

Autonomous self-pollination and insect visitation in a saprophytic orchid, *Epipogium roseum* (D. Don) Lindl.

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Abstract. Reproductive biology of saprophytic plants has been poorly studied. *Epipogium roseum* (D. Don) Lindl. is a small saprophytic orchid that is widely distributed in tropical and subtropical Asia, Australia and Africa. The floral biology and insect visitation of *E. roseum* were studied in Xishuangbanna, south Yunnan Province, China. *E. roseum* possesses an obligate self-pollination system, in which the degenerative rostellum has lost its function as a physical barrier separating the stigma and stamens (pollinia), allowing contact between the stigmatic secretions and the pollinia during bud development. Flowers of *E. roseum* usually open and successfully attract insect visitors. The Asian honey bee (*Apis cerana cerana*) was the only visitor observed, and regularly visited flowers of *E. roseum* for nectar. However, these bees did not carry pollinia away after visiting the flowers due to the absence of a viscid disk in *E. roseum*; the results of experiments also indicated that the Asian honey bee does not contribute to fruit set in *E. roseum*. The visiting frequency of Asian honey bees to flowers of *E. roseum* varied both spatially and temporally. *E. roseum* does not undergo outcrossing mediated by insects and is adapted to obligate self-pollination. We suggest that this may have evolved because of the uncertainty of pollinator services associated with its saprophytic lifestyle. Our current studies do not support the hypothesis that obligate autogamy is favoured by myco-heterotrophic plants due to resource limitations.

Additional keywords: obligate autogamy, Orchidaceae, pollinator services, relic floral features, saprophyte.

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Introduction

The great diversity of orchid species is widely considered to be attributed to adaptive radiation for specific pollinators driven by selection for outcrossing (Cozzolino and Widmer 2005; Tremblay *et al.* 2005). However, autonomous self-pollination is common in this family, and occurs in almost every tribe and subtribe, with a recent estimate that 31% of the orchid species in which pollination systems are known are capable of autonomous self-pollination (Arditti 1990; Peter and Johnson 2009). Autonomous self-pollination has often been explained as an evolutionary consequence of the lack of pollinators, providing reproductive assurance when the frequency of pollination is habitually low (e.g. Barrett 1985, 1996; Arditti 1990; Dressler 1993; Jacquemyn *et al.* 2005).

Autonomous self-pollination in orchids is facilitated by the close proximity of the anthers and stigma (Johnson and Edwards 2000), and a diversity of mechanisms has been reported in this family (Arditti 1990). Most mechanisms involve modification of the pollinarium and/or rostellum, allowing pollinia direct contact with the stigma of the same flower (Arditti 1990; Johnson and Edwards 2000). In the Orchidaceae, autonomous self-pollination occurs to different extents among species, and most retain the

potential for outcrossing by having functional pollinaria that will self-pollinate the stigma only if it is not removed by insects (Johnson and Edwards 2000). Only a few orchids are truly cleistogamous or apomictic (Arditti 1990).

Epipogium roseum is a widely distributed saprophytic orchid, and usually grows on very shaded forest floors. Although the reproductive biology of saprophytic plants has been poorly studied, a few studies have suggested that autonomous self-pollination is favourable for saprophytic plants that may encounter severe resource limitations (Takahashi *et al.* 1993; Zhang and Saunders 2000; Lehnbach *et al.* 2005). Jones (1985) observed and roughly elucidated the mechanism of autonomous self-pollination of *E. roseum* in Australia. As the bud develops, the stigma increases in size until it comes into direct contact with the pollinia and thus allows self-pollination. He also mentioned that *E. roseum* retains some relic floral features that are normally associated with cross pollination, such as flowers that have a musty scent, a spur full of nectar, and a callus on the labellum (Jones 1985). We were thus curious as to whether these relic floral features could successfully attract insect visitors, and if insect visitation could lead to outcrossing, as occurs in other orchids. We therefore set out to investigate the floral biology and insect

visitation of *E. roseum* in Xishuangbanna, one of the most orchid-rich areas in Southwest China, where the species occur naturally.

In this paper we report the results of studies concerning three aspects of the reproductive biology of *E. roseum*: (1) how does *E. roseum* accomplish autonomous self-pollination; (2) does this species successfully attract insect visitors; and (3) can this species accomplish outcrossing?

Materials and methods

Study species

Epipogium Gmelin et Borkhausen is a small genus containing three species of saprophytic orchids. *E. roseum* (D. Don) Lindl. is widely distributed in tropical and subtropical Asia, Australia and Africa (Luo and Chen 2002). It usually appears on thick humus on wet deciduous forest floors protected by a dense canopy.

Study site

Xishuangbanna is one of the most orchid-rich areas in China (Tsi and Chen 1995). However, little is known about the reproductive biology and pollination system of orchids in this area. We conducted our studies in the Green Limestone Forest Park (GLFP, 21°41'N, 101°25'E; alt. 580 m), which is part of Xishuangbanna National Nature Reserve, located in southern Yunnan Province, China. The GLFP covers 225 ha of tropical seasonal moist limestone rainforest with dominant trees including *Cleistanthus sumatranus*, *Tetrameles nudiflora*, *Pistacia weinmannifolia*, *Lasiococca comberi* var. *pseudoverticillata* and *Celtis wightii*. There is a pronounced rainy season (from May to October) and dry season (from November to March) with an average annual rainfall of 1200–1500 mm and an average annual temperature of 18–22°C in this area (Ma *et al.* 1998).

During investigation of the orchid diversity of the GLFP, 49 orchid species in 31 genera were recorded, most of which (40 species) are epiphytic. *E. roseum* is the only saprophytic orchid found in the GLFP. During its flowering season in May, *E. roseum* is commonly found in the deep shade on thick leaf-litter in the GLFP (Fig. 1a). This study was carried out during the flowering and fruiting period of *E. roseum* from 2009 to 2011.

Floral phenology and morphology

Since October 2008, we have been conducting long-term monitoring of the population dynamics and reproductive phenology of all orchids in the GLFP, in which we observed the phenology of 49 species, including *E. roseum* weekly. During the flowering and fruiting season, detailed observation of the flowering and fruiting phenology of *E. roseum* was conducted by recording the number of open flowers per inflorescence per day, the time of flower anthesis and withering, and fruit ripeness and seed dehiscence. Floral morphology was studied by measuring the aboveground inflorescence and the size of separated flower parts in the field. The structure of the flowers was photographed and described accordingly.

To determine the process of autonomous self-pollination in *E. roseum*, flowers at different developmental stages (estimated

by the positions on the same inflorescence) were examined under a binocular stereomicroscope, and the relative positions of the stigma and pollinia were described and photographed.

Thirty flowers from different individuals were randomly selected and bagged before anthesis in order to study nectar secretion. We used 10-μL SIGMA 'micro-cap' calibrated capillary tubes (Sigma Chemical Co., St Louis, MO, USA) to measure the nectar volumes of fully open flowers on 6 May 2009. The nectar sucrose concentration of each flower was measured using a hand-held, temperature-compensated refractometer (eclipse, Bellingham + Stanley Ltd, Tunbridge Wells, Kent, UK) at the same time.

Manipulation experiments

To assess the effects of autonomous self-pollination and floral visitors on fruit set, we conducted three manipulation treatments during the flowering seasons of 2 successive years: (1) bagging: 28 inflorescences in 2010 and 15 inflorescences in 2011 were bagged with nylon mesh at all times; (2) emasculation: 77 flower buds from 10 different inflorescences in 2010 and 78 buds from 15 different inflorescences in 2011 were randomly selected and the pollinia were carefully removed 2 days before anthesis, while maintaining access for insect pollinators; and (3) control: 10 inflorescences in 2010 and 20 inflorescences in 2011 were marked before anthesis and kept intact throughout the experiment. An additional test was conducted before the emasculation treatment to ensure that the process of pollinia removal had no effect on flower opening.

In all treatments, the number of open flowers was recorded every day. Fruit formation and maturation were observed and recorded every day, and fruit set was recorded accordingly. The difference in fruit set between the bagging and control treatment was tested using an independent samples *t*-test (performed in SPSS 13.0, SPSS Inc., Chicago, IL, USA).

Flower visitor observation

We recorded visitors to *E. roseum* from 0930 to 1730 hours discontinuously in 2009 (4–6 May) and 2011 (17–19 May) at the study site. In 2009, two patches (patch 1 and 2) ~10 m apart were selected to allow synchronous observation of visitors. In 2011, we conducted the same observations at another two patches (patch 3 and 4). Patch 3 was near patch 1 and patch 2, while patch 4 was ~3 km away from patch 3. The inflorescences involved in the observations made at the different patches are listed in Table 1. In total, ~96 h of observation were achieved at the four patches over 2 years. The duration and frequency of visits and the behaviour of all visitors were observed and recorded. All visitors were photographed. During the observations, 30 visitors were captured immediately after visiting a flower to determine whether there were pollinia of *E. roseum* stuck on their bodies. The captured insects were taken to the laboratory for species identification and morphological measurements.

Results

Floral phenology and morphology

E. roseum bore inflorescences aboveground at the beginning of the local rainy season in May. The racemes, which

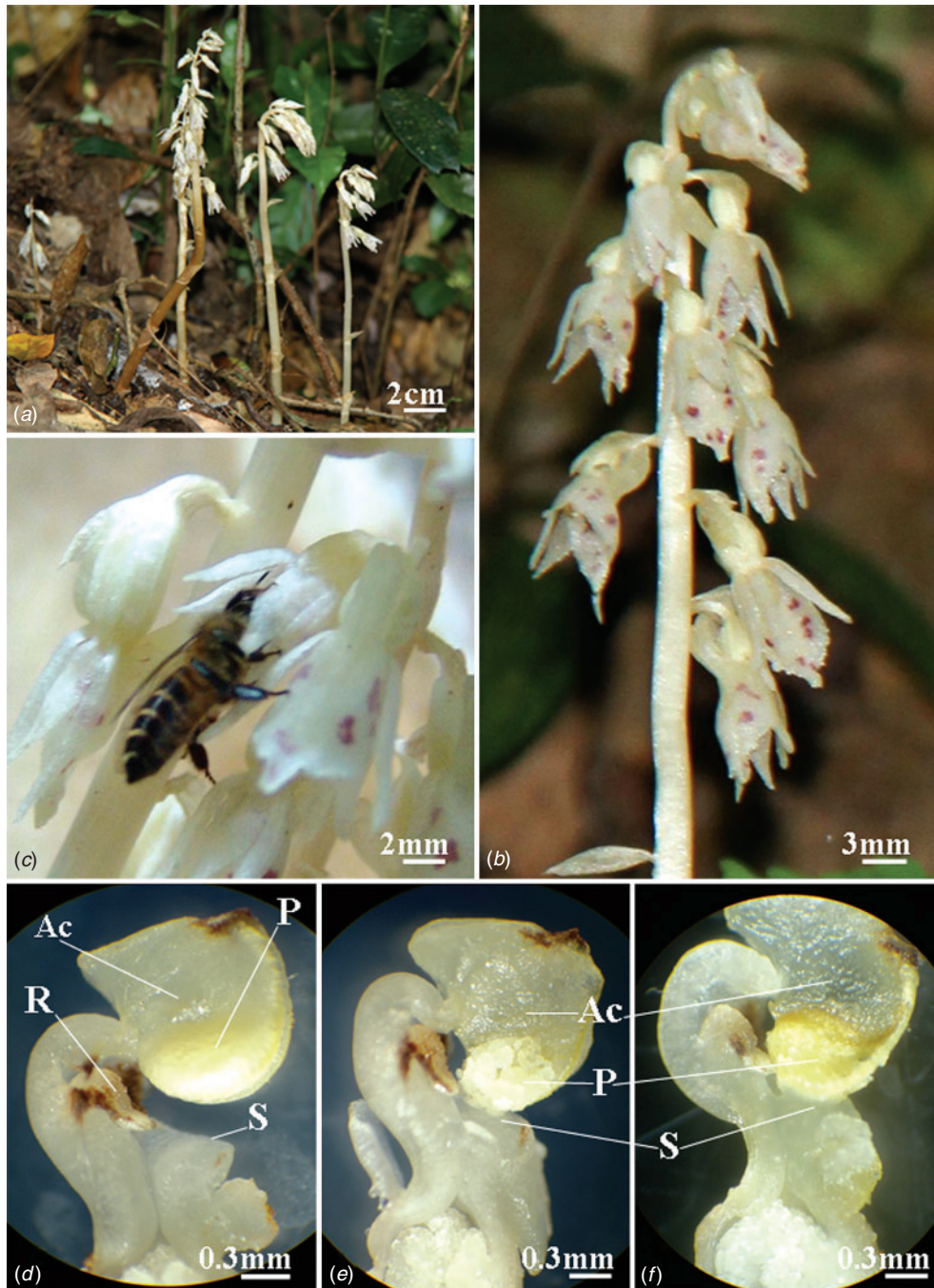


Fig. 1. Habitat, inflorescences, flowers, visitors, and the structure of the pollinarium and stigma of *Epipogium roseum*. (a) Inflorescences of *E. roseum* appear on highly shaded forest floors with thick leaf-litter in the Green Limestone Forest Park; (b) the inflorescence of *E. roseum* with many half-opened and loosely arranged flowers; (c) an Asian honey bee visiting a flower of *E. roseum*; (d–f) the structure of the pollinarium and stigma of *E. roseum*, showing the relative position of stigma and pollinia at the different stages of bud development. Ac, anther cup; R, rostellum; S, stigma; P, pollinia.

appeared translucent and fragile, were 15.49 ± 3.46 (mean \pm s.e., range 8–25, $n=30$) cm tall, and contained 8.79 ± 3.96 (range 2–17, $n=30$) flowers (Fig. 1a). These flowers were loosely arranged, and opened gradually from the bottom to the top of the inflorescence (Fig. 1b). The floral longevity was 3.80 ± 0.57 ($n=30$) days, and a flowering inflorescence lasted 7.76 ± 0.86

($n=30$) days. The fruits became mature and dispersed seeds 7.56 ± 0.90 ($n=30$) days after the flowers had opened. At our study site, the whole reproductive cycle, from the appearance of the first inflorescence to the disappearance of the last inflorescence, lasted 20, 23 and 29 days in 2009, 2010 and 2011, respectively.

Table 1. Observation data for *Apis cerana cerana* visiting flowers of *Epipogium roseum* at four patches in 2 different years

Observation patches	Year	Inflorescences involved	Total time of observation (h)	Total visits	Flowers visited per visit	Time spent on a single flower (s)
Patch 1	2009	14	24	105	8.35 ± 6.83 ($n=81$)	6.96 ± 3.37 ($n=266$)
Patch 2	2009	7	24	96	3.73 ± 2.37 ($n=96$)	6.75 ± 4.75 ($n=277$)
Patch 3	2011	9	24	10	2.70 ± 1.06 ($n=10$)	6.26 ± 3.53 ($n=27$)
Patch 4	2011	19	24	0	—	—

Flowers of *E. roseum* are dull white in colour with an obvious fragrance. The flower does not fully open and exhibits a semi-tubular appearance (Fig. 1b). The labellum is relatively broad and deeply concave with some purple speckles. The column is short and curved. The pollinia are covered by a large anther cap and placed right above the stigma (Fig. 1d). The tongue-shaped rostellum is situated at the apex of the column just below the anther cap. The two pollinaria, each consisting of a pair of pollinia united to a long slender caudicle, are tightly enclosed by the two flaps of the anther cap (Fig. 1d). Unlike other orchids, the rostellum of *E. roseum* is clearly degenerative and does not separate the stamen (pollinia) from the stigma. The viscid disk is absent. A large spur is positioned at the base of the labellum, containing $1.93 \pm 1.10 \mu\text{L}$ nectar with a sugar concentration of $4.7 \pm 1.22\%$ ($n=30$).

The results of our anatomical study revealed that, in *E. roseum*, autonomous self-pollination occurs at the bud stage before the flowers open. In the early bud, the stigma is separated from the pollinia (Fig. 1d). As the bud develops, the stigma expands and approaches the surface of the pollinia, while the pollinia are gradually released by the two flaps of the anther cap, and become swollen and incompact (Fig. 1e). Once the stigma and pollinia touch each other, self-pollination occurs (Fig. 1f). This usually happens during late bud development, 1 day before flower opening.

Manipulation experiments

In the manipulation experiments, no fruit was found in the emasculation treatments. The fruit set in the bagging and control treatments was $94.60 \pm 11.29\%$ ($n=28$) and $95.79 \pm 6.83\%$ ($n=10$) in 2010, and $93.06 \pm 12.11\%$ ($n=15$) and $94.41 \pm 9.41\%$ ($n=20$) in 2011, respectively. There was no significant difference in fruit set between the bagging and control treatments over the 2 years ($P=0.975$ in 2010, and $P=0.818$ in 2011, independent samples *t*-test). These results suggested that fruit set results from autonomous self-pollination, and has nothing to do with insect visitation in *E. roseum*.

Floral visitors

The Asian honey bee (*Apis cerana cerana*) was the only visitor of *E. roseum* observed during ~96 h of observation over 2 years. The bee landed directly on the labellum, adjusted its body direction and entered the flower (Fig. 1c). It remained in a single flower for an average of 6.83 ± 4.10 ($n=570$) seconds and visited 6.33 ± 5.58 ($n=165$) flowers during one visit. During an observation period of 24 h at each patches, we observed that Asian honey bees made 105 and 96 visits at patch 1 and patch 2, respectively, in 2009, and 10 and 0 visits at patch 3 and patch 4, respectively, in 2011 (Table 1). This

species regularly visited flowers of *E. roseum*, with the most frequent visits around midday (Fig. 2). However, the frequency of visits varied enormously between patches and different years (Table 1).

The Asian honey bee appears to match the flowers of *E. roseum* in terms of size, with a body length and width of 10.26 ± 0.26 mm and 3.53 ± 0.045 mm ($n=10$), in comparison with the length and width of the flower tube of 9.98 ± 0.89 mm and 4.84 ± 0.41 mm ($n=30$), respectively. However, of the 30 Asian honey bees captured immediately after visiting a flower of *E. roseum*, none was found with pollinia stuck on any part of its body.

Discussion

The mechanism of autonomous self-pollination in *E. roseum* observed in Xishuangbanna is the same as that described by Jones (1985) in Australia. The degenerative rostellum has lost its function as a physical barrier separating the stigma and stamens (pollinia), allowing contact between the stigmatic secretions and the pollinia during bud development. This constitutes cleistogamous selfing, which is rare in the Orchidaceae (Arditti 1990; Johnson and Edwards 2000; Peter and Johnson 2009).

Although self-pollination occurs at the bud stage 1 day before flower opening, flowers of *E. roseum* usually open and exhibit many characteristics of insect-pollinated flowers, such as flower

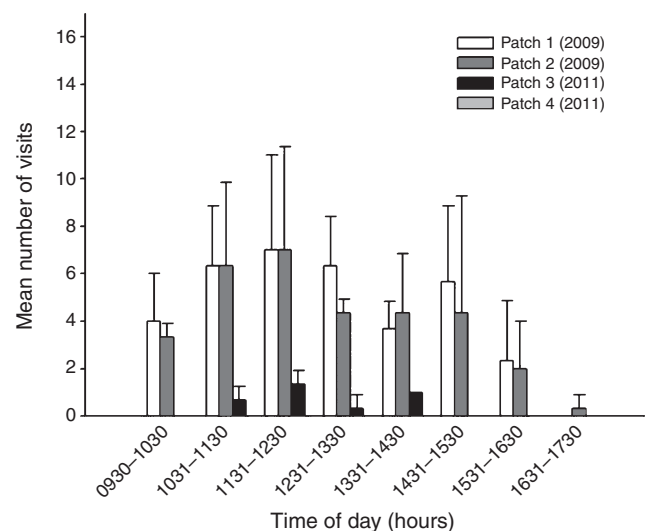


Fig. 2. The visitation frequency and patterns of Asian honey bees visiting flowers of *Epipogium roseum* at four patches in 2 different years (4–6 May 2009 at patches 1 and 2; 17–19 May 2011 at patches 3 and 4).

scent, a spur with nectar, and purple speckles on the labellum. Our observations also revealed that *E. roseum* was able to successfully attract insect visitors, and that Asian honey bees regularly visited flowers of *E. roseum* for nectar reward (Table 1; Fig. 1c). However, in the manipulation experiments, there was no significant difference in fruit set between the bagging and control treatments, and no fruit found in the emasculation treatment, suggesting that Asian honey bees visiting flowers of *E. roseum* do not contribute to fruit set. Moreover, none of the Asian honey bees had pollinia on their bodies immediately after visiting flowers of *E. roseum*. This may be due to the absence of a viscid disk in *E. roseum*. For the other two species in *Epipogium*, self-pollination is considered to be impossible due to the positioning of the pollinia below the stigma in the non-resupinate flower in *E. aphyllum*. Some bumblebees (*Bombus pascuorum*) were observed visiting flowers of *E. aphyllum*, but did not appear to have a role as pollinators as pollinia were not removed during visitation (Taylor and Roberts 2011). *Epipogium japonicum* has very similar flower features to *E. roseum* (Luo and Chen 2002), but nothing is known about its breeding system and pollinators so far.

Orchids with autonomous self-pollination usually retain the potential for outcrossing (Johnson and Edwards 2000). Unlike such orchids, *E. roseum* has lost the capacity for outcrossing by insects due to the absence of a viscid disk, and appears to be adapted to an obligate self-pollination strategy. *Epipogium roseum* is a saprophytic plant with a short sexual phase and grows in environments that are unfavourable for insects, i.e. under heavy shade. Furthermore, several authors have suggested that plants with short life histories favour inbreeding (Levin 1972; Bawa 1974; Solbrig 1976; Real 1983).

More than 400 species of angiosperms are myco-heterotrophic ('saprophytes'; Furman and Trappe 1971). Although the reproductive biology of myco-heterotrophic plants has been poorly studied, the limited studies suggest that a self-pollination system is favourable in saprophytic plants (Takahashi *et al.* 1993; Zhang and Saunders 2000). Those authors suggested that the pollination strategy of myco-heterotrophs would be affected by resource limitations as they have to depend on other organisms for nutrition; obligate autogamy is a highly economic form of sexual reproduction since it does not require extensive resource allocation for the production of pollen, or attractions such as nectar and the perianth (Takahashi *et al.* 1993; Zhang and Saunders 2000).

Epipogium roseum flowers produce scent and nectar, and can normally attract insect visitors, but the frequency of visitation by Asian honey bees varied enormously both spatially and temporally (Table 1). *Epipogium roseum* grows on very shaded forest floors, where insect pollination services are likely to be minimal and unreliable; moreover, the short sexual phase may increase the risk of lack of insect pollination services. Presence of floral scent, somewhat decorative labellum, and nectar may be traits inherited from the species' outcrossing ancestor that have not been lost with the development of cleistogamous pollination. These traits also suggest that sexual reproduction of the species is not limited by resources. Instead the strategy of autonomous self-pollination in *E. roseum* may have evolved because of the uncertain pollinator services

associated with its saprophytic lifestyle. Our study does not support the hypothesis that resource limitation is the only driver of obligate autogamy in myco-heterotrophic plants.

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