dispersal distance was longer in sparse populations than in dense populations, with estimated values of 21.7 ± 21.2 m and 10.5 ± 9.4 m, respectively. The actual mean distance of pollinia dispersal in dense populations may, however, be shorter than that estimated because bumblebees sequentially visited neighbouring flowers after one visitation. Similarly,

the actual mean distance of pollinia dispersal in sparse populations might be longer than that estimated because we captured a bumblebee that had carried microtag-marked pollinia from a donor located at a distance of 58 m.

DISCUSSION

POSITIVE DENSITY DEPENDENCE OF POLLINATION SUCCESS

The male pollination success of *C. amoena* was negatively related to population size over two seasons (Fig. 1). This result is consistent with the prediction that pollinator visitation rate and subsequent male pollination success to rewardless plants would be reduced in large populations because pollinators would learn to avoid such flowers after a few exploratory visits (Fritz & Nilsson, 1994; Alexandersson & Ågren, 1996; Smithson & Macnair, 1997; Ollason & Ren, 2002). A reduced pollinator visitation rate in large populations would result in a decrease in female pollination success. However, many studies have failed to demonstrate a significant relationship between female pollination success and population

Table 4. Analysis of phenotypic selection through three components of fitness on two phenological traits and a correlational trait in *Changnienia amoena* during two seasons

Traits <i>i</i>	$\beta' \pm \text{SE}$	95% Confidence interval	$\gamma' \pm \text{SE}$	95% Confidence interval
Male pollination success				
2002 (<i>N</i> = 73)				
Opening date	-0.303 ± 0.130*	-0.561, -0.045	-0.116 ± 0.132	-0.146, 0.378
Synchrony	0.291 ± 0.102*	0.087, 0.496	-0.117 ± 0.139	-0.396, 0.161
Opening date × Synchrony			-0.123 ± 0.087	-0.050, 0.297
2003 (<i>N</i> = 122)				
Opening date	-0.403 ± 0.130**	-0.561, -0.045	-0.330 ± 0.205*	-0.735, 0.076
Synchrony	0.263 ± 0.104*	0.469, 0.058	-0.246 ± 0.119*	-0.483, -0.010
Opening date × Synchrony			-0.054 ± 0.089	-0.230, 0.122
Female pollination success				
2002 (<i>N</i> = 73)				
Opening date	-0.521 ± 0.119**	-0.841, -0.031	-0.194 ± 0.325	-0.454, 0.842
Synchrony	0.346 ± 0.143	-0.139, 0.831	-0.064 ± 0.331	-0.725, 0.597
Opening date × Synchrony			-0.164 ± 0.192	-0.219, 0.546
2003 (<i>N</i> = 122)				
Opening date	-0.625 ± 0.223***	-1.067, -0.182	-0.385 ± 0.388*	-0.597, 0.793
Synchrony	0.340 ± 0.221*	0.977, 0.103	-0.395 ± 0.254*	-0.898, 0.107
Opening date × Synchrony			-0.228 ± 0.184	-0.137, 0.593
Total pollination success				
2002 (<i>N</i> = 73)				
Opening date	-0.445 ± 0.138**	0.776, 0.097	-0.004 ± 0.212	-0.419, 0.428
Synchrony	0.319 ± 0.072*	0.020, 0.617	-0.091 ± 0.204	-0.497, 0.316
Opening date × Synchrony			-0.144 ± 0.121	-0.098, 0.385
2003 (<i>N</i> = 122)				
Opening date	-0.661 ± 0.155***	-0.968, -0.354	-0.357 ± 0.254**	-0.045, -0.564
Synchrony	0.402 ± 0.136**	0.671, 0.132	-0.321 ± 0.156*	-0.631, -0.011
Opening date × Synchrony			-0.087 ± 0.114	-0.138, 0.312

Directional (β') and concave/convex (γ') selection gradients \pm standard errors (SE) are the direct effect of traits on relative fitness. Selection gradients shown in bold are significant after Bonferroni correction. (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

size in rewardless species (Steiner, Whitehead & Johnson, 1994; Alexandersson & Ågren, 1996). In *C. amoena*, a trend of increasing female pollination success with population size was observed; however, this was not statistically significant (Fig. 1). This paradoxical relationship between female pollination success and population size in rewardless plants may result from the impact of variation in population density. Some studies on rewarding species have indeed suggested that the density rather than the size of a population strongly influences pollinator behaviour and subsequent pollination success (Kunin, 1997; Mustajärvi *et al.*, 2001; Spigler & Chang, 2008).

In the present study, male pollination success were positively related to population density, consistent with the Allee effect (Allee, 1931) and the experimental result obtained for rewardless artificial flowers by Keasar (2000), although no effect of density on female

pollination success was found (Fig. 1). This result did, nevertheless, run counter to the results obtained for other rewardless species in which either male or female pollination success was higher in sparse populations than in dense populations (Gumbert & Kunze, 2001; Castillo *et al.*, 2002). Sabat & Ackerman (1996) demonstrated that rewardless *Tolumnia variegata* flowers with an intermediate number of conspecifics exhibited a greater probability of setting fruit (female pollination success) than those with fewer or more flowers. Rathcke (1983) hypothesized that pollinator visitation rate per reward-producing flower should first increase with plant density up to a maximum as a result of a facilitation effect, and then decrease with increasing density as a result of competition among plants for pollinator services. Similarly, by modelling pollinator learning curves and their effect on the rewardless plant population, Ferdyn *et al.* (1999) found

that the pollinator foraging behaviour induced a positive density dependence at low density, but a negative density dependence at a higher density. In *C. amoena*, we found a facilitation effect rather than a negative effect of plant density on male pollination success. The highest local density in the present study was 16 flowers per 10 m², which was considerably lower than that of other studied rewardless plants (Sabat & Ackerman, 1996; Gumbert & Kunze, 2001). This may indicate that the highest local density is not sufficiently high to induce a negative local density effect on pollination success. The lower pollinator visitation rate and subsequently lower pollination success in sparse populations might be attributed to the difficulties in sustaining pollinator interest (Peakall & Beattie, 1996; Johnson *et al.*, 2003), thus leading to a decreased availability of pollinators (pollinator limitation; Alexandersson & Ågren, 1996; O'Connell & Johnston, 1998; Sun *et al.*, 2006). This inference is supported by the previous study in which a significant increase in fruit set was observed when plants were hand-pollinated to *C. amoena* (Sun *et al.*, 2006).

THE ADVANTAGE OF HIGH FLOWERING SYNCHRONY FOR POLLINATION SUCCESS

It has been shown in rewardless species that plants with high flowering synchrony were at a disadvantage (Parra-Tabla & Vargas, 2007; Sun *et al.*, 2009) because pollinator visitations and pollination success depend on the frequency with which pollinators can be deceived (Gigord, Macnair & Smithson, 2001; Castillo *et al.*, 2002). Interestingly, in the present study, more than 80% of the marked flowers of *C. amoena* had a flowering synchrony value of greater than 0.7, and the phenotypic selection appeared to favour high flowering synchrony, which is reflected by the positive directional selection differential for total pollination success in 2003. This indicated that the *C. amoena* plants that flowered in high synchrony attracted more pollinators and thus increased pollination success through aggregation leading to large floral displays. For some rewardless species with highly synchrony flowering, variation in flower cues (morphology, scent, colour, etc.) is considered to be a mechanism of deception designed to increase pollination success (Ackerman, Meléndez-Ackerman & Salguero-Faria, 1997; Smithson & Macnair, 1997; Gigord *et al.*, 2001). However, such an explanation does not appear to be applicable to *C. amoena*, which exhibits little variation in morphology, colour, or scent (H.-Q. Sun, unpubl. data). We propose that density-dependent selection might explain the results obtained for *C. amoena* populations, supporting previous observations that density-dependent selection was one of the evolutionary strategies to increase

pollination success in rewardless species (Ackerman, 1986; Ferdy *et al.*, 1999; Johnson *et al.*, 2003). In a sense, flowering synchrony could be considered as a temporal concentration of flower density that enhances the pollination success of rewardless species (Augsburger, 1983; Méndez & Díaz, 2001). In *C. amoena*, male pollination success was positively related to population density, indicating that the spatial attractiveness of this single-flowered rewardless species was not sufficiently high to induce inexperienced pollinators to avoid them. High flowering synchrony, in common with the high density effect, is a facilitative factor that attracts pollinators to rewardless *C. amoena*, thereby increasing this plant's pollination success.

In *C. amoena*, directional selection favoured early flowering, which is reflected by the significant negative directional selection differentials and gradients. Many studies of rewardless species have also demonstrated phenotypic selection favouring early flowering (Sabat & Ackerman, 1996; O'Connell & Johnston, 1998) because pollinators eventually learn to avoid these species. This explanation also applies to *C. amoena*. However, *C. amoena* flowers that bloom during mid-season would have an advantage in that they would have a higher probability of being visited and pollinated than the flowers that bloom either early or late during the season, as demonstrated by the negative gradient values for three components of pollination success (stabilizing selection; Table 4). This result might arise from the flower opening date being negatively related to flowering synchrony and duration, and that the intermediately flowering individuals usually have higher synchrony with other conspecifics, and thus they could aggregatively attract more pollinators. High pollinator abundance during the period of flowering peak will lead to a higher pollinator visitation rate and, subsequently, a greater pollination success.

POLLINIA DISPERSAL IN REWARDLESS PLANTS

Pollen tracking was less conducted in rewardless orchids than in reward-producing plants, although it was easier for the former to label pollinia in the field. Many studies have demonstrated that pollen dispersal distance is shorter in reward-producing plants than in their rewardless counterparts (Peakall, 1989; Nilsson *et al.*, 1992; Peakall & Beattie, 1996; Alexandersson & Ågren, 2000; Johnson, Neal & Harder, 2005; Jersáková & Johnson, 2006). In the present study, the mean transfer distance of the pollinia of the rewardless *C. amoena* was 17.5 m, which is comparable to that of other rewardless plants in which pollinia were marked by the same methods (Peakall & Beattie, 1996; Alexandersson & Ågren, 2000), but

longer than for rewarding plants (Peakall, 1989; Nilsson *et al.*, 1992; Johnson *et al.*, 2005; Ishihama *et al.*, 2006). Long-distance pollen flow in the rewardless plants is presumably attributed to the brief visitation of pollinators to the flowers; as optimal foragers, pollinators usually leave populations of rewardless plants after visiting only a few flowers (Ollason & Ren, 2002). This prediction has been confirmed experimentally by the addition of nectar to the rewardless orchid *Anacamptis morio* (Johnson, Peter & Ågren, 2004), and also by our observations of the visits of pollinators to *C. amoena* flowers in the field. Bumblebees remained in empty flowers for only a short time, usually no more than 12 s, and they departed from the populations after visiting only one or two flowers (Sun *et al.*, 2006). Given the sequential visitation of most pollinators to adjacent flowers as a result of energy limitation (Harder, 1988; Chittka *et al.*, 1997), the distance that a pollinator moves and thus the pollen dispersal distance, tends to be longer in lower density populations. As expected, in *C. amoena*, the mean pollen dispersal distance was longer in sparse populations (21.7 m) than in dense populations (10.5 m).

Long-distance pollen dispersal has a substantial positive effect on the prevention of genetic differentiation between populations. In the sexual deceptive orchid *Caladenia tentaculata*, Peakall & Beattie (1996) detected a long distance pollinia dispersal (mean distance = 17 m; maximum = 58 m) and a low degree of genetic differentiation between populations ($F_{ST} = 0.037$). Similarly, in the rewardless orchid *Calypso bulbosa*, Alexandersson & Ågren (2000) found that pollen flow was restricted within populations but a substantial fraction of the pollen input originated from plants outside the patches, and that there was low to moderate genetic differentiation between populations ($F_{ST} = 0.072$). In our case, the pollinia of *C. amoena* dispersed long distance (mean distance = 17.5 m; maximum = 75 m); nevertheless, the population genetic study revealed a high degree of genetic differentiation between populations ($G_{ST} = 0.375$; Li & Ge, 2006). This consequence may be ascribed to a restricted gene flow by means of both pollinia and seeds, as a result of isolation between populations. In the present study, all study populations that were located at the different hillsides were separated by a distance of at least 3 km, except for two populations that were approximately 300 m apart. Such isolation distances appear to be sufficient to strongly reduce pollen flow between populations because pollen transfer of *C. amoena* occurred mainly within populations. Low population densities may be an accomplice of the isolation in the reduction of pollen flow between populations or almost all fertilizations being by plants from the same population.

The population densities (maximum: not more than 20 flowers per 10 m²) were far lower than those of most plant populations, which may lead to a high level of consanguineous mating because foraging pollinators may stay longer and visit more flowers within sparse populations (Kunin, 1997; Grindeland, Sletvold & Ims, 2005). Although direct measurements of seed dispersal are currently unavailable, seed dispersal in *C. amoena*, which takes place by wind, could be more restricted than that in many other orchids because the fruit stalk of this species is short (16.5 ± 9.5 cm; H.-Q. Sun, person. observ.) and wind speed might be reduced significantly in the shrub and forest vegetation that is a typical habitat of this species. Restricted gene flow accompanied by genetic differentiation between populations was not an exception for *C. amoena*. In an endangered plant *Silene tatarica*, Tero *et al.* (2003) found a clear subpopulation differentiation ($F_{ST} = 0.58$) and a low level of gene flow between the subpopulations. If small populations consist of closely-related plants, pollen transfer among individuals within populations could result in a higher level of inbreeding depression (Byers & Waller, 1999). Increasing the gene flow between the small populations by hand-pollination during the period of peak flowering therefore could be an effective way to reduce inbreeding depression of small populations and genetic differentiation between populations in the endangered plants when *in situ* conservation strategies are employed.

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