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Nocturnal hawkmoth and noctuid moth pollination of *Habenaria limprichtii* (Orchidaceae) in sub-alpine meadows of the Yulong Snow Mountain (Yunnan, China)

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Habenaria (Orchidaceae) is a species-rich genus, usually pollinated by members of Lepidoptera, but the pollination ecology of its sub-alpine species in the Himalayas remains under-explored. We focused on three populations of the endangered, nectar-secreting *H. limprichtii* on Yulong Snow Mountain (eastern Himalayas). Results showed that spurs of *H. limprichtii* contained 10.8 μl of nectar with 26.3% dissolved sugars at night. A hawkmoth (*Deilephila elpenor* subsp. *lewisii*) and two species of settling moths (*Cucullia fraterna* and *Trichoplusia intermixta*) were the dominant pollinators. The hawkmoths had long proboscides (26.3 mm in length), matching the spur length of the flowers, and carried one to 30 pollinaria on their eyes. In contrast, settling moths had short proboscides (15–16.4 mm) carrying fewer than nine pollinaria on their legs or thoraces. These insects may have been attracted by the floral scent dominated by two aromatic benzenoid compounds. Flowers are self-compatible, but pollinators were required to produce fruits. Hand-pollination experiments showed a high level of inbreeding depression (a low proportion of seeds with well-developed embryos in self-pollinated fruits), whereas insect-mediated (natural) rates of cross-pollination were also low (13%). This suggests that most fruits were fertilized following moth-mediated autogamy/geitonogamy. Fruit set was always higher than the rate of pollinaria removal, suggesting a higher pollination efficiency due to its sectile and friable pollinia. Fruit set in natural populations was highly variable among years (28.5–93.7%), doubling in 2016. Although this is only the fourth case of moth-pollination described in Chinese *Habenaria* spp., it highlights the potential importance of this syndrome at higher elevations.

ADDITIONAL KEYWORDS: Himalaya – pollinarium removal – reproductive success – Sphingidae.

INTRODUCTION

Literature on moth-pollination in Orchidaceae began with Darwin (1862, 1877). Historians of science are well aware of Darwin's correct prediction that *Angraecum sequipedale* Thouars was pollinated by a moth species in the family Sphingidae (Micheneau, Johnson & Fay, 2009). In fact, Darwin first addressed the pollination of *Anacamptis* (*Orchis*) *pyramidalis* (L.) Rich. noting it depended on butterflies and several species of diurnal, settling moths for pollinia transfer (Darwin, 1862; Edens-Meier & Bernhardt, 2014). Consequently, orchids pollinated by Lepidoptera in the allied genera *Habenaria* Willd., *Platanthera* Rich. and *Gymnadenia* R.Br. remain important model systems in modern evolutionary ecology for studying directional selection (Nilsson, 1983; Schiestl & Schluter, 2009).

Habenaria is no longer regarded as monophyletic (Jin *et al.*, 2014), but with *c.* 800 species it is one of the most species-rich lineages of terrestrial orchids. Brazil, southern and central Africa and East Asia are its centres of diversity (Dressler, 1993; Batista, Bianchetti & Miranda, 2006; Batista *et al.*, 2013).

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There are 54 *Habenaria* spp. in China, 19 of which are endemic (Chen & Cribb, 2009). With a few exceptions (Thien, 1969; Singer, 2001; Suetsugu & Tanaka, 2014), pollination by Lepidoptera appears to dominate in this genus, regardless of distribution. Past research often emphasized the length of the proboscides of the insects versus dimensions of the spurs and column architecture of the flowers (Smith & Snow, 1976; Nilsson & Jonsson, 1985; Singer & Cocucci, 1997; Singer, 2001; Singer *et al.*, 2007; Peter *et al.*, 2009; Pedron *et al.*, 2012; Ikeuchi, Suetsugu & Sumikawa, 2015; Xiong, Liu & Huang, 2015; Zhang & Gao, 2017). In contrast, the floral scent of only a few *Habenaria* spp. has been analysed to date (Peter *et al.*, 2009; Zhang & Gao, 2017), even though scent appears to be the key attractant in flowers pollinated by nocturnal moths (Raguso, 2001; Dobson, 2006). Most *Habenaria* spp. studied thus far are self-compatible, but they lack automatic self-pollination mechanisms in the absence of pollen vectors (Singer *et al.*, 2007; Pedron *et al.*, 2012). As members of this genus offer nectar rewards their flowers set fruit at a higher frequency than those of allied taxa pollinated by deceit (Tremblay *et al.*, 2005; Singer *et al.*, 2007; Peter *et al.*, 2009; Pedron *et al.*, 2012; Ikeuchi *et al.*, 2015; Xiong *et al.*, 2015; Zhang & Gao, 2017).

Incomplete records kept by *Fauna Sinica* suggest that *c*. 20% of all hawkmoth species (Sphingidae) occur in China (Zhu & Wang, 1997), but the roles of sphingids and settling moths (e.g. Noctuidae and Geometridae) as pollinators of the Chinese flora remains poorly documented (Johnson *et al.*, 2016) with only a few recent publications (Tian *et al.*, 2004; Xiong *et al.*, 2015; Zhang & Gao, 2017). Xiong, Liu & Huang (2015) found that three species of sphingid moths carried the pollinaria of *Habenaria glaucifolia* Bur. & Franch. at the bases of their proboscides but they were not able to observe moth visitations until the final three years of their impressive eight-year study. Reproductive success of *H. glaucifolia* varied seasonally with a maximum 46% conversion rate of flowers into fruit. Sphingid moths were also observed to pollinate sympatric populations of *H. davidii* Franch. and *H. fordii* Rolfe (Zhang & Gao, 2017).

In contrast, *Habenaria limprichtii* Schltr. is distributed through south-western China into Vietnam and northern Thailand (Chen & Cribb, 2009) from 100–3500 m but it is now found less frequently in forests and wet grasslands of south-western China due to increasing urbanization, deforestation and climate change (Liu *et al.*, 2015; Ren *et al.*, unpubl. data). A future conservation programme for any species requires dependable information on the decline of its remaining populations and that may include identification of factors that limit reproductive success (Bernhardt *et al.*, 2017). Here, we address the lack of basic information on the pollination ecology, fruit production and seed viability in *H. limprichtii* by asking the following questions. (1) What are the pollinators of this species? Does the pollen-vector spectrum include both sphingids and settling moths? (2) Is *H. limprichtii* self-compatible? If so, does this species experience inbreeding depression judging by the proportion of well-developed embryos in the seeds? (3) Does the flower emit a floral scent at night? If so, which floral scent compounds are present? (4) Does this nectar-rewarding orchid show a high rate of reproductive success?

MATERIAL AND METHODS

Study species

Habenaria limprichtii is found in wet grasslands, along margins of abandoned fields or overly grazed grasslands on the Yulong Snow Mountain range through Lijiang (City) in north-western Yunnan at 2300–3300 m (Fig. 1A). The population density in undisturbed wet grasslands is always higher than in sites disturbed by agriculture and grazing (Ren, unpubl. data). Each perennial herb produces a single inflorescence bearing multiple flowers. These flowers are greenish-white without any noticeable scent during the daytime, but they produce a strong and pleasant fragrance at night. Distinctive characters of the floral column include the great distance between the two pollinium sacs (locules) in the same anther (9.52 ± 0.86 mm; $N = 100$, mean ± SD; Fig. 1B) and the broad rostellum lobe contains two discrete viscidia and is, in turn, flanked by two receptive stigmatic lobes. Therefore, each column holds two, separate pollinaria (see below). The perianth segments either darken as these flowers age or following pollination. Flowering periods for populations in Lijiang occur from mid-July to mid-September and their mature fruits dehisce in October.

Study sites

Our study site is a wet meadow (Yushuizhai, YSZ) belonging to the Lijiang Forest Ecosystem Research Station (Lijiang Alpine Botanical Garden) and is operated by the Kunming Institute of Botany, Chinese Academy of Sciences (CAS). We selected this site for hand-pollination experiments and observations of floral foragers. It is located at 2725 m a.s.l., and the station is adjacent to a tourist and cultural centre for the Naxi people. Consequently, vegetation in the station has been disturbed occasionally by trampling from tourists, grazing by horses and browsing by

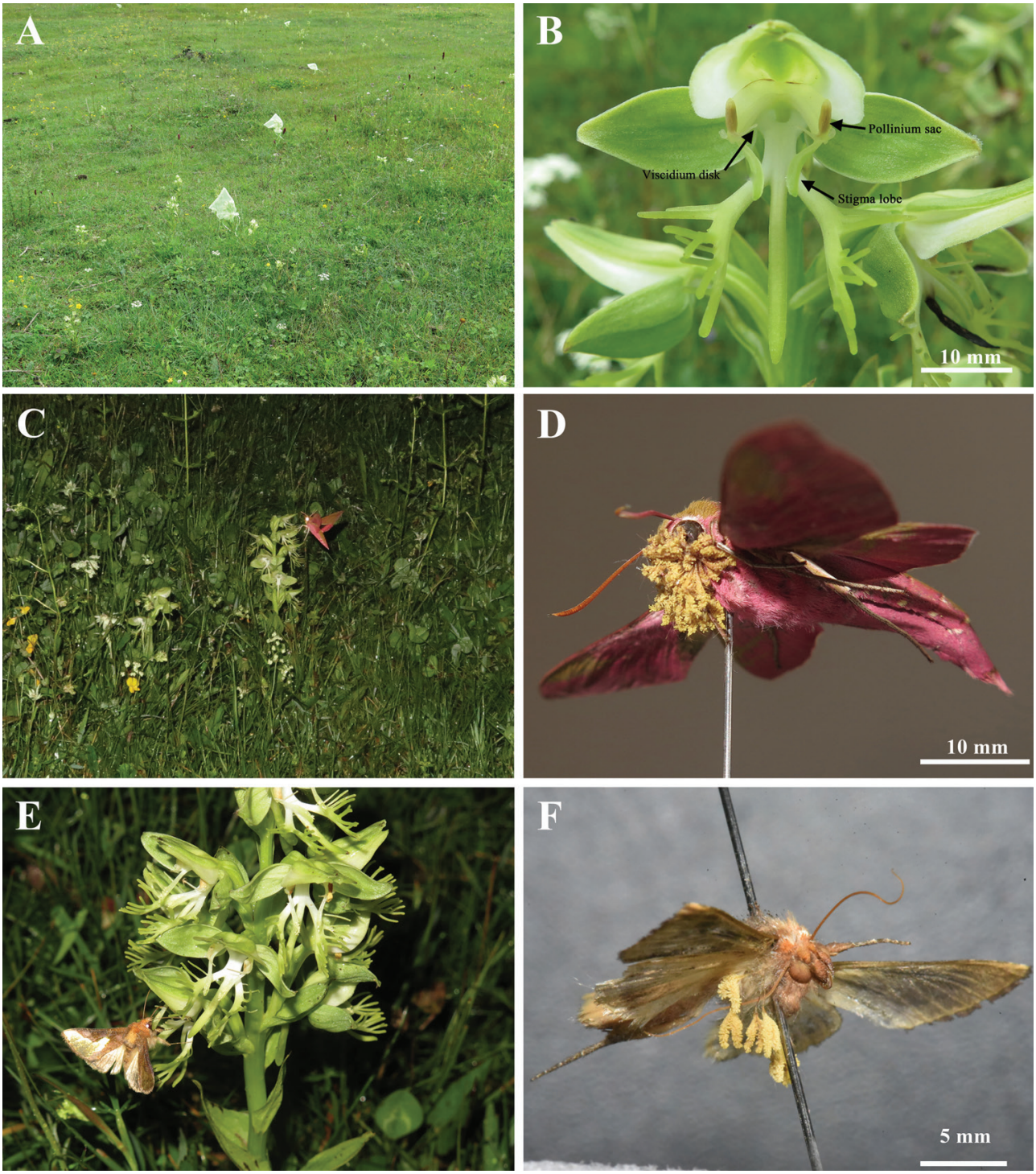

Figure 1. Habitat, inflorescence, flower and pollinators of *Habenaraia limprichtii* on Yulong Mountain, south-western Yunnan, China. A, Sub-alpine meadow of *H. limprichtii* at 2700 m showing individual inflorescences bagged for hand pollination in 2014. B, A flower of *H. limprichtii*, showing the great distance between the two anther sacs and two stigma lobes. C, *Deilephila elpenor* subsp. *lewisii* visiting flower of *H. limprichtii* photographed at 20:49 on 16 August, 2016. D, Pinned specimen of *Deilephila elpenor* subsp. *lewisii* carrying > 30 pollinaria on the left compound eye. E, Noctuid moth, *Trichoplusia intermixta* visiting an inflorescence (note deposition of pollen masses on several stigma lobes). F, Pinned specimen of *Trichoplusia intermixta* with pollinaria deposited on lateral-ventral side of its thorax. Photographs A, B, F by Dr. Zong-Xin Ren, and C, D, E by Hai-Dong Li, Kunming Institute of Botany, Chinese Academy of Sciences.

goats. However, the tourist centre closes at 17:00 so there is no light pollution or noise disturbance at night. Previous field studies *in situ* for over six seasons did not include any crepuscular or nocturnal pollinator observations (Li *et al.*, 2016; Zhao *et al.*, 2016). The late summer flora in this meadow is dominated by *Halenia elliptica* D.Don (Gentianaceae) and other white flowers in the Apiaceae and Asteraceae (see Zhao *et al.*, 2016). *Habenaria delavayi* Finet was sympatric and co-blooming with *H. limprichtii*, but *H. delavayi* produced much smaller flowers and was more common on upper, drier grasslands (Liu *et al.*, 2015; Ren *et al.*, unpubl. data).

We selected two other sites to compare fruit set among populations from 2014 to 2016. The Jiemeihu (YMH) site is 1 km away from YSZ at 2650 m a.s.l. It is also a wet meadow containing man-made ponds. Its vegetation is similar to YSZ. There were > 200 flowering stems of *H. limprichtii* in bloom in 2016 and we recorded fruit set at this site. The third site, Yuhu (YH) is > 10 km from YSZ. It is a dry meadow invaded by immature pine trees. The population density of orchids in this site consisted of < 100 flowering stems in a 1 km^2 area. Photographic vouchers from all sites are available from the authors.

Climate data were collected from the weather station in the Lijiang Alpine Botanical Garden at 3300 m. The horizontal distance of this station is < 1 km from YSZ. The annual precipitation in this region was 932.8–1302.4 mm from 2014 to 2016. In 2014 and 2015, Lijiang City experienced severe spring droughts with precipitation from January to June from 164.0 mm to 314.2 mm. Precipitation from January–June in 2016 was 407.6 mm.

Phenology

Phenological data were collected by recording the day the first flower opened in a population to the day in which we found that the last flower showed darkened and wilted perianth segments (N = 30). The longevity of an individual flower was recorded from the day the bud opened, and the contents of the spur was accessible to floral foragers, until the day we observed that floral segments had darkened and wilted (N = 20). We also recorded the number of days it took for a flower to wilt after it was hand pollinated.

Floral measurements

In 2016, we labeled 30 plants randomly and selected one flower on each scape at random in YSZ. We measured the following floral traits with digital calipers to an accuracy of 0.01 mm: (1) the circumference of the flower using the distance between the apices of the lateral sepals as length and the width measured from the terminus of the dorsal sepal to the terminus of its labellum, (2) length and width of the dorsal sepal and one of the lateral petals, (3) the length of the floral spur from its opening under the column to its terminus, (4) the distance between the spur opening and one of the two viscidia (see above), and the distance between the two viscidia, (5) the distance between the two, receptive stigma lobes and (6) the length of the caudicle and the length and width of its pollinium after withdrawing 15 pollinaria (one of two pollinia per flower, see above) using forceps.

Nectar meaurements

For nectar volume and sugar concentration measurements we selected 30 inflorescences with mature flower buds in YSZ. Each inflorescence was isolated under an organza bag. When all the flowers on an inflorescence opened, over a 3–5 day period, we selected two flowers at random on the inflorescence for a day (09:00 to 12:00) and a night (19:30 to 21:00) measurement (one for each measurement). The orifice of the spur is so narrow, < 1 mm in width that it is impossible to insert a micropipette through the orifice to reach nectar within the spur. Therefore, we cut off the tip of each spur with a pair of scissors, gently squeezed out the liquid contents and gathered up the liquid with a 20-µl micropipette (Drummond Scientific Company, Broomall, PA, USA). Total sugar concentration was measured by a handheld, sugar refractometer (0–50% Brix, Bellingham and Stanley Ltd, Basingstoke, UK).

Pollinator observations

From 2012 to 2016 at YSZ, we never saw anything visiting the flowers of this species during the daytime (from 09:30 to 17:30) over 18 days of observation (about 150 h). Night-time observations were discontinued in favour of observing nocturnal foragers from 19:30 to 22:00 and from 00:00 to 06:30. We completed 50 nocturnal, observation hours in August 2016. A red-light torch was used to observe floral foragers at night. Foraging behaviors of insects were recorded and photographed. All floral visitors observed belonged to Lepidoptera. Moths observed foraging on the flowers were collected in butterfly nets and killed in jars with ethyl acetate fumes. The proboscides of freshly killed specimens were carefully unrolled prior to pinning and measured using digital calipers to an accuracy of 0.01 mm. We also measured the width and depth of the thorax and the width of the head of the moth. Specimens were pinned, spread on butterfly spreading boards and labeled. We recorded the number and location of pollinaria attached to the bodies of each specimen. Photographs of sphingids were sent to the

Australian Museum, and photographs and pinned specimens were shared with entomologists in the Kunming Institute of Zoology, CAS for identification. Pinned vouchers are deposited in the Kunming Institute of Botany, CAS, Kunming.

Floral scent

We could not detect a floral scent until 19:00 after sunset. Due to difficulties of sampling in wet meadows at night, and to avoid contamination by other volatiles produced by the surrounding lush vegetation, we transplanted flowering plants into small plastic pots, and moved them to the field station for floral scent collection. Floral scents were collected as described by Edens-Meier *et al.* (2014). A headspace bag (Reynolds® Oven Bag; Reynolds, Inc., Richmond, VA, USA) cut to dimensions of 10 × 20 cm was used to cover each inflorescence bearing open flowers. The bag was sealed at the bottom using a twist tie. An adsorbent trap, prepared using a Pasteur pipette with 10 mg Porapak Q (80/100 mesh; SUPELCO, Bellefonte, PA, USA) packed between glass wool was attached to a battery-operated PAS-500 vacuum pump (Spectrex, Inc.) with Tygon tubing. The tip of the trap was then sealed within the top of the headspace bag with a second twist tie. Floral scent was collected for two hours from 19:30 to 21:30 on 30 August, 2016 at a standardized flow rate of 200 ml air/min. Ambient air controls were included to account for non-floral compounds. Upon completion of the fragrance collection, scent traps were eluted into 1.5 ml borosilicate glass autosampler vials using 300 µl of GC-MS grade hexane. Each vial was capped, labeled, wrapped with parafilm and stored at –20 °C. All collected sample vials were sent to the Kunming Institute of Botany, CAS for GC-MS analyses. We collected scent from eight replicate inflorescences with four to 12 flowers at the same time.

In the laboratory, floral headspace samples eluted in hexane were concentrated to 50 µl under a flow of nitrogen gas (N_2). An internal standard of 5 µl of a 0.03% solution of toluene (23.6 ng) in hexane was added to each sample. The volatiles were analyzed on a Hewlett-Packard Hp 6890 Series GC System coupled to a Hewlett Packard 5973 Mass Selective Detector. An Hp-5MS column (5% Phenyl-methylpolysiloxane; 30 m long; inner diameter 0.25 mm; film thickness 0.25 µm; Agilent, USA) was used for analyses. Each one µl sample was injected at 240 °C. Electronic flow control was used to maintain a constant helium gas flow of 1.0 ml/min. The GC oven temperature began at 40 °C and increased 3 °C per min to 80 °C, then increased 5 °C per min to 280 °C and held for 20 minutes. The MS interface was 250 °C and the ion trap worked at 230 °C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of one per scan from m/z 35 to 500. Component identification was carried out using NIST 05 mass spectral database, and Wiley 7n.1.

Breeding system

To test for mechanical self-pollination and vector-mediated, self-compatibility in this species, we conducted three treatments in YSZ in 2014. (1) Control bagged: inflorescences were bagged before flowering and remained bagged for the entire flowering season (N = 30 in 2014, N = 20 in 2016). Both hand self- and cross-pollinated flowers were also bagged during this experimental series and the bag was replaced after the following manipulations. (2) Hand self-pollination: one pollinium was removed with forceps and smeared onto both stigma lobes (see above) of the same flower over 60 seconds (N = 32 in 2014, N = 44 in 2016). (3) Hand cross-pollination: we repeated the procedure as in (2) using a pollinium taken from an open flower blooming at least 20 m away, and applied it to stigma lobes in the emasculated, experimental flower within 30 minutes after harvesting the pollinium (N = 45 in 2014, N = 44 in 2016). Each of these three procedures were repeated on > 30 flowers. To eliminate resource limitation, we hand-pollinated one to two flowers on the same scape. When two flowers were hand-pollinated on the same scape, one was a self-pollination and the second was a cross-pollination. We repeated the same experiment at YSZ in 2016. In both years, we returned to the sites at the end of September and the beginning of October to check for fruit. Bagged control plants produced no fruits, but each fruit produced by self- or cross-pollination was placed in its own paper bag to record embryonic development (see below).

Embryonic development and estimation of the proportion of insect-mediated cross-pollination

All the seeds in a capsule were placed on a Petri dish (N = 45 capsules for hand cross-pollination and N = 28 capsules for hand self-pollination) and embryonic development was checked under light microscopy with an Olympus BX51 (Tokyo, Japan) using the methods of Ren *et al.* (2014). We assigned seeds to four categories: large embryo, small embryo (the size of these embryos is half or less than half of large embryos), aborted embryo (collapsed, reduced and incomplete development) and no embryos (transparent seed coat lacks an embryo). We scored 331 ± 24.3 (N = 155 including 82 fruits of naturally pollinated flowers, see below) seeds in each capsule. An estimate of the inbreeding depression (δ) was calculated as $\delta = 1 - w_s / w_o$ where w_s and w_o are the fitness of selfed and outcrossed offspring,

respectively. In orchids, it is difficult to evaluate offspring fitness due to their obligate association with mycorrhizal fungi during seed germination. Therefore, the inbreeding depression index (δ) was modified and calculated following Suetsugu *et al.* (2015):

$$\delta = 1 - (\text{proportion of well-developed seeds after self-pollination / proportion of well-developed seeds after cross-pollination}).$$

We defined seeds with large embryos as well-developed seeds for both self- and cross-pollination.

We also collected 82 fruits (one or two fruits per plant) of naturally pollinated (open, insect visited) plants to check embryonic development. Using results of the hand cross- and self-pollinated fruits as a reference (see above), the proportion of seeds with large embryos was used to determine whether a fruit resulted from insect-mediated self- or cross-pollination (Peter & Johnson, 2009). Those fruits determined to be the result of insect-mediated cross-pollination followed the criterion that the proportion of seeds with embryos in cross-pollinated fruits should be larger than the maximum value of the proportion of seeds with embryos in the hand self-pollinated fruits. As this experiment removed viable seeds from the seed bank for the following year, we only did this experiment at YSZ once in 2014 due to conservation concerns.

Reproductive success

We measured male and female reproductive success in naturally pollinated (non-manipulated) inflorescences. In 2014, we checked 65 plants with 265 flowers at the YSZ population for pollinarium removal and fruit set. In 2016, we checked 46 plants (N = 296 flowers) at the same population for pollinarium removal and pollinia (pollen mass) deposition. Male reproductive success was evaluated by counting the number of flowers on each inflorescence and recording the natural rate of pollinarium removal over the flowering season. We also recorded the number of flowers in which pollinaria were not removed but insect debris (scales and hairs) was found on the stigmatic lobes and/or viscidia still attached to the rostellum. Female reproductive success was evaluated by the presence of pollinia fragments (e.g. pollen massulae) on the stigma lobes and by fruit set. We counted the total number of flowers on each tagged plant that were not used in the above hand-pollination experiments (N = 46–114, Table 5). We also recorded the ovaries on the same, naturally pollinated inflorescences that matured to become fruits by early October in 2014–2016. It was easy to discriminate between well-developed and empty fruits by gently squeezing them. Fruits containing seeds were dense and turgid, whereas empty fruit remained soft and hollow. Fruit set was calculated by the number of fruits on an infructescence divided by the original total number of flowers on the original inflorescence. The pollination efficiency (PE) was calculated following Scopece *et al.* (2010), PE = F_p/F_r, where F_p is the number of pollinated flowers. Here we used the number of fruits as an evaluation of pollinated flowers in 2014. F_r is the number of flowers found with one or both pollinaria removed.

Statistical analyses

The distance between the terminus of the spur to one of the two viscidia in each orchid flower and the length of pollinator proboscides were compared using a one-way analysis of variance (ANOVA). If significant differences were detected, a Tukey honestly significant difference (HSD) post hoc test was used to determine where the differences occurred. The width of the head of a hawkmoth, the width of the thorax of a settling moth and the distance between viscidia were compared by the Kruskal–Wallis test, a non-parametric analysis of variance. Volume of nectar between day and night failed to meet the assumption of normality, thus a Mann–Whitney U test was used to compare their differences. A student t-test was used to compare the difference between nectar concentrations in day versus night samples.

The difference in the number of pollinaria carried by different pollinator species was compared using a generalized linear model with a Poisson error distribution and a logit link function (number of pollinaria). Comparison of fruit set of hand self- vs. cross- pollination used a generalized linear model with a binomial error distribution and a logit link function (with or without fruit). We did not compare the bagged controls with the other treatments as controls produced no fruits. Significances of both comparisons were assessed using likelihood ratios in the R package 'car' (Fox & Sanford, 2011).

We compared the embryo development of cross-, self- and open insect-pollinated seeds using the Kruskal–Wallis test, followed by a series of Wilcoxon rank sum tests to do further paired comparison. A similar method was applied to compare fruit set among different years and populations. The difference between the number of flowers per inflorescence among years and populations was compared by a two-way ANOVA with year and site as independent variables followed by a post hoc Tukey HSD test. We compared pollinarium removal (the number of flowers with pollinaria removed divided by the total number flowers in an inflorescence) with pollinia deposition (the number of flowers bearing whole or fragmented pollinia on stigma lobes divided

by the total number of flowers in an inflorescence) by a Mann–Whitney U test due to these data failed to meet the normal distribution. All statistical analyses were performed in R (version 3.3.2, R Development Core Team, 2016).

RESULTS

Floral phenology, functional floral morphology and nectar measurements

The flowering period of individual inflorescences lasted from 13 to 15 days (N = 30). The longevity of a single flower was 11–13 days (N = 20). Three days after a whole pollinium or pollinia fragments were deposited on stigmas, regardless of whether they were self- or cross-pollinated, the lateral petals darkened while remaining floral organs darkened four to five days later (N = 20).

Each plant produces a single inflorescence with 2–15 flowers (7.03 ± 2.68 flowers/plant in YSZ, 2016, N = 100). The inflorescence height is 27.81 ± 6.00 cm (N = 100). The parameters of functional floral morphology are shown in Table 1. Each pollinarium can be removed independently and consists of one viscidium, one elongated caudicle and one sectile pollinium (*sensu* Dressler, 1993). Sectile pollinia fragment on contact with adhesive surfaces and each pollinium may pollinate more than one stigma lobe and more than one flower. The angle between the caudicle and its pollinium is *c*. 90 degrees.

Table 1. Floral morphology of *Habenaria limprichtii* in YSZ population on Yulong Snow Mountain, Lijiang, northwestern Yunnan

Trait	Replica (n)	Mean (mm)	SD
Length of flower	30	42.87	3.56
Width of flower	30	37.62	4.80
Length of lateral sepal	30	19.02	2.19
Width of lateral sepal	30	9.50	0.72
Length of lateral petal	30	18.96	1.38
Width of lateral petal	30	5.21	0.61
Length of spur	30	19.22	1.68
Distance between opening of spur to the viscidium	30	8.22	1.34
The distance between the terminus of the spur to the viscidium	30	27.5	2.15
Distance between the two stigma lobes	30	2.22	1.84
Distance between two viscidia	100	4.76	0.43
Length of caudicle	15	4.08	0.34
Length of pollinium	15	2.73	0.27
Width of pollinium	15	1.26	0.12

To compare with the length of a moth proboscis, the distance between the terminus of the spur to the viscidium reached 27.5 ± 2.15 mm (N = 30; Table 1). At its highest level, nectar filled half the length of the spur. During the day, nectar volumes varied in spurs, 8.44 ± 5.08 μl (N = 27) with sugar concentration at 29.22 ± 3.14% (N = 27). At night, nectar volume was slightly higher (10.81 ± 2.98 μl; N = 29), but this increment was not significant (Mann–Whitney U Test, P = 0.063). The total sugar concentration at night was 26.22 ± 2.93%, significantly lower than daytime readings (t-test, t = 3.692, P < 0.001).

Pollinators

We collected 93 floral foragers in three families of Lepidoptera (Lasiocampidae, Noctuidae and Sphingidae), but only three species carried pollinaria: a member of the Spingidae, *Deilephila elpenor* subsp. *lewisii*, and two nocturnal members of the Noctuidae, *Cucullia fraterna* and *Trichoplusia intermixta*. *Deilephila elpenor* subsp. *lewisii* and *T. intermixta* were the dominant floral foragers accounting for 89.2% of all collections. Both species carried pollinaria of *H. limprichtii*. We collected only four specimens of *C. fraterna* with three specimens carrying two to eight pollinaria (Table 2).

Deilephila elpenor subsp. *lewisii* visited orchid flowers from 19:30 to 21:00 (Fig. 1C). Of the 23 sphingid specimens collected, 15 carried one to 30 pollinaria on their eyes (Fig. 1D). *Trichoplusia intermixta* appeared to visit the flowers of orchids for most of the night (Fig. 1E), but only 46.7% carried one to six pollinaria on the lateral-ventral sides of their thoraces or on the femora of their middle and hind-legs (Fig. 1F). The numbers of pollinaria carried by these three species were significantly different (χ^2 = 50.35, d.f. = 2, P < 0.001), but there was no difference between the number of pollinaria carried by *C. fraterna* and *D. elpenor* subsp. *lewisii* (P = 0.638). The most common vector, *T. intermixta*, carried significantly fewer pollinaria than *C. fraterna* (P < 0.01). We observed that all three species repeatedly visited flowers from which pollinaria had been removed previously by other moths.

The proboscis of the sphingid was 26.28 ± 1.51 mm (N = 16). The proboscides of the two noctuid species were about half this length [15.00 ± 1.00 mm (N = 39) for *T. intermixta* and 16.43 mm (N = 1) for *C. fraternal*]. The lengths of the proboscides of the sphingids, the two settling moth species and the distance between the terminus of the floral spur and the viscidium disk on the column of the orchid showed a significant

Table 2. Moths collected on the flowers of *Habenaria limprichtii*, length of their proboscides and the number and location of pollinaria carried

Species	Number of specimens	Number of specimens with pollinaria	Number of pollinaria carried	Length of proboscis (mean ± SD mm)	Location of pollinaria
Lasiocampidae					
Dendrolimus angulata	1	0			
Noctuidae					
Agrotis segetum	1	0		9.87 (N = 1)	
Anadevidia hebetata	2	0		18.75 (N = 1)	
Cucullia fraterna	4	3	2–8	16.43 (N = 1)	latero-ventral side of thorax
Ochropleura ellapsa	1	0			
Trichoplusia intermixta	60	28	1–6	15.00 ± 1.00 (N = 39)	lateral-ventral side of thorax and on the femora of middle and hind-legs
Noctuidae sp.	1	0			
Sphingidae					
Deilephila elpenor subsp. *lewisii*	23	15	1–30	26.28 ± 1.51 (N = 16)	Eyes

difference (one-way ANOVA, F = 609.8, d.f. = 2, 82, P < 0.001; Table 2). The proboscis length of the sphingid was shorter than the distance between the terminus of the floral spur and the viscid disk, but the difference was not significant (Tukey HSD test, P = 0.0507). The width of the head of the sphingid was equal to the width of the thoraces of the two noctuids (Kruskal–Wallis test, P > 0.05). Both parameters were significantly greater than the distance between the two viscidia at either ends of the same, lobed anther (P < 0.001).

To suck nectar, the two species of noctuids, with shorter proboscides, had to push their bodies into the flowers (Fig. 1E). Moth scales and hairs (judged by their colour) were left on the viscidium disk when these insects failed to remove either pollinarium. We also found that most of the pollinaria on the eyes of sphingids showed erosion of more than half their pollinium mass. In contrast, pollinaria on the bodies of the noctuids showed little sign of erosion.

Floral scent

After deducting the molecular compounds from the air control, we identified 15 compounds from eight headspace samples of whole inflorescences. The total floral scent emission was high at 31 962.33 ± 19 738.65 ng per inflorescence per hour (N = 8). The dominant compounds were aromatic benzenoid molecules, including benzyl acetate (emission rate: 17 043.52 ± 12 421.05 ng/inflorescence/hour, N = 8) and benzaldehyde (4517.12 ± 4102.73 ng/inflorescence/hour, N = 8). One sesquiterpene, α-farnesene, was also detected in all the samples (Table 3).

Breeding system, seedset and estimation of cross-pollination rates

All flowers in control bags failed to produce fruits in either year (Table 4). Both hand self- and cross-pollinations produced high fruit set ranging from 95.6 to 100% with only a few ovaries failing to produce fruits. There were no significant differences between results for cross- and self- pollination or between years (GLM model, χ^2 = 2.93, d.f. = 3, P = 0.4153; Table 4). However, the proportion of seeds with large embryos in cross-pollinated fruits (62.9 ± 19.6%, N = 45) was significantly higher than the proportion in self-pollinated fruits in 2014 (22.8 ± 11.2%, N = 28; cross- vs. self- pollination, Wilcoxon Rank Sum Test, P < 0.001; Fig. 2A and Table 4). We detected a significant difference among the three treatments (cross-, open- and self-pollination; Kruskal–Wallis test, χ^2 = 56.52, d.f. = 2, P < 0.001). The proportion of seeds with large embryos in open (insect-mediated) flowers was low, 27.4 ± 21.3% (N = 82, Fig. 2A) in 2014. This measure was significantly lower than for seeds in cross-pollinated fruits (P < 0.001) but showed no significant difference from seeds produced by

Table 3. Analysis and the total emission rate (ng per inflorescence per hour) of floral scent using GC-MS from headspace samples of *Habenaria limprichtii* (volatiles are listed in order of increasing retention time). Sample values represent the percentage of total emission for each compound except for total emission rate and the number of compounds

Compound	Retention time	Samples							
		1	2	3	4	5	6	7	8
Benzaldehyde	10.25	25.76	9.02	15.96	4.79	6.90	3.79	39.86	14.20
3-Carene	12.34	0	1.58	0	0	0	0	0.38	0
Limonene	13.19	0.23	8.93	0	0.13	0	0	0	0
Benzyl alcohol	13.60	1.05	0	5.24	0	0	0	0	1.79
2-Phenylacetaldehyde	13.91	0.23	0	0	0	0	0	1.35	0
Methyl benzoate	16.05	0	0	0	0	0	0	0.60	0
Linalool	16.27	0.26	0	0.19	0	0	0	0	0
Benzyl acetate	18.68	46.17	40.00	59.73	58.63	67.71	61.44	22.41	58.47
Methyl salicylate	19.67	0.79	0	0.09	3.53	0.77	0	16.08	6.95
Caryophyllene	26.33	0.46	0	1.92	0	0.44	0	0.43	0
Geraniol	27.24	0.21	0	0	0.15	0	0	0	0
Alloaromadendrene	27.42	0.49	0	0	0	0	0.76	0.62	0.19
Germacrene-D	27.95	5.69	0	12.74	1.99	6.29	26.89	9.50	6.63
(E,E)-alpha-farnesene	28.56	18.18	40.46	3.66	29.88	17.90	7.13	7.30	11.77
Germacra-1 (10), 5-dien-4-ol	30.29	0.47	0	0.47	0.89	0	0	1.46	0
Total emission rate		54 382.38	6400.69	29 007.21	55 231.38	50 764.25	8192.00	29 605.62	22 115.11
Number of compounds		13	5	9	8	6	5	11	7

self-pollinated fruits ($P = 0.6044$). The proportion of small embryos among three treatments showed no significant differences ($\chi^2 = 7.45$, d.f. = 2, $P = 0.0242$; Fig. 2B). In contrast, self-pollinated fruits produced a higher proportion of seeds with aborted embryos (27.9 ± 11.8%, $N = 28$) and empty seeds (38.9 ± 14.3%, $N = 28$). Both values were significantly higher than seeds with aborted and empty seeds in the cross-pollinated fruits ($P < 0.001$, Fig. 2C, D). Results of embryo tests in 2016 were similar to those in 2014 with cross-pollinated fruits producing a higher proportion of seeds with large embryos compared to self-pollinated fruits ($\chi^2 = 19.003$, d.f. = 1, $P < 0.001$; Table 4). In 2014, self-pollinated fruits showed a higher proportion of empty seeds and seeds with aborted embryos than in 2016 ($P < 0.001$). Comparing embryo development between cross- and self-pollination, the inbreeding depression index (δ) was 0.36 in 2014 and 0.45 in 2016 in the YSZ population.

Using the percentage of large embryos in each fruit to estimate natural rates of cross- vs. self-pollination showed a significant difference ($P < 0.001$, Fig. 3A). The range of large embryos was 4.66–86.4% with a mean of 64.0% for cross-pollination. The range for self-pollination, was 5.18–52.8% with a mean of 23.4%. Of the 11 open-pollinated fruits collected, seeds with large embryos comprised > 52.8% of the contents. This number of insect-mediated, potentially, cross-pollinated fruit accounted for 13.4% of the total number of open-pollinated fruits tested (Fig. 3B).

Reproductive success

The number of flowers per inflorescence among populations was significantly different (two-way ANOVA, $F = 157.6$, d.f. = 2, 428, $P < 0.001$). There was a slight but significant difference among years ($F = 3.968$, d.f. = 2, 428, $P < 0.05$; Table 5). At YH, flower numbers on inflorescences showed no variation among years (Tukey HSD test, $P = 0.503$) but all of them were significantly higher than the two other populations regardless of year ($P < 0.001$). Flower number in YSZ did not vary for three years. The number of flowers at JMH in 2016 did not differ from YSZ in 2014 ($P = 0.1909$) and 2015 ($P = 0.5319$), but differed significantly from YSZ in 2016 ($P < 0.001$, Table 5).

The number of flowering plants differed among populations growing in wet meadows (YSZ and JMH), and their population sizes doubled in 2016 (Table 5). Fruit set showed significant differences among populations and years ($\chi^2 = 239.89$, d.f. = 5, $P < 0.001$), but fruit set for YSZ and YH sites in 2014 did not differ (Wilcoxon test, $P = 1$). There was no significant difference between fruit set in 2014 and 2015 in YSZ ($P = 0.12$). However, all three sites showed a significantly higher fruit set in 2016 than in previous

Table 4. Results of hand-pollination experiments and embryo development of hand-pollinated fruits of *Habenaria limprichtii* (Yulong Mountain, north-western Yunnan, China)

Treatments	Number of plants	Number of flowers	Number of fruits	Fruit set (%)	Seed containing large embryos (%)
Bagged control					
2014	30	131	0	0	0
2016	20	20	0	0	0
Self-pollination					
2014	32	32	31	96.9	22.8 ± 11.2 (N = 28)
2016	44	44	43	97.7	27.8 ± 18.5 (N = 24)
Cross-pollination					
2014	45	45	43	95.6	62.9 ± 19.6 (N = 45)
2016	44	44	44	100	62.0 ± 26.0 (N = 27)

years (all $P < 0.001$). The fruit set in YH at 2016 was significantly lower than the fruit set at the other two sites in the same year ($P < 0.001$, Table 5).

The percentage of pollinaria removed at the YSZ population in 2014 was 25.2%. This was significantly lower than fruit set in the same year and site (42.56%; Mann–Whitney test, $P < 0.01$) with a pollination efficiency (PE) of 1.66. In 2016 the pollinarium removal rate in this population reached 74.0%, but it was still lower than the rate of whole pollinia or fragments deposited on stigmatic lobes at 86.9% ($P < 0.05$). Pollination efficiency (PE) in 2016 was 1.19. We checked flowers on 100 inflorescences in 2016 for moth scales and hairs on intact viscidia that had been 'loosened' but not removed from their rostellum by visiting insects. We found that 67 flowers retained moth debris on their rostella in the absence of full viscidia removal. These flowers accounted for 19.49% of all flowers examined.

DISCUSSION

Comparative pollination ecology of *Habenaria limprichtii* and other sphingophilus orchids

Previous but recent studies on the pollination of *Habenaria* spp. in China also found that fruit set of *H. glaucifolia* (Xiong *et al.*, 2015) and sympatric populations of *H. davidii* and *H. fordii* (Zhang & Gao, 2017) were also limited by season and the foraging habits of one to three species of Sphingidae. We note that our *H. limprichtii* and these three *Habenaria* spp. all show the classic suite of traits associated with sphingophily (*sensu* Faegri & van der Pijl, 1979; Haber & Frankie, 1989; Proctor, Yeo & Lack, 1996). Specifically, all four species produce greenish-white flowers that are presented horizontally on their scapes. Nectar secretion is restricted to the interior of an elongated spur derived from a modified petal. In all four species, primary pollinators forage at night. In the case of *H. limprichtii*, floral scent is discernible during crepuscular and nocturnal periods accompanied by copious nectar secretions with sugar concentrations of 26–29%. Similar sugar concentrations were found in other *Habenaria* spp. (Peter *et al.*, 2009; Pedron *et al.*, 2012; Zhang & Gao, 2017).

As most moths and butterflies are covered with deciduous scales, their bodies lack sites that provide a continuously smooth or bare spot for visicidium deposition. For example, Xiong *et al.* (2015) observed deposition of pollinaria of *H. glaucifolia* at the bases of sphingid proboscides, whereas we observed deposition only on the eyes of our only sphingid species. In both species, this corresponds with mechanisms of pollinarium dispersal in other moth-pollinated, *Habenaria* spp. investigated on other continents (Singer & Cocucci, 1997; Singer *et al.*, 2007; Pedron *et al.*, 2012). Therefore, the long rostellum arms and two, widely spaced viscidia, found in sphingophilous *Habenaria* spp., may be co-adaptive paralleling the dimensions of the large, compound eyes of the moths and/or the distance between those eyes (Pedron *et al.*, 2012).

As this suite of adaptations is indicative of a sphingophilous syndrome, it is not, of course, restricted to *Habenaria* or even to Orchidaceae. Darwin (1877) was among the first to address the problem of sites for pollinarium deposition on the furry/scaly body of moths with a simple experiment using the flowers of *Angraecum sesquipedale* Thouars (Orchidaceae). Sites for attachment on the moth must be broad enough to permit viscidia to adhere, and morphometrics related to floral tube/spur length vs. proboscis length have since been used to identify those precise sites leading to pollinarium dispersal and deposition. However, pollinarium dispersal for the orchid occurs only when the proboscis of the moth is slightly shorter than the spur of the orchid.

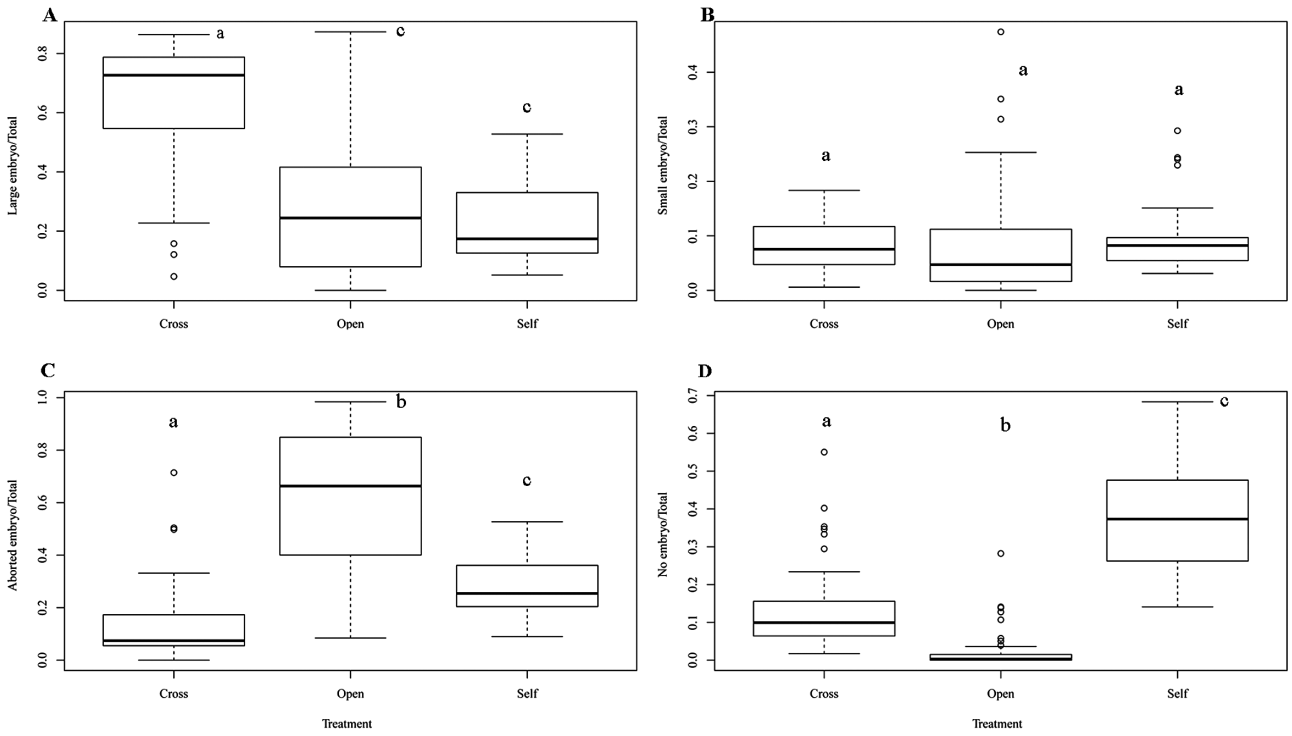

Figure 2. Comparative embryonic development in seeds produced by cross-pollination vs. open-pollination (insect-mediated) vs. self-pollination. The embryos were assigned to four categories: large (A), small (B), aborted (collapsed; C) and empty (no embryos; D). All lower case letters indicate significant differences ($P < 0.001$).

This forces the insect to push further into the flower aligning the few smooth parts of the pollinator under the rostellum (see Nilsson, 1988; Pedron *et al.*, 2012; Johnson *et al.*, 2016).

What was unanticipated in our study was the possible role of two species of settling moths (Noctuidae) as secondary vectors of pollinaria of *H. limprichtii*. These insects had shorter proboscides. To suck nectar in the spur, the noctuids we observed must insert their bodies even further under the wide space of the rostellum, explaining the ventral deposition of the pollinaria on their thoraces and also on their middle pairs of legs. This is consistent with other reports of pollination by settling moths (Proctor *et al.*, 1996; Barthelmess, Richards & McCauley, 2006; Macgregor *et al.*, 2014; Hahn & Brühl, 2016), in general, but the only other report of leg placement of pollinaria on a moth was found previously in *Habenaria epipactidea* Rchb.f. (Peter *et al.*, 2009). We also note that one of our noctuids, *Trichoplusia intermixta*, was also recorded as a pollinator of *Platanthera hologlottis* Maxim. in Japan. The pollinaria of *P. hologlottis* were deposited at the bases of the proboscides of *T. intermixta* (Van Der Cingel, 2001).

Comparative significance of floral scent analyses

Crepuscular or nocturnal sphingids and settling moths rely on scent to locate their flowers at considerable distances (Raguso, 2001; Dobson, 2006). Although we identified 15 scent components, different inflorescences of the same species produced different scent combinations and volumes releasing from five to 11 identifiable molecules (Table 3). Benzaldehyde and benzyl acetate were dominant molecules in all eight inflorescences, whereas detectable amounts of linalool, so common in the scents of other orchids and unrelated angiosperms (see Kaiser 1993, 2010), were restricted to only two inflorescences. Peter *et al.* (2009) analysed the scents released by two inflorescences of moth-pollinated, South African, *H. epipactidea*; they detected lower proportions of benzaldehyde, but only trace amounts of benzyl acetate. In contrast, the scent of *H. epipactidea* was dominated by methyl benzoate. The same molecule dominated the scent of *H. davidii*, whereas the fragrance of sympatric *H. fordii* consisted primarily of linalool (Zhang & Gao, 2017). Methyl benzoate, in particular, has been shown to elicit a strong antennal response from the sphingid *Hyles lineata* (Raguso, Light & Pickersky, 1996) and neural responses in the antennal lobes of *Manduca sexta* (Riffell *et al.*, 2013). In flowers dominated by sphingid

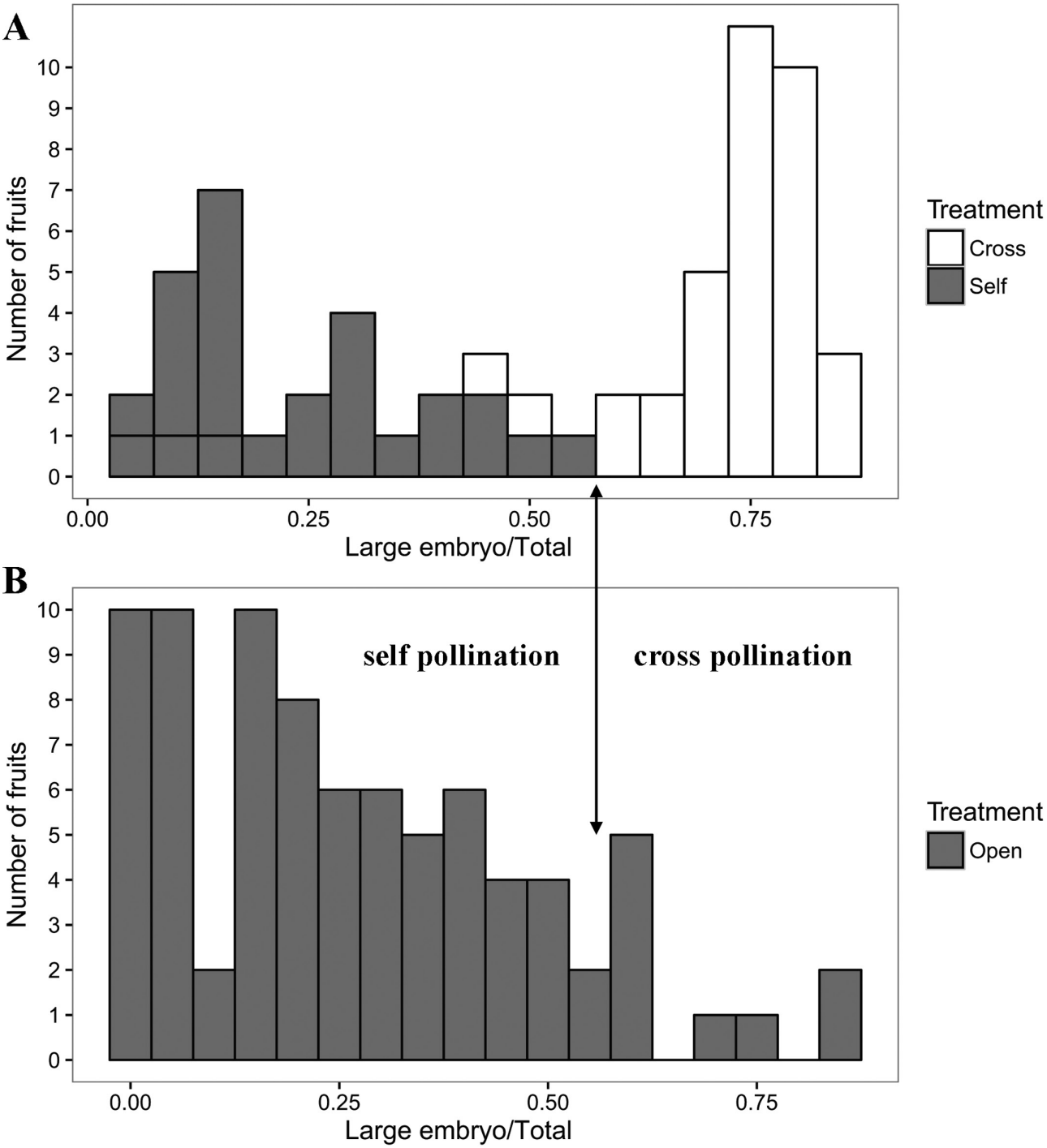

Figure 3. Estimation of cross-pollination rate in open (moth-pollinated) flowers based on proportions of large embryos in seeds per fruit. A, Bimodal distribution of the frequency of seeds with large embryos in the fruits of self- and cross-pollinated pistils (N = 28 for self-pollination and N = 45 for cross pollination). B, Frequency distribution of different proportions of fruits with large embryos (N = 82).

pollinators we also expect some fatty acid-derived esters and N-compounds (Knudsen & Tollsten, 1993; Dobson, 2006). However, chemical profiles of floral scents in settling moth and sphingid flowers are more likely to be characterized by benzenoids (benzaldehyde, esters) and some terpenoids. Therefore, we should

Table 5. Reproductive success rates (mean ± SD) in three populations of *Habenaria limprichtii* 2014 to 2016 (standard deviation in parentheses). All replicants are equal to the number of inflorescences investigated excluding pollinaria removal and pollinia deposition in 2016 at YSZ. The number of flowers per inflorescence and fruit set in three populations were compared. Different superscript, given in lower case letters, indicates significant differences ($P < 0.001$)

	Yushuizhai (YSZ)			Jiemeihu (JMH)	Yuhu (YH)	
	2014	2015	2016	2016	2014	2016
Number of flowering plants	150	150	> 400	200	50	100
Number of inflorescences investigated	65	80	114	101	28	46
Number of flowers per inflorescence	6.29 (1.77)^{ab}	6.16 (2.32)^{ab}	7.13 (2.97)^{b}	5.62 (2.18)^{a}	13.43 (5.82)^{c}	15.48 (5.30)^{c}
Number of fruits per inflorescence	2.68 (2.16)	2.55 (2.56)	6.71 (3.10)	5.17 (2.33)	3.68 (3.08)	12.46 (5.64)
Fruit set (%)	42.56 (32.20)^{a}	37.94 (34.03)^{a}	93.70 (15.90)^{c}	91.12 (19.79)^{c}	28.52 (23.25)^{a}	79.73 (19.83)^{b}
Pollinarium removal (%)	25.20 (25.20)	NA	74.00 (28.50)*	NA	NA	NA
Pollinium deposition (%)	NA	NA	86.90 (20.10)*	NA	NA	NA

*N = 46 for pollinaria removal and pollinia deposition in 2016 at YSZ, we did not count both measures for all investigated inflorescences.

not be surprised when other orchid species with night-blooming, tubular-spurred, white flowers show similar scent profiles. Kaiser (1993) detected methyl benzoate and benzyl benzoate in two, presumably, moth-pollinated, *Platanthera* spp. Additional benzenoid compounds are identified in other tropical, presumably, moth-pollinated orchids in the larger subfamily Epidendroideae. In fact, we find similar profiles in many night-blooming, moth-pollinated species in other angiosperm families (Kaiser, 2010). None of the compounds discussed above is exclusive to nocturnal, moth-pollinated flowers. Benzaldehyde, for example, was found in the flowers of 64% of flowering plant families studied by Knudsen *et al.* (2006).

Breeding system, inbreeding depression and estimation of cross-pollinated flowers

We expected a high conversion of ovaries into fruit in hand-mediated, self-pollinated flowers of *H. limprichtii* as pre-zygotic self-incompatibility is uncommon in the Orchidaceae and more likely to be described in the subfamily, Epidendroideae (Tremblay *et al.*, 2005; Millner, McCrea & Baldwin, 2015). All *Habenaria* spp. studied previously are self-compatible, but they lack developmental mechanisms ensuring self-pollination (Singer *et al.*, 2007; Peter *et al.*, 2009; Pedron *et al.*, 2012). Conversely, a high level of fruit set following self-pollination in *H. limprichtii* ended in a low production of seeds with fully developed embryos. This occurs in hand self-pollinated seeds of some other orchids (Peter & Johnson, 2009; Ren *et al.*, 2014), suggesting that inbreeding depression in some species is based on one or more post-zygotic, self-isolation mechanisms. Of far greater importance, this study shows that high fruit set following foraging by primary pollinators does not lead automatically to the production of higher frequencies of viable seed. Long-distance foraging by moths is expected to result in higher frequencies of cross-pollinated individuals (Nilsson, Rabakonandrianina & Pettersson, 1992; Barthelmess *et al.*, 2006), but in this study we estimated that the actual cross-pollination rate was only 13%. This suggests a far higher rate of moth-mediated self-pollination despite the extraordinary numbers of pollinaria carried on the eyes of the primary pollinator. The rate of out-crossing in any species is influenced by a number of factors including the demographics of the plant population over time. For example, in the nectar rewarding, bee-pollinated, epidendroid orchid *Acrolophia cochlearis* (Lindl.) Schltr. & Bolus., Peter & Johnson (2009) found that the rates of cross-pollination ranged from *c.* 10% in a small, sparsely distributed population to 66% in a large and dense population. Other studies on orchids, based on monitoring their pollinators, also suggest that part of the observed fruit set is a consequence of pollinator-mediated geitonogamy (Pedron *et al.*, 2012; Sanguinetti *et al.*, 2012; Sanguinetti & Singer, 2014), i.e. pollinators visit several flowers on the same inflorescence or return to the same inflorescence during the same foraging bouts. Why was there such a high-proportion of sterile or undeveloped seed produced by sphingophily in *H. limprichtii*? If each of our Himalayan populations represent descendants of one or a few founders (Tremblay *et al.*, 2005), then genetic variation may be low. A multi-flowered stem with spurs half-filled with nectar (see above) must encourage moths to linger on the same genotype and

forage on the same inflorescence after caudicles dry and reposition their pollinia. This encourages vector-mediated modes of self-pollination leading to a decline in reproductive fitness that may represent a trade-off. Nights at these elevations are cold (< 10 °C). Calorific rewards must be substantial to encourage moth foraging, but this may occur at the expense of encouraging moths to 'trap-line' and forage between more isolated but compatible genotypes.

Orchid cross-pollination rates and the cost of secreting nectar

To explain the out-crossing hypothesis of deceptive (non-rewarding) orchids, some authorities argue that cross-pollination rates should be higher in food mimics (Jersáková, Johnson & Kindlmann, 2006). When a pollinator has a negative experience foraging for edible rewards in an 'empty' orchid flower (food mimic), it is less likely to return to the same flower or visit a second flower on the same self-compatible inflorescence (Tremblay *et al.*, 2005; Jersáková *et al.*, 2006). For example, higher rates of cross-pollination rates in food deceptive *Calanthe yaoshanensis* Z.X.Ren & H.Wang (Epidendroideae) are anticipated, although it produces multi-flowered inflorescences and is pollinated by hoverflies and bumblebees (Ren *et al.*, 2014). The rate of cross-pollination in *C. yaoshanensis* was 32% (Ren *et al.*, unpubl. data) and almost three times higher than in nectar-secreting *H. limprichtii* (see above).

An unanswered question in this study is whether sphingid or settling moths are the most important pollinators of this species? Another way of asking the same question is which moth group was responsible for the higher frequency of vector-mediated self-pollination in this species? Settling moths carried fewer pollinaria and carried them on scaly body parts where the viscidium may not have adhered properly. Likewise, although sphingids carried a far greater number of pollinaria on their eyes this could also mean they affected a greater number of self-pollinations.

Population dynamics, climatic factors and conservation policies

We recorded a dramatic increase in natural fruit set in all three populations in 2016 and this coincided with a season marked by higher rainfall. The 2016 increase in fruit in *H. limprichtii* may have been due, in part, to the increase in precipitation in 2016 providing enough water to increase vegetative growth in terrestrial orchids doubling flowering populations in one season and increasing nectar reserves for pollinators.

Based on optimal foraging theory (*sensu* Pyke, 2016) large-bodied sphingids need more energy to forage efficiently for nectar resources. The stability and availability of a seasonal nectar flow plays an important role in maintaining moth populations as nectar is their only nutritional source (Willmer, 2011). The serious drought in the Himalayan region over the past decade (Bernhardt, 2016a, 2016b), combined with urban expansion in Yunnan, may have caused a population decline in some Lepidoptera. Climate change is proposed as one factor in current declines in numbers of nocturnal moths in Europe in addition to habitat degradation and fatalities caused by various types of urban lighting at night (Conrad *et al.*, 2006; Groenendijk & Ellis, 2011; Macgregor *et al.*, 2017).

Conserving any orchid species means conserving a life history dependent on other organisms including symbiotic fungi, a sympatric and co-blooming flora and pollinator guilds. In particular, Dixon (2009) and Bernhardt *et al.* (2017) have warned that it is unlikely that we can conserve and/or restore an orchid species *in situ* if their pollinators have already suffered regional extinction. Consequently, fluctuations in pollinator populations and diversity (Mayer *et al.*, 2011) must also be recorded in field studies on the pollination ecology of all orchid species.

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