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REPRODUCTIVE BIOLOGY OF THE RARE PLANT, DYSOSMA PLEIANTHA (BERBERIDACEAE): BREEDING SYSTEM, POLLINATION AND IMPLICATIONS FOR CONSERVATION

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Abstract

Dysosma pleiantha is an endangered and endemic species in China. We have reported the flowering phenology, breeding system and pollinator activity of the species distributed in Tianmu Mountain (Zhejiang Province) nature reserves. Flowering occurred during the months of early April to late May, with the peak in the middle of the April, and was synchronous across all four subpopulations. The anthesis of an intact inflorescence lasted from sixteen to twenty-three days with eight to eleven days blossom of an individual flower. In *D. pleiantha*, the morphological development of flowers and fruit leading to the development of mature seeds takes place over a period 3–5 months from flowering. The average of pollen-ovule ratio (P/O) was 18 898.7. The pollen transfer in this species was mainly performed by flies, *Hydrotaea chalcogaster* (Muscidae). Controlled pollination experiments indicated *D. pleiantha* was obligate xenogamyous and self-incompatible, and pollination was pollinator-dependent. Controlled pollination experiments showed that the mean fruit set (%) under the natural condition (17.1%) was markedly lower than that of manual cross-pollination (75.6%). It was concluded that pollen-limitation and mate limitation were responsible for the low fruit set of *D. pleiantha* in the field. Thus, the identification and translocation of compatible mating types to create reproductively viable populations were essential for the recovery of the rare species.

Key words: Breeding system, *Dysosma pleiantha*, Pollination ecology, Self-incompatibility.

Introduction

The continued existence of plants in changing habitats depends a great deal on their reproductive biology. In order to avoid extinction, endangered species must ensure a viable population size that can be maintained through reproduction (Pandit & Babu, 2003). The reproductive success of plants is often limited by pollen availability (Burd, 1994; Larson & Barrett, 2000; Ashman *et al*., 2004; Knight *et al*., 2005). This occurs when the quantity of pollen that a plant receives during pollination is insufficient to fertilize available ovules, resulting in a reduction in fruit and/or seed production (Burd, 1995; Aizen & Harder, 2007). For self-incompatible (SI) species, if reduced population size is accompanied by the loss of S-allele richness, mate availability and fecundity may be limited (DeMauro, 1993; Vekemans *et al*., 1998), and fruit set can be affected by mate limitation as well as pollinator limitation (Glemin *et al*., 2008; Young & Pickup, 2010; Leducq *et al*., 2010; Young *et al*., 2012). Therefore, knowledge of both pollination biology and breeding systems of rare and endangered species is crucial to our understanding of the causes of rarity, and it is important for successful management and recovery programs of rare plant taxa.

The Podophyllaceae [formerly taken as a separate family but now included in Berberidaceae APG Ⅲ (2009)] comprises six genera: *Achlys*, *Diphylleia*, *Dysosma*, *Jeffersonia*, *Ranzania* and *Podophyllum*. Among the six genera, *Dysosma* Woodson is native to China, and consists of seven diploid herbaceous species. The rhizomes of *Dysosma*, also known as “Guijiu”, are used extensively in traditional Chinese medicine. The active constituent of “Guijiu” is podophyllotoxin, which has cytotoxic and antitumour properties and has been used for cancer treatment (Jackson & Dewick, 1985). *D. pleiantha* (Hance) Woodson, the focal species of the present study, is a constituent of montane temperate-deciduous forest habitats and is now limited to a few isolated populations in Southeast China (mainland) and Taiwan island. Due to a rapid demographic decline, this species has been treated as ‘threatened’ in Chinese mainland (Wang & Xie, 2004; Zong *et al*., 2008) and is classified as vulnerable in Taiwan (List of the rare and endangered plants in Taiwan, 2007). Recent anthropogenic activities including deforestation, habitat destruction and overcollecting for medicinal applications have led to the reduction of population size and serious habitat fragmentation of *D. pleiantha*. In the field, *D. pleiantha* grows in small, isolated populations and these populations are characterized by very low densities and patchy distribution. Most of the *D. pleiantha* populations have individuals less than a thousand. Tianmu (TM) Nature Reserves is the largest habitat of the rare species, comprising more than 1500 individuals based on our investigation and estimation (Bi-cai Guan, unpubl. data). Moreover, preliminary field observations indicated that although adult plants flower almost every year, fruit production of *D. pleiantha* is limited. Previous genetic diversity data (Qiu *et al*., 2005; Zong *et al*., 2008) showed a substantial heterozygosity deficiency in all analyzed *D. pleiantha* populations, and propagules appeared to occur mainly through vegetative means or plant selfing. However, further study has seldom been carried out to elucidate the reproductive biology of *D. pleiantha* in the field, which is important for the management of the rare plant.

In the present work we studied the reproductive biology of *D. pleiantha*, addressing the following issues: (1) the floral biology and flowering phenology, (2) the breeding systems and pollination ecology. Furthermore, these data may be useful to assess reproductive strategies, and the results also provide implications for conservation.

Materials and Methods

The species and site studied: The study was conducted in TM nature reserves of Zhejiang Province (30º19′ 04″ N, 119º26′50″ E) at an altitude of 200–340 m. The climate during the observation period was cool rainy days, alternated with sunny days. Annual average temperature was 21.2°C, and the average temperature in the coldest January and in the hottest month was 2.7°C and 31.3°C, respectively. In an individual day during the investigation, the air temperature was about 22°C from 0530 am to 0730 am. At the 1300 pm, the highest air temperature recorded during a day was 29°C.

D. pleiantha is a diploid (2*n* = 12; Ying *et al*., 2011). Individual plants mainly grow from the rhizomes and reach 30–110 cm in height, with a single stem bearing one leaf or two alternately arranged leaves. The leaves, 1 or 2, are centrally peltate, rounded, and 4–9-lobed with finely dentate margin. Plants remain in a juvenile phase for 4–5 years. When mature, they produce a terminal cyme with 1–30 drooping red-purple flowers in April. Plants reproduce asexually by rhizomatous growth and sexually by seed. But previous field observations have indicated that although adult plants blossom almost every year. Yet the seed production is limited by poor seedling establishment (Ma, 2000; Zong, *et al.,* 2008).

D. pleiantha in TM grows in rocky and humus soils on hillsides, and primarily appears in mixed evergreen and deciduous forests. The forest habitats of *D. pleiantha* are dominated by subtropical and temperate woody species such as *Cryptomeria japonica*, *Ginkgo biloba*, *Cunninghamia lanceolata*, *Phyllostachys edulis*, *Quercus acutissima*, *Castanopsis eyrei*, *Alangium chinense* and *Cyclobalanopsis multinervis* (B.C. Guan, pers. obs.). We divided the area, where species were located, into four subpopulations (ZLC, HM, GLJ and ZL), according to the landscape and the dominant vegetation. All the subpopulations in TM are mainly patchy, and each patch consists of 5–71 ramets, separated from neighboring patches by tens to hundreds of meters.

Flowering phenology and floral biology: In the field, Flowering individual was monitored daily, and the date of flower opening and wilting was recorded. A flower was defined as ‘opening’ when its calyxes and petals became loose and visiting insects could enter the flower (Fig. 1a). When the anthers were lost, the flower was considered as ‘wilting’. The floral biology was studied in detail using 74 flowers from 35 tagged individuals, including morphological changes, flowering period, flower lifespan, anther dehiscence, odor and stigmatic status. Among randomly selected 45 flowers, we measured corolla length, corolla width, style length and stamen length.

The pistils and stamens were fixed in a solution of 95% ethanol and glacial acetic acid (3:1) and stored in 70% ethanol. Scanning electron microscopy was used to examine the surface of pollen grains in detail. Specimens for scanning electron microcopy (SEM) were dehydrated in an ethanol-amyl acetate series, critical-point dried, coated with gold palladium and observed with XL-30E SEM (Philips). Samples were dehydrated through a graded ethanol series, infiltrated and embedded in Historesin according to Yeung & Law (1987). Serial, 3 µm sections were cut by glass knives using Teichert Autocut microtome, stained according to periodic acid-Schiff’s procedure and counterstained with TBO (Yeung, 1984). The stained sections were examined, and photographs were obtained using Olympus microscope.

P/O ratio: The pollen-ovule ratio (P/O) was estimated based on randomly selected individuals from TM following the method of Cruden (1977) and Dafni (1992). In total, 45 pre-anthesis flowers were collected, and stored in 70% ethanol. The anthers were crushed using a miniature pestle until there were no visible anther particles and suspended in a 25 ml solution of three parts lactic acid to one part glycerol. The solution was mixed with a vortex for 3 min to ensure even suspension of pollen grains. The numbers of pollen grains per flower (in a solution) was counted under a microscope using a hematocytometer. In addition, ovules were counted under a dissecting microscope. The number of pollen grains and ovules in each flower were calculated, and finally an average and standard errors was estimated.

Observation of flower visitors: Observation of flower visitors were carried out during 10 consecutive days (from 0800 h to 1800 h), spreading across two flowering seasons from 2010–2011 (from 11th to 20th April in 2010 and 15th to 24th April in 2011). We measured the activity of flower visitors of *D. pleiantha* by making observations in haphazardly chose four patches from delineated subpopulations. The patches contained, respectively, 47, 57, 60 and 65 tagged open flowers, which were divided into 3 groups according to individuals for tallying visitors. All observations occurred in daylight. Visitation rates (visits/flower/h) were calculated by dividing the total number of observed visits of a given insect by the total number of open flowers, and the total observation time of two years. The captured insect visitors were brought to the laboratory for identification and to examine them for the presence of pollen.

Breeding system analyses: To prevent insect visits, we bagged flowers for hand-pollination treatments before anthesis and rebagged them after pollination. Bags were made of fine bridal veil and remained on the treated flowers until they senesced or fruit had set. Additionally, virgin flowers were emasculated and left unbagged (No. of flowers = 186) to be tested for their presumedly decreased attractiveness to pollinators. Naturally (open) pollinated flowers (No. of flowers = 291) were used as controls. Artificial pollination was accomplished by directly brushing the stigmas of recipient flowers with pollen from the donors. Breeding systems for autogamy, geitonogamy and xenogamy, were tested through controlled pollinations. Hand-selfed within-flower pollination (No. of flowers =

99) was conducted to determine active autogamy, while spontaneous self-pollination (bagging) was studied by bagging intact flowers (No. of flowers = 109) before they opened. Hand-selfed within-plant pollination (No. of flowers = 180) was performed to detect geitonogamy. Xenogamy was tested by outcrossed hand-pollination (No. of flowers = 179) with pollen from flowers of other individuals. Because the species is capable of vegetative spread via rhizomes, the distance between pollen donor and receptor was more than 15 m to make sure that they were derived from the different genets.

Index of self-incompatibility: Index of self-incompatibility (ISI) was calculated to determine the breeding system. The ISI ratio was obtained as the percentage fruit set resulting from hand self-pollination over that from hand cross-pollination. Species with ratios < 0.25 are considered as self-incompatible and those with ratios > 0.25 as self-compatible (Bawa, 1974).

Data analyses: To determine whether there were significant differences in fruit set for pollination treatments and in visitation frequency for subpopulations, one-way ANOVA and contrast tests (Duncan) were used. The statistical techniques were based on Sokal & Rohlf (1995). All analyses were carried out using the statistical package SPSS 17.0 (SPSS 2009). In the text, mean values were cited with their standard errors.

Results

Flowering phenology and floral biology: The red-purple, drooping flowers of *D. pleiantha* were fasciculedly arranged on extra-axillary umbels containing 8.4 ± 0.41 flowers (No. of plants = 35). Only 1–6 of the flowers in an inflorescence bloomed firstly (Fig. 1a). The flowering period of *D. pleiantha* in TM natural reserve population started in the beginning of April and lasted until the end of May. The peak flowering time was in the middle of April. The lifespan of an intact inflorescence is about 16 to 23 days with about 8 to 11 days blossom of an individual flower. During the anthesis, six petals generally wrapped around the six stamens, and anthers were a little introrse toward pistil. After the flower blossomed 2–3 days, the anthers dehisced and uncovered yellowish pollen grains. Though the flower was lacking nectar but it smelled fetid after the anthers dehisced. No more than four days later, the pollen grains gradually turned gray from the top of anther to the base. More than two months were needed for the fruits to mature. The morphological development flowers and fruit leading to the development of mature seed takes place over a period 3–5 months from flowering.

In SEM studies we found that maturing gynoecium with an open loculus (Fig. 1b) exhibited a complex convoluted stigmatic surface (Fig. 1c), which constituted the receptive portion (Fig. 1b). According to the classification of Heslop-Harrison & Shivanna (1977), it was wet papillate type. The tract was lined with transmitting tissue composed of glandular cells, which extended from the receptive surface of the stigma through the stylar canal somewhat less than half the distance to the loculus (Fig. 1d). Pollen grains, with finely pitted exine, were spherical and tricolpate, and the floor of the furrow was flecked with minute granulations (Fig. 1e).

Six floral morphological characteristics of *D. pleiantha* were measured (Table 1). The length of anther was found to vary from 0.94 to 1.65 cm, with an average of 1.24 ± 0.02 cm. However, the length of the stamen varied from 1.40 to 2.91 cm, with an average of 1.91 ± 0.03 cm. The pistil was about 0.72 cm shorter than the stamen (mean of pistil length was 1.23 ± 0.03 cm). The length and width of petal ranged from 1.69 to 2.93 cm and from 0.79 to 1.49 cm with an average of 2.41 ± 0.05 cm and 1.11 ± 0.03 cm, respectively. The mean number of ovules per flower was 46 ± 7, ranging from 25 to 74.

Observations of flower visitors: *Hydrotaea chalcogaster* (Muscidae) was the most common visitor and the only insect that we calculated for the visit frequency (Fig. 1f). Besides, occasional ants and spiders (Not identified) were found visiting flowers in four subpopulations, but were excluded from observations, because the visits to flowers were so infrequent that timed watches were not practical. Voucher specimens of the insect visitors had been deposited in the Herbarium of Nanchang University (JXU).

The mean visitation rate for flies (*H. chalcogaster*) in TM natural reserves was 0.43 ± 0.03 (mean ± SE) (visit/flower/hour) (Table 2), and the difference in fly visitation frequency among subpopulations was not significant ($F_{3,8}$ = 0.567, P = 0.652). The flies favored visiting the flowers with dehiscent anthers. We observed that the flies crept on the corollas, and then moved into the flowers (sometimes flied away after staying on the flowers for a moment). Almost all the flies in the flower crawled around and occasionally touched the stigma with abdomen and antennae before stationed on a stamen. After staying a few minutes (usually no more than ten minutes), the insects left the flower with pollen grains stuck to the setae thereafter left the patch or visited another inflorescence in the same patch. *H. chalcogaster* usually appeared and visited the flowers, starting at about 1000 h during daytime. The peak of visitation was from 1100 h to 1300 h.

Table 1. Floral traits measured on *D. pleiantha*.

Observation	Flowers	Min. (cm)	Max. (cm)	Variation	Average (cm)	SE
Length of petal	45	1.69	2.93	1.24	2.41	0.0476
Width of petal	44	0.79	1.49	0.70	1.11	0.0292
Length of stamen	45	1.40	2.91	1.51	1.91	0.0329
Length of anther	45	0.94	1.65	0.71	1.24	0.0234
Length of pistil	44	0.81	1.7	0.89	1.23	0.0291
Amount of single flower ovules	45	25	74	49	46	7

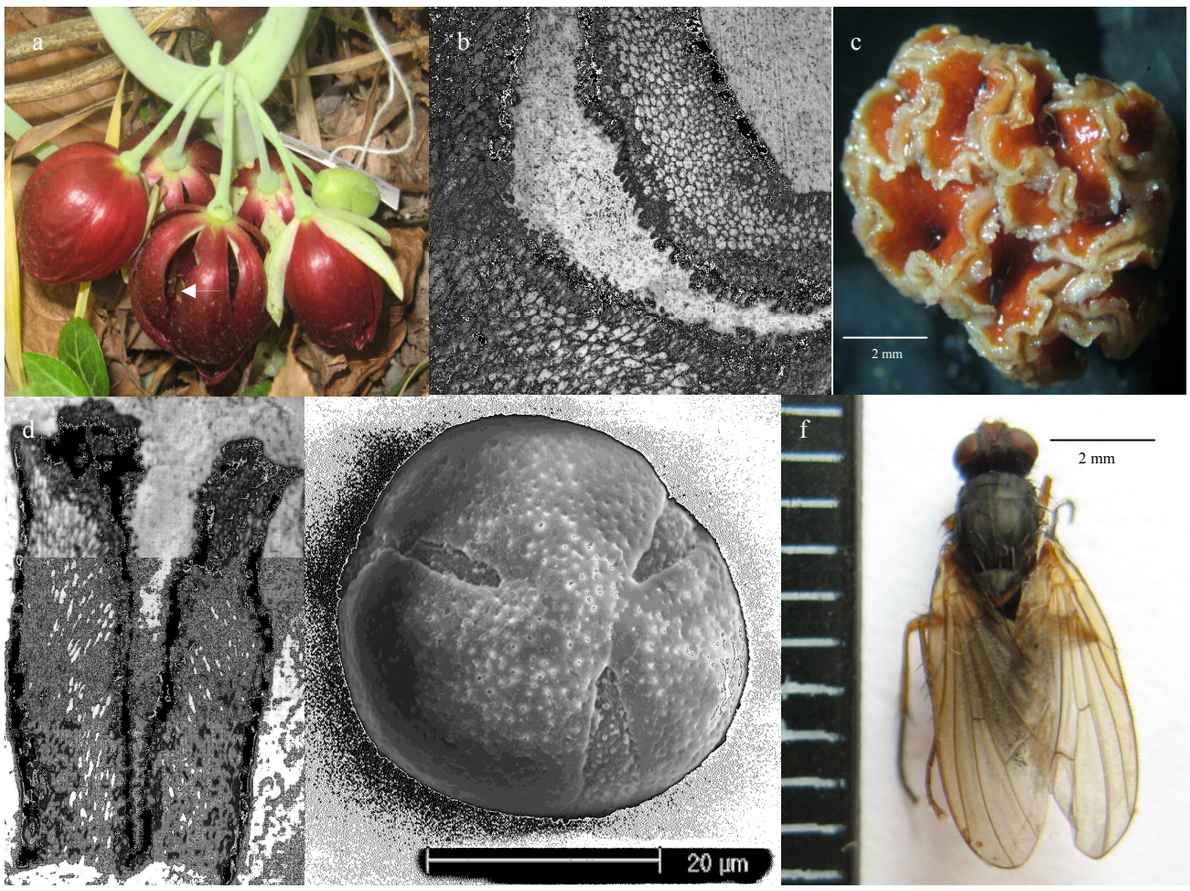

Fig. 1. Floral biology and flower visitor: a) The flowers of *D. pleiantha* are fascicledly arranged on extra-axillarels. Only 4 flowers in an inflorescence bloom firstly. Showing a fly in a open flower (arrow); b) Light microscopic micrograph of stigmatic transection showing papillae ×200; c) Complex convoluted stigmatic surface; d) Longitudinal section through stigma and partial style, lined in the upper portion with transmitting tissue composed of glandular cells ×100; e) SEM of tricolpate pollen with finely pitted; f) *Hydrotaea chalcogaster* captured from a flower of *D. pleiantha*.

Table 2. *Hydrotaea chalcogaster* visitation (visit/flower/hour) to four Subpopulations of *D. pleiantha.* Means with the same letter do not differ significantly at the 5% level (Duncan tests).

Subpopulations	No. of flowers (individuals)	No. of visitors	No. of hours	Frequency of visits	Mean in subpopulation	Mean in TM population
ZLC	16.00 (5)	1638.00	182.00	0.56		
	16.00 (5)	1274.00	182.00	0.44	$0.44^{a} \pm 0.07$	
	15.00 (4)	910.00	182.00	0.33		
HM	19.00 (6)	1780.00	178.00	0.53		
	18.00 (6)	1246.00	178.00	0.39	$0.44^{a} \pm 0.05$	
	20.00 (7)	1424.00	178.00	0.40		
GLJ	21.00 (7)	1260.00	180.00	0.33		0.43 ± 0.03
	21.00 (7)	1800.00	180.00	0.48	$0.47^{a} \pm 0.08$	
	18.00 (6)	1980.00	180.00	0.61		
ZL	22.00 (7)	1584.00	176.00	0.41		
	23.00 (7)	1408.00	176.00	0.35	$0.37^{a} \pm 0.02$	
	20.00 (5)	1232.00	176.00	0.35		

Breeding systems analyses: By counting, the mean numbers of pollen grains and ovules per flower (±SD) were 870 236 ± 37 996 and 46 ± 7, respectively. The pollen-ovule (P/O) ratio was 18 898.7 ± 794.89, falling within the range for obligate outcrossing species (2 108–195 525; Cruden, 1977 & 2000).

The percentage fruit set for different pollination treatments in each of the four subpopulations of *D. pleiantha* from TM nature reserves was summarized in Fig. 2, together with results from one-way ANOVAs (Duncan's test) for treatment differences in mean fruit set across subpopulations. The fruit set under the natural condition (control) was 17.1 ± 1.2%. Hand-selfed within-plant ("geitonogamous") pollinations resulted in only four fruits, and the fruit set was 2.4 ± 0.3%, which was markedly lower than that of outcrossed ("xenogamous") hand-pollinations (75.6 ± 2.4%). In emasculated flowers with no bagging, the fruit set was 4.6 ± 0.3%. However, both flower-bagging and hand-selfed within-flower pollinations did not yield any fruits, indicating no potential for either autonomous or enforced selfing. Duncan's tests indicated that the difference in overall fruit set between outcrossed and natural pollinations was significant ($F_{3, 12}$ = 753.379, P = 0.000) (Fig. 2). In contrast, overall fruit set was not significantly different from zero after either open-emasculated or geitonogamous pollinations ($F_{2, 9}$ = 1.709, P = 0.166) (Fig. 2). The calculated index of self-incompatibility was 0.03, suggesting that *D. pleiantha* was mostly self-incompatible (Zapata & Arroyo, 1978).

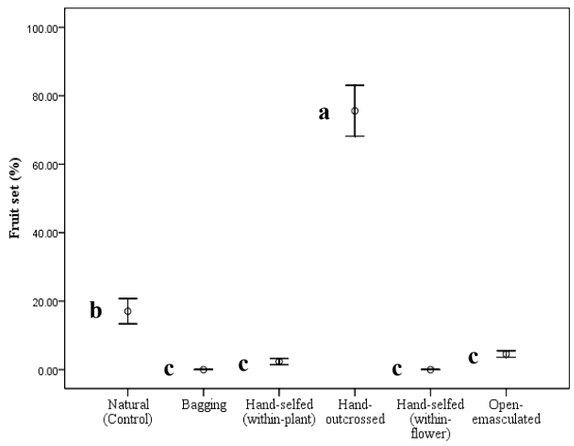

Fig. 2. Mean (± SE) fruit set (%) by *D. pleiantha* under natural conditions (control) and following different pollen transfer treatments: hand outcrossed, bagging, hand-selfed within-plant, hand-selfed within flowers, emasculation. Means with the same letter do not differ significantly at the 5% level (Duncan tests).

Discussion

According to the low index of self-incompatibility calculated (0.03) and the high pollen to ovule ratio observed (ca. 19 000), we believe that *D. pleiantha* is mostly self-incompatible (Zapata & Arroyo, 1978) and obligatory outbreeding (Cruden, 1977 & 2000). This, together with the presence of "wet" stigma (Fig. 1b and 1c) and binucleate pollen in *Dysosma* (Huang *et al*., 2001), suggest that incompatibility may be gametophytic, and the incompatibility may occur in style (Heslop-Harrison, 1975; De Nettancourdt, 1997). Conspicuous transmitting tissue extends over the stigmatic surface and lines the canal extending deeply downward into the style. The transmitting tissue is composed of glandular stigmatic cells that appear similar to the glandular stigmatic cells described by Demaggio & Wilson (1986) for *Podophyllum peltatum*. Although the function of the transmitting cells in style is not fully understood, they may play a role in pollen grain recognitions, determining the path of the pollen tube and supplying energy-yielding compounds to the growing pollen tube. Nevertheless, whether stylar self-incompatibility barriers occur in *D. pleiantha* would be worth further investigating using histochemical methods. However, the fact that at least some fruits developed in *D. pleiantha* after geitonogamous hand-pollination (ca. 2.4%) interestingly shows that self-incompatibility may break down in *D. pleiantha*. Similar dissolution of self-incompatibility has been observed previously for other plants, including *P. peltatum* (Policansky, 1983; Stone *et al*., 2006). The genetic mechanisms and physiological changes in the pollen or stigma could be responsible for the breakdown of gametophytic self-incompatibility systems (GSI) (Whisler & Snow, 1992; Stone *et al*., 2006).

The factors that affect the foraging behavior of a pollinator include both intrinsic and extrinsic factor. Extrinsic factors include floral color, shape, wind velocity, the distribution of nectar rewards, density, number and distribution of the plant (Waddington, 1981). Our observation revealed that *H. chalcogaster*, the most common visitor to *D. pleiantha*, might be attracted by the malodour of pollen grains and floral color. Fruit set from naturally pollinated control flowers in *D. pleiantha* was higher compared to open-emasculated pollinations (p<0.05; Fig. 2), which further suggested that flowers with removed anthers were less attractive to potential pollinators than intact ones. Moreover, lack of nectar, low densities and patchy distribution could be responsible for making *D. pleiantha* less attractive to more pollinators. As showed by many previous findings, pollen-limitation or failure is thought to be more likely in plants that are pollinated by one or few insect species (Bond, 1994; Kearns & Inouye, 1997; Johnson & Steiner, 2000; Spira, 2001). The significant difference (p<0.05, Fig. 2) between outcrossed and natural (control) fruit sets clearly indicated that the fruit set in *D. pleiantha* is strongly pollen-limited. On the other hand, it turned out that seed production was accomplished primarily by the outcrossing that resulted from visits by flies (*H. chalcogaster*). However, field observations had revealed that *H. chalcogaster* sometimes visited individuals in the same patch, and previous allozyme and ISSR markers studies have demonstrated that there was low genetic heterogeneity and a ramets in the wild generally shared identical or similar genotypes in the species (Qiu *et al*., 2005; Zong *et al*., 2008). Thus, the current study, together with the results of genetic diversity investigations, implied that mating partially took place among genetically related and geographically close individuals and/or intraclone ramets. For the species with gametophytic self-incompatibility, the pollen from

ramets of a genet will have little importance in the reproduction, and sexual reproduction requires pollen from different genets (Weekley & Race, 2001). It was also supported by Wolf & Harrison's findings 2001 that fruit set of the clonal self-incompatible morning glory, *Calystegia collina*, was limited by the availability of compatible mates (Wolf & Harrison, 2001). In conclusion, the limited or failed sexual reproduction in *D. pleiantha* could have been caused mainly by pollen-limitation as well as mate limitation. Thus, a further investigation should aim at quantifying the effects of both pollen-limitation and mate limitation on reproductive success and population viability in future. Furthermore, shortage of reward sought by its pollinators may be selectively favored because it encouraged pollinators to leave the patch after visiting one or a few flowers, thus promoting outcrossing (Dressler, 1981; Laverty, 1992). Therefore, we suggest that lack of nectar is an adaptive response to the combination of self-incompatibility and clonal growth, and pollen-limitation is simply a by-product of this adaptation. Certainly, the hypothesis needs to be verified by future studies.

Fragmented small populations of self-incompatible species face an increased short-term risk of extinction, which may be due to alteration in reproductive output caused by lack of interaction between patches. Moreover, they are also less capable of adapting to environmental changes (Kéry *et al*., 2000) and may eventually be prone to genetic drift and inbreeding depression (Knight *et al*., 2005). Depending on the vitality of a given genotype, isolated non-breeding populations may persist through vegetative reproduction for a protracted time, but it is essentially an evolutionary dead end (Luijten *et al*., 2000; Weekley & Race, 2001). A conclusion common to previous studies is that genetic factors, which have immediate effects on fitness or population viability in small or fragmented populations, does or at least has the potential to alter these effects (Wolf & Harrison, 2001; Lennartsson, 2002; Aigner, 2004). Therefore, the creation of viable populations necessitates artificial transplanting of individuals among isolated populations or different scattered plots within a population. In addition, a good strategy to encourage fruit set, with improvement of seedling recruitment, needs to be considered. Hence, an ecological manipulation (e.g. supplemental transfer of pollen by hand) would be an appropriate management method. Furthermore, demographic data should also be used to model the long-term viability of the species. In conclusion, an integrated conservation strategy based on breeding systems, demographic, ecological and genetic aspects should be prepared. By these ways, we hope that the future of this medicinally important and severely threatened species will be guaranteed.

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