

Reproductive isolation and pollination success of rewarding *Galearis diantha* and non-rewarding *Ponerorchis chusua* (Orchidaceae)

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• **Background and Aims** Increasing evidence challenges the conventional perception that orchids are the most distinct example of floral diversification due to floral or prezygotic isolation. Regarding the relationship between co-flowering plants, rewarding and non-rewarding orchids in particular, few studies have investigated whether non-rewarding plants affect the pollination success of rewarding plants. Here, floral isolation and mutual effects between the rewarding orchid *Galearis diantha* and the non-rewarding orchid *Ponerorchis chusua* were investigated.

• **Methods** Flowering phenological traits were monitored by noting the opening and wilting dates of the chosen individual plants. The pollinator pool and pollinator behaviour were assessed from field observations. Key morphological traits of the flowers and pollinators were measured directly in the field. Pollinator limitation and interspecific compatibility were evaluated by hand-pollination experiments. Fruit set was surveyed in monospecific and heterospecific plots.

• **Key Results** The species had overlapping peak flowering periods. Pollinators of both species displayed a certain degree of constancy in visiting each species, but they also visited other flowers before landing on the focal orchids. A substantial difference in spur size between the species resulted in the deposition of pollen on different regions of the body of the shared pollinator. Hand-pollination experiments revealed that fruit set was strongly pollinator-limited in both species. No significant difference in fruit set was found between monospecific plots and heterospecific plots.

• **Conclusions** A combination of mechanical isolation and incomplete ethological isolation eliminates the possibility of pollen transfer between the species. These results do not support either the facilitation or competition hypothesis regarding the effect of nearby rewarding flowers on non-rewarding plants. The absence of a significant effect of non-rewarding *P. chusua* on rewarding *G. diantha* can be ascribed to low levels of overlap between the pollinator pools of two species.

Key words: *Galearis diantha*, *Ponerorchis chusua*, rewarding and non-rewarding, mechanical isolation, ethological isolation, pollinator limitation, fruit set.

INTRODUCTION

Reproductive isolation is a central theme in evolutionary plant biology and involves a number of pre- and postzygotic mechanisms that form barriers to gene flow between distinct plant phenotypes, lineages and species (Charlesworth and Charlesworth, 2000). One important prezygotic mechanism is floral isolation (Grant, 1949), in which interspecific pollen transfer among sympatric flowering plants is prevented by differences in floral traits (mechanical isolation) and pollination by different pollinator taxa (ethological isolation). The most common type of floral isolation is the adaptation of sympatric species to different taxa of pollinators with different body sizes and shapes (Grant, 1994). Even when two or more sympatric species share the same pollinator, prezygotic reproductive isolation can be achieved by depositing the pollen of different plant species on different parts of the body of the pollinator (Dressler, 1968; Yang *et al.*, 2007).

When sympatric species share the same pollinator, their pollination success may be increased because the presence of simultaneously flowering species can facilitate attraction of sufficient pollinators (pollination facilitation) (Laverty, 1992; Johnson *et al.*, 2003). However, reproductive output may be diminished due to pollination competition (Levin and Anderson, 1970), if neighbouring co-flowering plants with superior rewards draw pollinators away, or if the sharing of pollinators results in reproductive interference through the receipt of heterospecific pollen or the wasted export of pollen to heterospecific stigmas (Waser, 1983; Johnson *et al.*, 2003).

Floral isolation between species is considered to be widespread in angiosperms with specialized animal-pollinated flowers (Grant, 1994; Schemske and Bradshaw, 1999; Charlesworth and Charlesworth, 2000). Orchids often show highly species-specific plant–pollinator relationships and therefore have been commonly considered to be the most

distinct example of floral diversification due to floral or prezygotic isolation (van der Pijl and Dodson, 1966; Dressler, 1993; Cozzolino and Widmer, 2005a). High levels of natural hybridization and low pollinator specificity, however, have been found among some species and genera of European orchids (Cozzolino et al., 2005; Cozzolino and Widmer, 2005b; Bateman et al., 2008), indicating the need for a reassessment of the importance of pollinator specificity or pollinator sharing for prezygotic reproductive isolation in orchids (Cozzolino and Widmer, 2005a).

Many orchids, referred to as **non-rewarding or deceptive species exhibit a pollination system involving deceit**, they offer no reward for pollinators but can attract and deceive pollinators by mimicking nectariferous flowers growing in the same habitat or by exploiting the innate preference of naive pollinators for large, brightly coloured flowers (Ackerman, 1986; Nilsson, 1992). These orchids often show low pollination success compared with their rewarding counterparts (Gill, 1989; Neiland and Wilcock, 1998), which has mainly been ascribed to pollinator limitation (Zimmerman and Aide, 1989; Calvo and Horvitz, 1990; Tremblay et al., 2005). Non-rewarding plants, however, may benefit from growing in close proximity to co-flowering rewarding plants (magnet effect; Lavery, 1992; Alexandersson and Ågren, 1996; Johnson et al., 2003; Internicola et al., 2007; Peter and Johnson, 2008). Most studies have focused on the effect of rewarding plants on pollinator visitation or on the pollination success of non-rewarding species; however, few studies have investigated whether co-flowering, non-rewarding plants affect the pollination success of rewarding plants. Non-rewarding flowers usually have a larger floral display compared with rewarding flowers, which might have a negative influence on the reproduction of rewarding plants. Examining the mutual relationships between rewarding and non-rewarding plants may offer insights into the ways in which co-flowering plants in a community interact to maximize pollination success.

The Huanglong National Nature Reserve, in south-west China, harbours a great diversity of orchid species that differ widely in floral morphology and pollination system (Li et al., 2005). However, among the 34 orchid species found, two intriguing species, *Galearis diantha* and *Ponerorchis chusua*, show similar morphological characteristics despite being classified into different genera on the basis of DNA evidence (Bateman et al., 2003) and differences in vegetative parts (Cribb, 2001; Wood, 2001). Both species produce purplish-red flowers, with two petals and a dorsal sepal forming a hood covering two erect anthers with two parallel thecae containing the pollinia (see Fig. 2B, D). These floral traits indicate that these orchids might share the same pollinator. It should be noted that *G. diantha* is nectariferous, whereas *P. chusua* is nectarless. They are particularly well-suited species for the investigation of reproductive isolation in plants that share floral shape and colour. Because recent studies have revealed that non-rewarding flowers with a colour similar to rewarding ones receive more visits than those with dissimilar colours (Internicola et al., 2007; Peter and Johnson, 2008), the effects that rewarding and non-rewarding plant with the same floral colour have on one another also merit examination.

This study addressed the topics of reproductive isolation and pollination success by posing the following specific questions: (a) Does temporal isolation occur between *G. diantha* and *P. chusua*, or do the two species flower in an overlapping manner? (b) Do the two orchids share pollinators, or does ethological isolation minimize pollen transfer between the two species? (c) Are there substantial differences in floral morphology that obstruct pollen transfer between the two species, or does mechanical isolation occur between the two species? (d) Are the two species artificially hybridizable? (e) Is non-rewarding *P. chusua* more pollinator-limited than rewarding *G. diantha*? (f) **Does non-rewarding *P. chusua* benefit from growing in close proximity to rewarding *G. diantha*?** (g) **How does non-rewarding *P. chusua* affect the pollination success of rewarding *G. diantha*?**

MATERIALS AND METHODS

Study species and populations

Galearis diantha and *Ponerorchis chusua* are perennial herbaceous orchids that commonly occur in the alpine meadows, alpine shrublands and forests of the eastern Himalayas and the Hengduan Mountain region of China, Russia, Korea and Japan (Lang, 1999). *Galearis diantha* produces an inflorescence with one to four flowers (mean number of open flowers per plant = 2.1, $n = 109$) at the top of a 6- to 10-cm-tall stalk emerging from the underground rhizome, whereas *P. chusua* produces an inflorescence with 1–20 flowers (mean number of open flowers per plant = 4.6, $n = 111$) at the top of a 10- to 25-cm-tall stalk emerging from the underground tuber. The flowers of both species are purplish-red, with dark purple spots on the trifold labellum of *P. chusua* and the non-divided labellum of *G. diantha*. The two petals and the dorsal sepal of both species form a hood over the anthers, which are erect with two parallel thecae (Fig. 2B and D) that contain two pollinia per flower. The stigma of *G. diantha* consists of two elliptical lateral lobes and lies on both sides of the anther, whereas that of *P. chusua* is a cavity and lies above the entrance of the spur. Both orchids flower for 3–4 weeks beginning in middle or late June.

Two subpopulations (A and B) in the Huanglong National Nature Reserve (32°41' to 32°54' N, 103°44' to 104°3' E, altitude 3100–3570 m a.s.l.) in Sichuan province, south-west China, were selected in 2005. In these two subpopulations, which were separated by a **distance of at least 500 m**, two species grew either monospecifically or heterospecifically in a calcareous, relatively nutrient-rich sparse coniferous forest (with *Picea purpurea*, *Abies faxoniana* and *Abies ernestii*) mixed with some shrubs (*Betula utilis*, *Salix tetrasperma*, *Berberis polyantha* and *Dasiphora fruticosa*) (Huang et al., 2008). During the study period, there were a few concurrently blooming insect-pollinated species dispersed within or in the vicinity of the studied communities, including *Polygonum sphaerostachyum*, *Allium prattii* and one species of *Caltha*. These co-flowering plants were different from the two species of orchid in floral colour and morphology.

Flowering phenology

Flowering phenological traits for each species were studied in subpopulation A. At least 100 individual flowering plants of each species were selected randomly and marked with plastic tags before flowering. A flower was judged as 'opening' when the labellum was spread and visiting insects could enter the flower. A flower was judged as 'wilting' when its colour or shape changed and it thereby lost its role in the pollination process. The flowering period at the individual level was measured as the number of days elapsed between the opening of the first flower and the wilting of the last flower within an inflorescence. The flowering period at the subpopulation level was measured as the number of days elapsed between the opening of the first flower in the subpopulation and the wilting of the last flower in the subpopulation. Every flower of each marked plant was visited every 2 or 3 d (except on rainy days), and opening and wilting were recorded.

Pollinator observations

The composition of the pollinator assemblage, flower constancy and visitation frequency of the pollinators were investigated between 1000 and 1600 h (the peak activity hours of insects) on sunny days during the peak flowering period in subpopulation B. Individual pollinators visiting the two orchids were carefully observed to ascertain their pollinating behaviour. Particular attention was paid to how the insect approached and landed on the flower. The flower constancy of pollinators was determined by tracking at least 20 pollinator individuals (regardless of species) during a whole flying bout and recording whether the pollinator visited other flowers before or after visiting the focal orchid. Visitation frequency was estimated as the total number of times that a pollinator approached but did not land, landed after visiting other flowers or landed directly on the flowers of each orchid in an area that the observers were able to monitor during a 1-h observation period (flower numbers ranged between about 50 for *P. chusua* and 30 for *G. diantha*). At least six randomly selected areas were used, with a total observation time of over 60 h. For each pollinator, the number of inflorescences visited in a visiting bout, the number of flowers visited per inflorescence and the time spent on each flower and inflorescence were also recorded. Some insects visiting the two species were captured immediately after visiting the flower for identification. Insects carrying pollinaria of orchids were defined as pollinators. Whether the collected pollinators loaded the pollinaria of the two species and non-conspecific species ($n > 10$) was also examined to assess pollinator constancy and potential interspecific pollen transfer for each of the two species.

Measurements of the functional characteristics of flowers and pollinators

The entrance to the spur of *P. chusua* is situated below the rostellum and the stigma, whereas that of *G. diantha* is situated below the rostellum and between two lateral stigmas. Variations in the spur might be the key functional characteristics that influence mechanical isolation between the two

species. Three functional characteristics that closely relate to pollen transfer (spur length and the horizontal and vertical diameter of the spur entrance) were measured in 30 randomly selected flowers from 30 different individuals of *P. chusua* in subpopulation A. In addition, spur length was measured in 30 randomly selected flowers from 30 individual plants of *G. diantha* in subpopulation A (in those specimens, the spur entrance was too small to be measured). Also measured were five characteristics of the collected pollinators that closely relate to pollen transfer: the width and height of the head, the height of the mesothorax and the length of proboscises. All of these variables were measured to the nearest 0.1 mm using a digital caliper.

Artificial experiments for breeding system and pollinator limitation

Hand-pollination experiments were conducted to detect the breeding system and whether pollination success is limited by pollinator visitations. For each of the following treatments, at least 20 flowers each of *P. chusua* and *G. diantha* were selected randomly from subpopulation A and enclosed with fine nylon-mesh (6 cm × 8 cm in size) netting before the opening of the flowers. Each experimental flower was assigned to receive one of four treatments early in the flowering season: self-pollination ($n = 20$ flowers), intraspecific cross-pollination ($n = 20$ flowers), no manipulation (to test for spontaneous autogamy; $n = 20$ flowers) and open without treatment (control; $n = 56$ – 58 flowers). Pollen donors for cross-pollination were selected from plants growing at least 10 m away. All individuals were checked for fruit set at the end of the reproductive season.

Although no hybrids between the two species have been found in nature, interspecific hand-pollinations between the two species were conducted in both directions in subpopulation A to determine which factors prevent the formation of hybrids. Twenty flowers from 20 individuals of *G. diantha* were paired with 20 flowers from 20 individuals of *P. chusua* as mutual pollen donors and recipients (all of which had previously been enclosed in fine nylon-mesh netting) for use in interspecific hand-pollination experiments during the period of highest simultaneous flowering. All of the experimental plant pairs were checked for fruit set at the end of the reproductive season and the percentage of seeds with embryos was calculated in the laboratory.

Pollination success

At the study site, the two species of interest were found growing either in separate monospecific patches or in heterospecific patches (mixtures of rewarding and non-rewarding orchids). To census pollination success within the two groups of patches where the habitats were similar, at least four plots for each category were established within a 1-m radius (median of patch size) in subpopulations A and B. To minimize the effect of flower density on pollination success, flowering individuals within all plots were pruned to 30 flowering plants (a commonly found number). Heterospecific plots were arranged to have the same number of flowering individuals for each of the two species to avoid a frequency-dependent

effect on pollination success. Fruit set was used to evaluate pollination success. At least 4 weeks after the flowering period, when the fruits had matured, the number of fruits per individual was recorded. The fruit-set rate of an individual was calculated as the percentage of flowers that set a fruit. The fruit-set rate of a plot was calculated as the average percentage of individuals that set fruit within the plot. Floral display size (number of flowers on an individual plant) was counted prior to fruit maturation.

An univariate analysis of variance (ANOVA) model was used to examine the mutual effects of the rewarding *G. diantha* and the non-rewarding *P. chusua* on pollination success. Pollination success (fruit-set rate) was the dependent variable and dispersion patterns (monospecific or heterospecific) were the independent variables. All analyses simultaneously included display size as a covariate. Fruit-set rate was arcsine-square root transformed prior to the analysis to meet the requirements for parametric tests.

RESULTS

Temporal variation in blooming period for the two species

The anthesis of an individual flower was not significantly different between the two species: about 14 d ($n = 291$, s.d. = 0.72) in rewarding *G. diantha* and 15 d ($n = 370$, s.d. = 0.67) in non-rewarding *P. chusua*. Similarly, the anthesis of an individual plant was not significantly different between the two species: about 20 d on average, ranging from 7 to 27 d ($n = 104$, s.d. = 0.70) in *G. diantha* and about 22 d on average, ranging from 12 to 33 d ($n = 146$, s.d. = 0.89) in *P. chusua*. The anthesis period of *G. diantha* within the subpopulation was about 5 weeks, whereas that of *P. chusua* was about 7 weeks. Flowers of *P. chusua* opened about 10 d before those of *G. diantha*; however, the peak blooming periods of the two species overlapped (Fig. 1).

Pollinator composition for the two species

On observing *P. chusua* for 61 h, it was found that 11 anthophilous insect species visited this non-rewarding species. However, only four *Bombus* (bumblebee) species (workers of *B. rufofasciatus* and *B. consobrinus*, queens of *B. lucorum* and males of *B. pyrosoma*) were the effective primary pollinators, as they were found to carry the pollinaria of *P. chusua*.

On observing *G. diantha* for 59 h, it was found that ten insect species visited this rewarding species. However, only one bumblebee species (workers of *B. rufofasciatus*, which were also pollinators of *P. chusua*) was found carrying the pollinaria of *G. diantha* and thus regarded as the effective pollinator. Workers of *B. pyrosoma* and *B. lepidus* were considered potential pollinators because they were observed to visit these rewarding flowers but no pollinia were found on their bodies.

Pollinator behaviours

During the field observation, three visiting patterns were observed for bumblebees visiting the rewarding or non-rewarding flowers. First, some bumblebees tended to approach the orchid flowers but immediately turn away and fly out of the vicinity of the flowers. Secondly, some bumblebees landed directly on the labellum and immediately started foraging the orchid flowers. Thirdly, some bumblebees visited other concurrent flowering plants such as *Pedicularis* spp. (louseworts) before landing on and visiting the orchid flowers. The frequency of these behaviours (in terms of visits per hour) differed between the two orchid species. In *G. diantha*, the frequency of approaching the orchid was far higher than that of direct landing. In *P. chusua*, the frequency of direct landing and visiting the orchid was higher than that of approaching (Table 1). However, the frequency of bumblebees visiting other plants before landing on the orchid flowers was low in both orchid species (Table 1). The frequency of approaching was higher in *G. diantha* than in *P. chusua*, but the frequency of landing either directly on the orchids or after visiting other plants was far higher in *P. chusua* than in *G. diantha*, indicating that the bumblebees appeared to be more attracted to non-rewarding *P. chusua* with its large floral display than to rewarding *G. diantha* with its small floral display.

The behaviour of bumblebees when removing the pollinarium and pollinating the flower was similar in the two species. When a bumblebee was engrossed in exploring the spur (Fig. 2A–E), the proboscis of a *G. diantha* pollinator or the mentum of a *P. chusua* pollinator contacted the viscidium, resulting in the removal of the pollinarium from the flower as the bumblebee withdrew after finishing its foraging. Pollinia deposition occurred when a pollinarium-carrying bumblebee visited another flower and repeated its movements, thereby depositing pollinia on the stigma.

Bumblebees stayed inside both rewarding and non-rewarding orchid flowers for a short time, about 6 s ($n = 70$, s.d. = 0.03) per flower in *P. chusua* and only 2.5 s ($n = 22$,

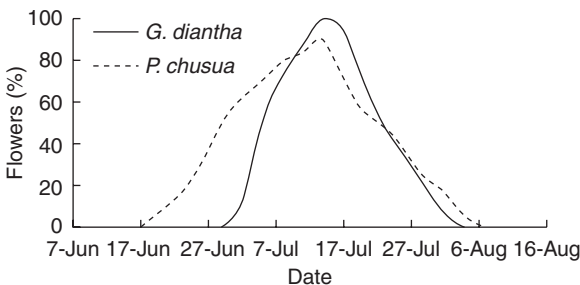


FIG. 1. Flowering phenology of rewarding *Galearis diantha* ($n = 291$ flowers from 104 individuals) and non-rewarding *Ponerorchis chusua* ($n = 370$ flowers from 146 individuals).

TABLE 1. Frequency of pollinators visiting rewarding *G. diantha* and non-rewarding *P. chusua* (times per hour)

Visiting pattern	<i>G. diantha</i>	<i>P. chusua</i>
Approaching, but immediately flying away	0.83	0.70
Direct landing	0.29	0.90
Landing after visiting other flowers	0.08	0.25

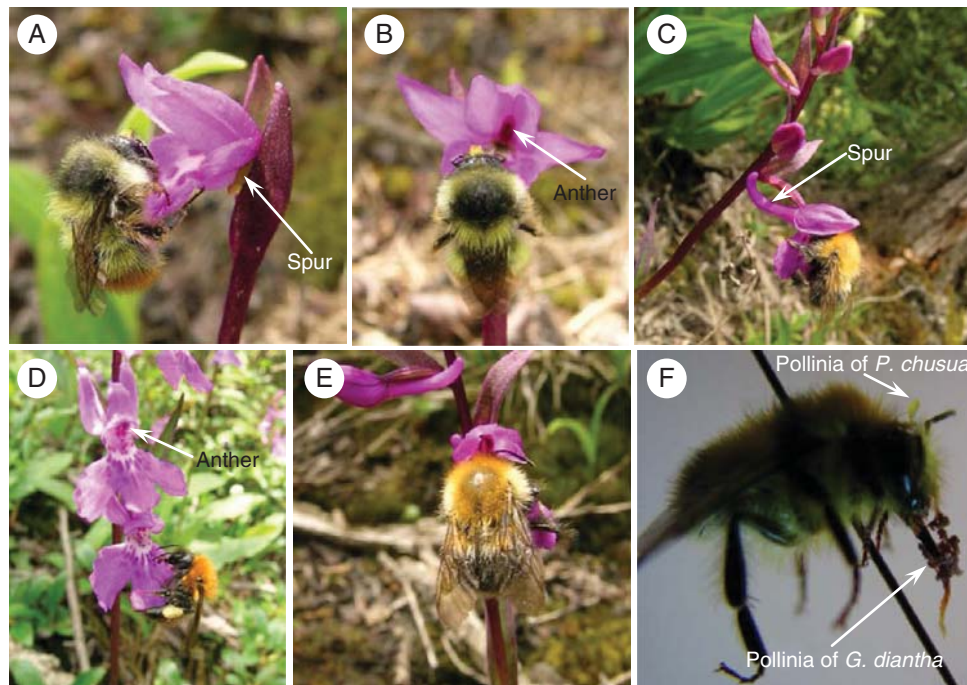


FIG. 2. *Galearis diantha* and *Ponerorchis chusua* and their pollinators: (A, B) pollinators visiting the flower of *G. diantha*; (C–E) pollinators visiting the flower of *P. chusua*; (F) *Bombus rufofasciatus*, the shared pollinator of *G. diantha* and *P. chusua*, with pollinia from two species sticking to different parts of its body.

s.d. = 0.12) per flower in *G. diantha*. Before flying out of the subpopulation, bumblebees usually visited only one and occasionally two flowers per plant for one to three plants in *P. chusua* and only one or two flowers per plants for one to five plants in *G. diantha*. During the field observation, no bumblebee was observed visiting flowers of both *P. chusua* and *G. diantha* during a single visiting bout to a subpopulation. However, two *B. rufofasciatus* workers were caught carrying the pollinia of both *P. chusua* and *G. diantha* (Fig. 2F).

Morphological traits of the orchids and pollinators

The differences in the size and morphology of the flower spurs of the two species have an important effect on pollen transfer. For *G. diantha*, the spur points downward (Fig. 2A) and is significantly shorter than both the spur of *P. chusua* ($t = 14.54$, $n = 30$, $P < 0.05$) and the proboscis of bumblebees ($t = 5.04$, $P < 0.05$; Table 2). In *P. chusua*, the spur points upward or horizontally (Fig. 2C) and is much longer than both the *G. diantha* spur and the proboscis of bumblebees ($t = 10.02$, $P < 0.05$; Table 2). This indicates that the bumblebee proboscis could partly enter the spur of *G. diantha* flowers but could completely enter the spur of *P. chusua* flowers. In addition, the size of the spur entrance (mouth) varied significantly between the two species. The width and height of the spur entrance of *P. chusua* were significant larger than those of *G. diantha* and than the head width and height of the bumblebees (2.15 ± 0.12 mm and 2.05 ± 0.4 mm, respectively) but were smaller than the thorax width and height of bumblebees (Table 2), resulting in entry into the spur by the head of the bumblebee but not its thorax. In *G. diantha* flowers, the size of the spur entrance was too small to be measured; neither the proboscis nor the thorax of the bumblebees could

enter the spur. A consequence of these differences in floral morphology was that the pollinaria of the two species were deposited onto different parts of the bumblebee: the pollinarium of *G. diantha* was deposited on the proboscis of the bumblebee, whereas that of *P. chusua* was deposited on the mentum (Fig. 2F).

Pollinator limitation and crossability between the two species

All *G. diantha* and *P. chusua* flowers that were bagged before opening failed to develop fruits (Table 3), indicating that spontaneous autogamy does not occur in either species. Of the flowers that were cross-pollinated by hand, 65 % of the *G. diantha* flowers and 70 % of the *P. chusua* flowers set fruit. Of those that were self-pollinated by hand, 80 % of the *G. diantha* flowers and 85 % of the *P. chusua* flowers set fruit. These percentages were significantly higher than that of the control flowers (Table 3; control vs. crossing *G. diantha*: $F = 22.61$, $P < 0.001$; control vs. selfing *G. diantha*: $F = 83.09$, $P < 0.001$; control vs. crossing *P. chusua*: $F = 24.84$, $P = 0.002$; control vs. selfing *P. chusua*: $F = 64.43$, $P < 0.001$). These results indicate that pollinator limitation was prevalent in both species. However, no significant difference was observed between rewarding *G. diantha* and non-rewarding *P. chusua* in the average fruit set of the control flowers ($F = 0.662$, $P = 0.417$) (Table 3).

Interspecific artificial pollination between *P. chusua* ♀ × *G. diantha* ♀ resulted in 100 % fruit set, with 67 % of seeds having embryos ($n = 20$, s.d. = 5.8 %), whereas interspecific artificial pollination between *G. diantha* ♀ × *P. chusua* ♀ resulted in only 90 % fruit set, with 73 % of seed having embryos ($n = 20$, s.d. = 7.7 %).

TABLE 2. The size of key morphological traits of two orchid species and their pollinators (mm, mean \pm s.d.)

Floral morphology	<i>G. diantha</i>	<i>P. chusua</i>	<i>Bombus</i>	Pollinator morphology
Height of spur entrance	–	2.34 \pm 0.21	4.47 \pm 0.53	Thorax height
Width of spur entrance	–	2.79 \pm 0.26	12.02 \pm 0.38	Thorax width
Spur length	2.63 \pm 0.52	12.71 \pm 1.93	6.27 \pm 1.93	Proboscis length

Floral traits were measured from 30 flowers; bumblebee sizes were measured from 15 individuals. The size of spur entrance of *G. diantha* is too small to be measured.

TABLE 3. Percentage of flowers that set fruit after four treatments for determining breeding system and pollinator limitation in rewarding *G. diantha* and non-rewarding *P. chusua*

Treatments	<i>G. diantha</i>	<i>P. chusua</i>
Autogamous pollination	0 % (20)	0 % (20)
Out-crossing pollination	65 % (20)	70 % (20)
Self-pollination	80 % (20)	85 % (20)
Control	40.1 % (58)	34.6 % (56)

The numbers within the parentheses were the numbers of flower sampled for experiments.

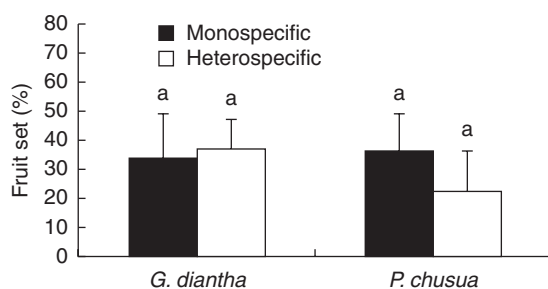


FIG. 3. Fruit set of rewarding *G. diantha* and non-rewarding *P. chusua* in monospecific and heterospecific plots. The letters show significant ($P < 0.05$) differences between dispersion patterns (Tukey *post-hoc* comparison test).

Pollination success of rewarding and non-rewarding orchids

In rewarding *G. diantha*, no significant difference in fruit set was found between the two patterns of dispersion (monospecific vs. heterospecific) (Fig. 3), indicating that co-flowering, non-rewarding *P. chusua* did not affect the pollination success of *G. diantha*. A similar result was observed in non-rewarding *P. chusua*: the fruit set of *P. chusua* did not differ between the individuals growing within monospecific plots and heterospecific plots (Fig. 3), indicating that rewarding *G. diantha* had no effect on the pollination success of non-rewarding *P. chusua*.

DISCUSSION

Reproductive isolation of two orchids

The field observations in Huanglong National Nature Reserve showed that the two sympatric orchids, *G. diantha* and *P. chusua*, were pollinated by *Bombus* species and that workers of *B. rufofasciatus* pollinated both species. The

potential hybrids, however, were not found in nature. Therefore, reproductive isolation mechanisms must serve to eliminate the possibility of interspecific pollen transfer between them. Here, the reproductive isolation (especially floral isolation) between *G. diantha* and *P. chusua* is discussed.

Temporal isolation is an important prezygotic mechanism in which interspecific pollen transfer among sympatric species is prevented by flowering at different times (Grant, 1992; Grant, 1994). Flowering periods could differ in time (Raven, 1962) or season (Sun et al., 2009), or they could partially overlap (Yang et al., 2007). The difference in peak blooming period is considered to be especially important because, in general, flowers in the peak blooming period tend to have a greater chance of being fertilized (Willson, 1983). In *G. diantha* and *P. chusua*, however, the peak blooming periods overlapped (Fig. 1), which could not result in reproductive isolation. Considering sympatry, pollinator sharing and overlapping flowering periods, other mechanisms are thus expected to prevent interspecific pollen transfer between these two orchids.

Some sympatric co-flowering species attract different pollinating species to prevent heterospecific pollen transfer (van der Pijl and Dodson, 1966; Dressler, 1993; Cozzolino and Widmer, 2005a). In the present study, *P. chusua* was pollinated by four *Bombus* species, among which *B. rufofasciatus* was also the exclusive pollinator of *G. diantha*. This indicates that isolation via unique pollen vectors was not a means of preventing pollen transfer between these two species. When the taxa pollinating different species overlap, individual pollinators may visit only one (or a few) plant species (floral constancy) to eliminate interspecific pollen transfer (Waser, 1986; Chittka et al., 1999). Flower constancy and/or floral preference is considered to be a very common type of ethological isolation and is found in many other taxa (Grant, 1994; Yang et al., 2007). Bumblebees usually exhibit a high level of flower constancy in their foraging behaviour because individual bees tend to go from plant to plant of the same flower type, skipping over plants with different flower traits (Heinrich, 1976). In *G. diantha* and *P. chusua*, bumblebee pollinators of both species display some degree of constancy in visiting each orchid species (Table 1). However, they also visited other flowers, including other orchid species, before foraging the focal orchids, indicating that ethological isolation between the two orchid species is not complete. The interspecific visits of bumblebees may be ascribed to the fact that *P. chusua* offers pollinators no nectar and *G. diantha* offers only meagre nectar. The spurs of *G. diantha* contain an average one-time nectar secretion of 1.2–1.6 μ L; after bumblebee harvesting, the flowers are nearly empty (H.-Q. Sun, pers. obs.), and thus bumblebees only visit a few of these flowers before leaving the patches, or approached but

immediately flew away (Table 1). The fact that bumblebees stayed in the flowers for only short time and visited a few flowers of both species in a foraging bout indicates that both species have difficulty in maintaining bumblebee interest and flower constancy. This incomplete ethological isolation may function in combination with other mechanisms, such as mechanical isolation, to prevent interspecific pollen transfer, as has been found in other sympatric co-flowering taxa (Nilsson, 1983; Nilsson et al., 1987; Yang et al., 2007).

In the most-common type of mechanical isolation, substantial variation in floral structures in two or more sympatric species usually leads them to attract different groups of pollinators with different body sizes and shapes (Grant, 1994). This form of mechanical isolation does not occur in the two study species because both are pollinated by bumblebees. In some congeneric species, flowers exhibit more similarities in structure or other traits; however, only a few variations are needed to affect pollinator taxa and plant reproduction. Floral spurs, for instance, have been proposed to be a key factor in diversification of *Aquilegia* (Hodges, 1997) and to provide a prezygotic reproductive isolating mechanism via differential pollinator visitation (Hodges and Arnold, 1994; Hodges, 1997; Whittall and Hodges, 2007). In the present study, the sizes of petals, sepals and lips differed significantly between *G. diantha* and *P. chusua* (H.-Q. Sun, unpubl. res.). The spur was, nevertheless, the key trait that affected interspecific pollen transfer between the two species. The spur of *P. chusua* was longer than the proboscis of the bumblebees, and the height of the spur entrance was larger than the head height, but smaller than the thorax height, of bumblebees (Table 2), resulting in the dispersion of the pollinarium onto the mentum of the bumblebee (Fig. 3F). In *G. diantha*, the spur was far shorter than the proboscis of the bumblebee, resulting in the attachment of the pollinarium to the proboscis of the bumblebee (Fig. 3F). The deposition of the pollen of sympatric species onto different regions of the body of a shared pollinator because of substantial differences in key floral traits is not unique to *G. diantha* and *P. chusua*; it is one of two forms of mechanical isolation, *Pedicularis*-type, which is well-known in *Pedicularis* (Grant, 1994; Yang et al., 2007, and references therein) but has also been found in other sympatric co-flowering orchids, such as *Platanthera* (Nilsson, 1983), *Angraecum* (Nilsson et al., 1987) and *Ophrys* (Kullenberg, 1961; Borg-Karlson, 1990; Cortis et al., 2009).

Reciprocal crosses of *G. diantha* and *P. chusua* produced a high percentage of fruit set and a high percentage of seeds with embryo, suggesting that an internal isolation mechanism between the two species was weak or lacking. The crossability seen in the two studied species is not unusual and has been found frequently in other sympatric orchids (Cozzolino and Widmer, 2005b). However, no hybrids were found in nature, indicating that, compared with postzygotic isolation, floral isolation (primarily a mechanical mechanism in combination with an ethological mechanism) is the most important factor in maintaining the integrity of the two species studied.

Pollinator limitation and pollination success of the two orchids

Low pollinator availability has been found to be a crucial cause of low natural fruit-to-flower ratios in some non-rewarding plants (Gill, 1989; Neiland and Wilcock, 1998;

Tremblay et al., 2005). In the present study, however, rewarding *G. diantha* suffered significant pollinator limitation to fruit set, as did non-rewarding *P. chusua*. This result contrasts with the prediction that the rewarding species should attract more pollinators and so have lower pollinator limitation, but it is consistent with the experimental result of a study on two non-rewarding orchids and one rewarding orchid by Smithson (2006). The fact that non-rewarding *P. chusua* experienced pollinator limitation, despite four bumblebee species being involved in pollen transfer, may be ascribed to the fact that it provides no nectar for bumblebees and thus has difficulty in sustaining pollinator interest, as has been found with other non-rewarding plants (Peakall and Beattie, 1996; Johnson et al., 2003). The reason that rewarding *G. diantha*, which offers nectar to pollinators, also attracted limited numbers of bumblebees may be that it only offers a one-time secretion of meagre nectar to pollinators. After harvesting by a bumblebee, the flower is nearly empty, so only a few flowers are visited before the bumblebee leaves the patch or switches to other flowers. A high frequency of approaching but not landing on *G. diantha* (Table 1) seems to support this explanation. Another reason for pollinator limitation in the two species may be the abundance of these pollinators fluctuating greatly in space or time (Knight et al., 2005; Cosacov et al., 2008). Pollinator limitation within a season is also found in meagre-nectar-producing *Comparettia falcata* (Meléndez-Ackerman et al., 2000) and other rewarding plants (Ågren, 1996; Smithson, 2006).

It has been shown that compared with rewarding species, non-rewarding plants usually experience low fruit set (Gill, 1989; Neiland and Wilcock, 1998). Non-rewarding plant species, however, may benefit from close proximity to co-flowering rewarding species (Thomson, 1978; Laverty, 1992; Alexandersson and Ågren, 1996; Internicola et al., 2007). In the non-rewarding, bumblebee-pollinated orchid *Anacamptis morio*, for example, pollination success was significantly higher for individuals translocated to patches of nectar-producing plants than for individuals placed outside such patches (Johnson et al., 2003). In contrast, the pollination success of the deceptive orchid *Dactylorhiza incarnata* was adversely affected by the experimental addition of nectar-producing *Viola* flowers (Lammi and Kuitunen, 1995). In the present study, the presence of nearby rewarding flowers resulted in neither the facilitation effect nor the competition hypothesis of rewarding flowers on the pollination success of non-rewarding plants. The fruit set of non-rewarding *P. chusua* growing in heterospecific plots was not different from that of monospecific plots (Fig. 3). This result may be attributed to the floral display effects and to the meagre nectar of *G. diantha*. *Galearis diantha* has a smaller floral display than *P. chusua* and usually produces two (occasionally three or four) flowers at the top of a 6- to 10-cm-tall stalk, whereas *P. chusua* produces from one to more than 20 flowers at the top of a 10- to 25-cm-tall stalk. For foraging bumblebees, *P. chusua* seems to be more attractive than *G. diantha*. The observation of four bumblebee species pollinating *P. chusua* and the higher frequency of direct landing (Table 1) appear to support this explanation. Additionally, the meagre nectar in the short spur of *G. diantha* is harvested after only one or few visits, after which the flower is nearly

empty and might be regarded as being just as deceptive as that of *P. chusua*. All of these characteristics might result in the lack of a facilitation or competition effect of *G. diantha* on *P. chusua*.

Compared with the rewarding species, non-rewarding plants generally have flamboyant or larger floral displays (Nilsson, 1992), which might have a negative influence on the pollination success of neighbouring rewarding species. This hypothesis, however, has rarely been investigated. In the present study, no such negative effect was found; the fruit set of *G. diantha* growing in plots mixed with non-rewarding *P. chusua* was not different from that in monospecific plots (Fig. 3). This result might be ascribed to the early flowering of *P. chusua* and the low overlap in the pollinator pool of the two species. Although their peak blooming periods did overlap, *P. chusua* flowered about 10 d earlier than *G. diantha*, which offered some opportunity for reduced competition with *G. diantha*. In addition, if two or more species compete for pollinators, a highly overlapping pollinator pool will facilitate this competition. In the two orchids studied, however, four bumblebee species transferred the pollinaria of *P. chusua*, among which one species also pollinated *G. diantha*. Logically, the degree to which *P. chusua* competes with *G. diantha* for pollinators might be low. The definite conclusion, nevertheless, remains to be determined because the significance of the shared pollinator *B. rufofasciatus* in the whole pollinator pool of *P. chusua* was not measured and will be investigated in a future study.

Conclusions

Although some species and genera of Orchidaceae show high levels of natural hybridization and low pollinator specificity (Cozzolino *et al.*, 2005; Cozzolino and Widmer, 2005b), floral or prezygotic isolation is still crucial to minimize pollen transfer between sympatric co-flowering species. Such isolation can occur by various means, including differences in key morphological traits that result in the deposition of pollen onto the different parts of shared pollinators in some orchids (including the species used in the present study; Nilsson, 1983; Nilsson *et al.*, 1987) and the specific floral odours that attract specific pollinators in some sexually deceptive species of *Ophrys* (Paulus and Gack, 1990; Schiestl and Ayasse, 2002; Mant *et al.*, 2005). The effect of nearby rewarding flowers on the pollinator visitation and pollination success of non-rewarding species has been investigated in many studies (Laverty, 1992; Lammi and Kuitunen, 1995; Alexandersson and Ågren, 1996; Johnson *et al.*, 2003; Internicola *et al.*, 2007; Peter and Johnson, 2008); however, few looked at the effect of non-rewarding flowers on the pollination success of rewarding species, especially those with the same flower colour. The findings of the present study reveal that non-rewarding *P. chusua* has no significant effect on the fruit set of rewarding *G. diantha*. Nevertheless, the study did not census removal of pollinia, which is an important parameter in assessing male reproductive success. Therefore, studies are still needed to gain a better understanding of the relationship between sympatric co-flowering species in general and between rewarding orchids and non-rewarding orchids in particular. Strong visual similarity between two

sympatric species, especially when one is an orchid, has led some authors to argue *a priori* for floral mimicry (Dafni and Ivri, 1981; Dafni, 1984; Roy and Widmer, 1999; Johnson, 2000; Johnson *et al.*, 2003). The two species of orchids studied, to the human visual system, have the same floral colour (Fig. 2); however, whether floral mimicry occurs between the two species remains to be tested because the data on visual cues (e.g. reflectance) are currently unavailable.

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