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Research

Cypripedium subtropicum (Orchidaceae) employs aphid colony mimicry to attract hoverfly (Syrphidae) pollinators

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Summary

- In Orchidaceae, pollination is mostly animal-mediated, and one-third of species have evolved a deceptive pollination mechanism without rewards. *Cypripedium* is a representative lineage of nonrewarding orchids restricted to temperate regions. *Cypripedium subtropicum* flowers are pollinated by hoverflies and have hairy tufts that visually resemble an aphid colony covered with honey dew.
- We recorded the behavior of hoverflies on the flowers, determined the breeding system of the species and the structure of hairy tufts, and investigated the roles of hairy tufts and floral volatiles in this specialized pollination by using pollination experiments, scanning electron microscopy, bioassays and chemical analyses.
- The white hairy tufts covering the sidelobes of the labellum provide edible rewards and serve as crucial visual lures for hoverflies. The flowers emit primarily (*E*)-β-farnesene and a smaller amount of β-pinene that were found to attract hoverflies.
- Our results suggest that *C. subtropicum* uses both visual mimicry of an aphid-colonized labellum with a reward and chemical mimicry of aphid alarm pheromones to attract hoverflies for pollination. This is the first described example of a rewarding mimicry system in plants, where the models are animals with their secretions and the reward is similar in nutrients to that of the model mimicked.

Introduction

A fascinating feature of orchids is their incredible diversity of floral design that has evolved to enhance pollinator attraction and visitation. An estimated one-third of orchid species feature deceptive pollination mechanisms such as mimicry of food sources, brood sites, and sexual deception or prey, without offering a reward (Van der Pijl & Dodson, 1966; Dafni & Bernhardt, 1990; Nilsson, 1992). The genus *Cypripedium* is recognized by its slipper-shaped pouch (modified labellum) flowers, which are adapted to attract and trap pollinators (primarily bees) by deceit (Bernhardt & Edens-Meier, 2010). In southwestern China, species of the *Trigonopedia* section of *Cypripedium* use food deception to exploit different flies to achieve pollination. For example, *C. fargesii* recruits a syrphid fly, *Cheilosia lucida* by mimicking fungus-infected foliage (Ren *et al.*, 2011). Flowers of *C. bardolphianum* and *C. micranthum* produce a fruity odor to attract fruit flies for pollination (Li *et al.*, 2012).

The mountains of southwestern China have a high level of biological diversity that is considered the center of diversity for *Cypripedium* (Cribb, 1997). In addition to the section *Trigonopedia*, a unique species of the section *Subtropica*, *C. subtropicum*, was first described in 1986 from the herbarium specimens collected in the subtropical forest of Medog County in southeastern Tibet (Chen & Lang, 1986). However, no one had seen the living plants until its rediscovery in 2009 in southeastern Yunnan and northern Vietnam (Jiang & Liu, 2009; Rankou & Averyanov, 2014). This species has distinctive characteristics of several simultaneously opening flowers emerging from a tall stem with evergreen leaves (Fig. 1a). The dark brown flowers have contrasting white hairy tufts covering the sidelobes (Fig. 1b,c) and emit a strong fruity odor. The use of rotting fruit mimicry is known from a mycoheterotrophic orchid, *Gastrodia similis* (Martos *et al.*, 2015). Its flowers primarily emit fatty-acid esters to attract a drosophilid fly species for pollination in the dark and deep rainforest.

In our preliminary field investigations in Malipo County from 2010 to 2013, we observed that a number of hoverflies visited *C. subtropicum* flowers. They landed on the labellum to eat the white hair tufts around the sidelobes (Supporting Information Video S1). Such hair-like structures on the labellum are rare among *Cypripedium* species, but they are known from a few

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Fig. 1 *Cypripedium subtropicum* and its flower morphology. (a) Natural habitat of *C. subtropicum*. Bar, 10 cm. (b) Front view of flowers. Bar, 12 mm. (c) Microscopic view of white hair tufts. Bar, 2 mm. (d) Cryo-scanning electron microscopy (SEM) image of multicellular trichomes in white hair tufts around the sidelobes of labellum. Bar, 100 μm.

orchids in the subfamily Epidendroideae (Pansarin & Maciel, 2017). These food hairs are shown to be rich in starch grains, protein and lipoidal bodies (Davies & Turner, 2004b). Adult hoverflies are important pollinators of many plants. The larvae of hoverflies feed on aphids, thrips and scale insects, whereas the adults mainly feed on pollen, nectar or honeydew (Klecka *et al.*, 2018). Some orchids attract hoverflies for pollination by providing nectar in flowers (e.g. *Epipactis veratrifolia* (Stökl *et al.*, 2011) or by mimicking pollen clusters (e.g. *Govenia utriculata* (Pansarin, 2008)).

Aphids and their honeydew and aphid alarm pheromones (e.g. (*E*)-β-farnesene, α- and β-pinene) are important visual and olfactory cues for hoverflies to locate aphid colonies on the plant (Budenberg & Powell, 1992; Verheggen *et al.*, 2008). We wondered whether the floral white hair tufts of *C. subtropicum* mimic an aphid colony and provide edible rewards to hoverflies. So far we have no evidence that flowers of any *Cypripedium* species offer a reward to their pollinators.

In order to elucidate the mechanism of floral mimesis of *C. subtropicum* and to interpret the function of white hair tufts on its labellum, we first performed pollination experiments to identify the breeding system and examined embryo viability of cross- and self-pollinated seeds. Then we examined the morphology of white hair tufts by cryo-scanning electron microscopy, and analyzed their nutrient content. Do the flowers of *C. subtropicum* emit floral scents characteristic for aphids? To test this hypothesis, we collected the floral scent in the field by headspace solid-phase microextraction and identified its components by GC-MS. To test whether the presence of white hair tufts plays a role in attracting hoverflies, we investigated the changes of pollinator behaviors after removing the tufts from the labellum, and gluing them back. We further tested the behavioral responses of hoverflies present in the forest habitat to natural flowers and floral scents presented both individually and in blends.

Materials and Methods

Study species and site

Our study was conducted during six consecutive flowering seasons, from 2014 to 2019, in Malipo County, Yunnan Province, China. At the study site, we investigated a population of *c.* 100 blooming plants with > 300 flowers of *Cypripedium subtropicum* in subtropical broadleaf forests at an elevation of 1200 m above sea level. The flowers bloom from early to late July. We recorded the number and behavior of visitors from the moment they entered the vicinity of the flower until they left, between 08:00 h and 17:00 h. Field observations totaled 180 h in six consecutive years. We used the aperture-clogging method to catch trapped visitors inside the labellum (Case & Bradford, 2009). The exit holes of the labellum were covered by tape to retain visitors. When visitors carried the *C. subtropicum* pollen mass, we kept them dry in silica gel at 4°C to identify the attachment site and describe the morphological characteristics of head and body. Hoverflies were identified by Dr K. Zhang of the Institute of Zoology, Chinese Academy of Sciences, Beijing, and confirmed by DNA barcoding with cytochrome c oxidase I. Vouchers were deposited in Yunnan Academy of Forestry, Kunming.

Breeding system experiments

In order to determine natural fruit sets and test whether flowers of *C. subtropicum* are self-compatible and required vector-mediated pollination to produce fruits and seeds, we conducted

pollination experiments. In each experiment, 60 flowers were enclosed by nylon bags before anthesis and divided into three treatments (20 flowers in each treatment): (1) control: flowers enclosed by nylon bags and never exposed to insects; (2) artificial self-pollination: stigmas hand-pollinated with pollen masses from their own flowers; (3) artificial cross-pollination: flowers of plants pollinated with pollen masses from other flowers ≥ 5 m away. Other flowers were not enclosed by bags, but during their whole lifespan exposed to insects in order to record natural pollination. Fruit-set of treated flowers and untreated natural flowers was recorded in October of each year. To examine the seed viability, dehiscent fruits were harvested in late October, and mature seeds were treated with 1% NaOCl solution (w/v) + 0.1% Tween-20 (v/v) for 1 h, and then incubated with 1% 2,3,5-triphenyltetrazolium chloride solution at 27°C for 5 d as described by Lee *et al.* (2005). Under a dissecting microscope, the embryos remaining yellow were considered unstained (dead), and those turning orange to red were considered stained (viable). The staining tests were replicated three times with 120–150 seeds in each replicate. One-way ANOVA was used to test for differences in mean number of cross-pollinated seeds and self-pollinated seeds. Post-hoc testing involved using Tukey's honestly significant difference (Tukey's HSD) test.

Cryo-scanning electron microscopy (cryo-SEM)

In order to learn more about the cell morphology of white hair tufts and to check if a few cells from white hair tufts remained on the mouthparts of a trapped hoverfly, fresh flowers and mouthparts of trapped hoverflies were dissected and loaded onto stubs for observation. At least three biological samples were collected for the observation. Samples were frozen by using liquid nitrogen slush, then transferred to the preparation chamber at −160°C. The samples were etched for 10 min at −85°C, sputter-coated with gold at −130°C, then observed at −160°C by cryo-SEM (FEI Quanta 200 SEM; FEI Co., Hillsboro, OR, USA).

Analysis of water and nutrient content of white hair tufts

At anthesis, white hair tufts were collected from the labellum by using fine forceps, immediately frozen in liquid nitrogen, and stored at −70°C. All measurements were replicated three times with 100 mg white hair tufts collected from four to five flowers in each replicate. In total, > 80 flowers were collected for measurements of water and nutrient contents. For measuring water content, 100 mg white hair tufts were dried at 70°C for 48 h, and water content was estimated as percentage weight loss: (FW − DW)/FW. The soluble carbohydrate content in white hair tufts was determined by the anthrone reagent method (Hansen & Moller, 1975), and the absorbance of the reaction solution was measured at 620 nm by using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). For calibration, glucose (Sigma-Aldrich) was used as the standard. The content of free amino acids was determined by the ninhydrin method (Doi *et al.*, 1981) and measured at 570 nm with a microplate reader. For calibration, L-cysteine (Sigma-Aldrich) was used as the standard.

Identification and relative quantification of volatile components

The headspace solid-phase microextraction (HS-SPME) method was used for volatile collection. One flower of *C. subtropicum* was enclosed in a gas-collecting bag (15 cm × 25 cm, 1050-TK-3 MT passive bag; GL Sciences, Tokyo, Japan), and a 50/30 μm divinylbenzene (DVB)/Carboxen® (CAR)/polydimethylsiloxane (PDMS) fiber and a manual SPME holder (Supelco Inc., Bellefonte, PA, USA) was placed in the bag for 30 min, then the sample was injected into the GC-MS system. The negative control was collected from an empty gas-collecting bag. The experiment was replicated three times with one flower in each replicate. The volatiles were analyzed on an Agilent 7890B Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a DB-1 capillary column (60 mm × 0.25 mm inner diameter, 0.25-μm film thickness; Agilent Technologies) coupled to an Agilent 5977 N MSD mass spectrometer. The injector temperatures were maintained at 250°C, in splitless mode. Oven temperature was programmed from 40°C for 1 min, increased to 150°C at 5°C min^{-1} and held for 1 min, then increased from 150 to 200°C at 10°C min^{-1} and held for 11 min. The injector temperature was maintained at 250°C. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The electron energy was 70 eV at 230°C. Kovats indices were calculated for the separated components relative to a C5-C25 n-alkane mixture. The compounds were identified by matching spectra with those recorded in the MS library (Wiley 7n), and confirmed by authentic pure standards and reported linear retention index (Kovats) values in the literature. Relative quantification of volatile components was performed by the external standard technique as described by Lee *et al.* (2014). Relative quantification of each volatile compound was calculated by using its extracted ion peak area divided by the peak area of the extracted ion peak area of an internal standard (I.S.), 8 ng g^{-1} of cyclohexyl acetate (Sigma-Aldrich, ≥ 99%).

$$\text{Concentration}\left(\frac{\text{ng}}{\text{g}}\right) = \frac{\text{extracted ion peak area}}{\text{extracted ion peak area of I.S.}}\left[\text{I.S.}\left(\frac{8\ \text{ng}}{\text{g}}\right)\right].$$

In addition to the flowers, volatiles of the aphid *Brevicoryne brassicae*, a common species in the local area, were collected. For each colony, *c.* 200 individuals of adult stage were placed in a glass air-collection vial (10 ml) and volatiles were again collected using the same fiber as above HS-SPME for 1 h. Identification and quantification were performed by GC-MS analyses as described above. The measurement was replicated three times with three aphid colonies and three controls (i.e. an aphid-free vial; see also Fig. S2c later).

Hoverfly response to the removal of white hair tufts

In order to test whether the white hair tufts played a significant role in attracting hoverflies, in the first treatment, we removed the tufts from the sidelobes of the labellum of six flowers (six inflorescences; six plants). In the second treatment, we glued the white

hair tufts back onto the labellum with agarose gel (olfactory neutral). We selected six intact flowers (six inflorescences; six plants) as the control. During a test period of 8 h (between 08:00 h and 16:00 h), the number of hoverflies landing on each flower was counted and collected to avoid counting an individual potentially more than once. The experiments were replicated three times (3 d) with six flowers in each replicate. One-way ANOVA was used to test for differences in mean number of hoverflies landed between treatments. Post-hoc testing involved using Tukey's HSD test.

Hoverfly responses to volatiles

In order to test the attractiveness of major volatile compounds emitted by *C. subtropicum* flowers to hoverflies, we performed attraction experiments with olfactory sticky traps in the forest habitat at Malipo County. The trap consisted of a 10-ml glass vial placed in the bottom of a polyethylene terephthalate bottle (67 mm diameter and 120 mm high) with a sticky glue layer on the inner surface to catch hoverflies. We placed 5 ml of a solution of each of the following volatile compounds into 10-ml glass vials: 2911 ng ml^{-1} geranyl acetone (Sigma-Aldrich, ≥97%), 265 ng ml^{-1} (*E*)-β-farnesene (Sigma-Aldrich, ≥90%), 143 ng ml^{-1} β-citronellol (Sigma-Aldrich, ≥98%), 43 ng ml^{-1} β-caryophyllene (Sigma-Aldrich, ≥98.5%), 33 ng ml^{-1} α-humulene (Sigma-Aldrich, ≥96%), 17 ng ml^{-1} β-pinene (Chemservice, ≥99.3%), 15 ng ml^{-1} β-citronellal (Merck, ≥94%), 0.01 ng ml^{-1} limonene (Sigma-Aldrich, ≥95%), 0.01 ng ml^{-1} anisaldehyde (Sigma-Aldrich, ≥95%) and 0.01 ng ml^{-1} citral (Sigma-Aldrich, ≥95%) dissolved in pentane (Sigma-Aldrich, ≥99%). The synthetic mixture (5 ml solution) consisted of all volatile compounds dissolved in pentane. The qualitative and quantitative compositions of individual volatile compounds and the synthetic mixtures were the same as the natural scent composition, as collected volatiles, released from the vials by HS-SPME and verified by GC-MS analyses. The glass vial containing just solvent (pentane) served as negative control, and a vial with a natural flower served as positive control. The traps including synthetic single compound, mixtures and controls (*c.* 4 m apart each other) were placed on the forest floor in five different locations between 08:00 h and 16:00 h. The experiments were replicated three times (3 d) with five locations in each replicate. One-way ANOVA was used to test for differences in the mean number of hoverfly responses to synthetic compounds, their mixtures and controls. The post-hoc test was performed with Tukey's HSD test.

Results

Floral morphology and pollination process

The flower of *C. subtropicum* is dark brown (Fig. 1b) and not as vivid in coloration as most *Cypripedium* species described (Cribb, 1997). The reddish-brown sidelobes of the labellum are covered with contrasting white hair tufts (Fig. 1c). Cryo-scanning electron microscopy revealed that the white hair tufts consist of a number of multicellular trichomes that look like strings of beads (Fig. 1d). Our field observations showed that *C. subtropicum* flowers were attractive to various hoverflies (Table S1). Of the observed hoverfly visitations, 70.5% (122 of 173) of captured specimens were female. Hoverflies first approached the flowers with an undulating flight and landed on the labellum to eat the white hair tufts around the sidelobes of the labellum (Fig. 2a,c). When hoverflies ate the white hair tufts, they easily fell into the labellum pouch and were trapped (see Video S1). They tried to escape by the entrance of the labellum, but most failed because of its curved margin. They could only escape by crawling up the back of the labellum under the stigma. They contacted one of two dehiscent anthers as they exited the flower from one of the basal apertures (Fig. 2b,d), as occurs for most *Cypripedium* species. Fig. 2(e) shows the route of escape. As hoverflies struggled past an anther, they carried an entire pollen smear on the dorsum of their thorax (Fig. S1). During the entire observation period, both genders could carry pollen smear, and we neither observed hoverflies attempting to oviposit on or in the flowers, nor found eggs on the flowers.

The breeding system

Natural fruit set ratio was 18.18% in 2014, 14.28% in 2015, 17.65% in 2016 and 16.09% in 2017. Both hand cross-pollination and self-pollination flowers produced 100% capsules. The seed viability tests showed no significant difference between cross- and self-pollinated seeds. None of the control flowers produced capsules, which indicates no autogamous self-pollination (Table S2).

Nutrient and water contents of the white hair tufts

In order to determine whether *C. subtropicum* rewarded its pollinators, we measured the nutrient content of white hair tufts. The extracts of white hair tufts contained a mean ± SD of 83.24 ± 0.58 mg g^{-1} FW sugar, 9.43 ± 0.07 mg g^{-1} FW amino acids and 873.5 ± 26.81 mg g^{-1} FW water.

Flower and aphid volatiles

Cypripedium subtropicum flowers emitted a strong fruity odor to the human nose. The GC-MS analyses revealed that the volatile components of *C. subtropicum* flowers were dominated by geranyl acetone (2911.01 ± 805.6 ng g^{-1} FW), (*E*)-β-farnesene (264.9 ± 74.1 ng g^{-1} FW) and β-citronellol (142.9 ± 62.2 ng g^{-1} FW). The remaining volatile components consisted of a small amount of β-caryophyllene (42.6 ± 6.7 ng g^{-1} FW), α-humulene (33.2 ± 10.2 ng g^{-1} FW), β-pinene (17.2 ± 3.3 ng g^{-1} FW), β-citronellal (14.5 ± 5.4 ng g^{-1} FW) and a number of trace compounds (< 0.01 ng g^{-1} FW) (Table 1; Fig. S2a). The headspace samples released from aphid colonies of *B. brassicae* consisted mainly of (*E*)-β-farnesene (Fig. S2b).

Hoverfly responses to the white hair tufts

We demonstrated that the white hair tufts played a significant role in attracting hoverflies. Hoverfly response significantly differed among treatments (one-way ANOVA, $F_{2,51} = 73.76$, $P = 8.89 \times 10^{-16}$), with similar numbers of hoverflies landing on

Fig. 2 A pollination system in *Cypripedium subtropicum*. (a) *Eristalinus arvorum* eating hair tufts. Bar, 3 mm. (b) The body of *E. arvorum* has contacted the anther. Bar, 3 mm. (c) *Eupeodes confrater* eating hair tufts. Bar, 3 mm. (d) The body of *E. confrater* has contacted the anther. Bar, 3 mm. (e) Dissected flower with half the labellum removed. The dotted line indicates the pollination route and direction of fly movement. Bar, 10 mm. (f) Cryo-scanning electron microscopy (SEM) image of a few cells from white hair tufts (arrow) on the mouthparts of a trapped hoverfly. Bar, 100 μm.

the nontreated flowers (mean ± SD 5.7 ± 1.9 hoverflies per flower) and flowers to which the tufts were replaced after having them removed (mean ± SD 5.2 ± 1.0 hoverflies per flower). No flies landed on flowers with removed tufts. We observed approaching flies in all treatments (Fig. 3).

Hoverfly responses to volatiles

Bioassays with flowers and synthetic scents revealed strong differences in the number of trapped flies among the treatments (one-way ANOVA, $F_{12,182} = 71.47$, $P < 2 \times 10^{-16}$). Tukey's HSD test indicated that single flowers (the positive controls) and the synthetic mixture attracted the most, and similar numbers of, hoverflies ($n = 4.73 \pm 1.44$ and 3.87 ± 1.41, respectively), followed by (*E*)-β-farnesene ($n = 2.8 \pm 1.37$) and β-pinene ($n = 1.8 \pm 1.08$). The other compounds did not attract significantly more hoverflies than the negative control (the solvent pentane only; Fig. 4; Table S3).

Discussion

Cypripedium is considered a lineage of food-deceptive orchids (Bernhardt & Edens-Meier, 2010). Here we report a new pollination system in *C. subtropicum*. This species provides edible rewards – hairy tufts – to attract hoverflies, which are involved in eliciting landing responses in the flies (Fig. 3).Such edible multicellular trichomes as found on the labellum of *C. subtropicum* are not observed in other *Cypripedium* species (Bernhardt & Edens-Meier, 2010). However, cell specialization to attract pollinators also has

Table 1 The contents of floral volatile compounds emitted from *Cypripedium subtropicum*.

No.	RI	Compound	Contents (ng g^{-1} FW)
1	979	β-pinene	17.2 ± 3.3
2	1028	Limonene	< 0.01
3	1133	β-citronellal	14.5 ± 5.4
4	1214	β-citronellol	142.9 ± 62.2
5	1218	Anisaldehyde	< 0.01
6	1246	Citral	< 0.01
7	1428	β-caryophyllene	42.6 ± 6.7
8	1435	geranyl acetone	2911 ± 805.6
9	1452	(*E*)-β-farnesene	264.9 ± 74.1
10	1464	α-humulene	33.2 ± 10.2

Data are mean ± SD of three independent measurements. RI, retention index.

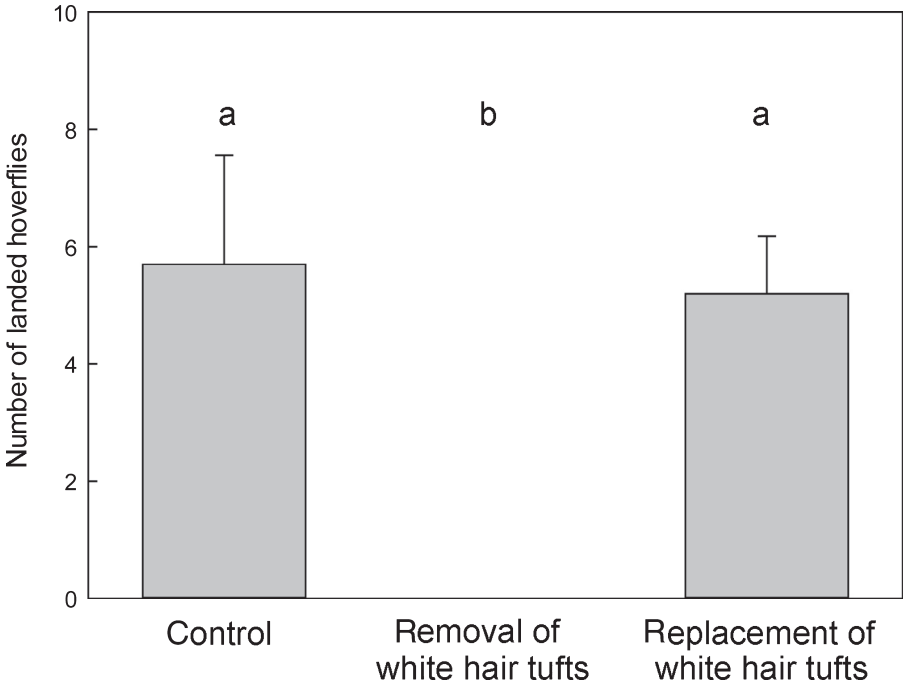

Fig. 3 Effect of the presentation of white hair tufts on hoverfly landing, including *Episyrphus balteatus*, *Eristalinus arvorum*, *Eupeodes confrater* and *Korinchia nova* (mean number of hoverflies ± SD). Different letters indicate significant differences (one-way ANOVA with Tukey's honestly significant difference (HSD) test).

been reported in *C. fargesii*, in which the mimicry of fungus-infected foliage deceives a syrphid fly for pollination (Ren *et al.*, 2011). More widespread are edible trichomes in epidendroid subtribes, such as Catasetinae (Pansarin & Maciel, 2017), Dendrobiinae (Davies & Turner, 2004b), Eriinae (Davies & Turner, 2004a), Maxillariinae (Davies *et al.*, 2003) and Polystachyinae (Davies *et al.*, 2002). Such floral trichomes are usually rich in protein bodies, oil droplets or starch grains (Davies & Turner, 2004b) and are collected by bees as a nutritive reward. In the subfamily Orchidoideae, *Satyrium microrrhynchum* provides nectar on long floral trichomes to attract beetles and wasps for pollination (Johnson *et al.*, 2007). These data suggest that edible trichomes may evolve independently in the orchid family (Pansarin & Maciel, 2017).

Adult hoverflies are known to feed on honeydew secreted by aphids, and honeydew provides an important visual signal for adult hoverflies to locate the aphid colony (Scholz & Poehling, 2000). Our results indicate that the nutrient content of white hair tufts is comparable to nutrients of honeydew secreted by aphids, with a sugar content of 14–68 mg g^{-1} and an amino acid content of *c.* 5 mg g^{-1} (Auclair, 1963; Sabri *et al.*, 2013). Therefore, the white hair tufts may mimic aphid colonies with abundant honeydew for attracting hoverflies. This provides a good example of a rewarding mimicry system, a rare mimicry system among plants (see Johnson & Schiestl, 2016), in which hoverflies do not find aphid colonies, but still get an appealing reward.

Analysis by GS-MS revealed that the volatile components contained a large amount of (*E*)-β-farnesene and a small amount of β-pinene, which are commonly used alarm pheromones produced by aphids (Pickett & Griffiths, 1980; Almohamad *et al.*, 2008) (Table 1; Fig. S2a). (*E*)-β-farnesene alone or associated with other natural molecules such as α/β-pinene, β-myrcene and limonene provides olfactory cues for hoverflies to locate aphid colonies (Francis *et al.*, 2005). The pheromone of the local aphid *Brevicoryne brassicae* primarily consists of (*E*)-β-farnesene (Fig. S2b). Our results show that the aphid alarm pheromones emitted by *C. subtropicum* flowers are important volatile components in mimicry to attract hoverflies for pollination.

This observation can be compared to the strategies of chemical mimicry used by *Epipactis veratrifolia* for pollination. This hoverfly-pollinated species, which offers a small amount of nectar, also produces aphid alarm pheromones (i.e. α/β-pinene, β-myrcene and β-phellandrene) (Stökl *et al.*, 2011; Jin *et al.*, 2014). In previous studies of the volatile profile of *Cypripedium* species (Nilsson, 1979; Bergstrom *et al.*, 1992; Barkman *et al.*, 1997; Li *et al.*, 2006, 2008a,b; Ren *et al.*, 2011; Zheng *et al.*, 2011), only a small amount of (*E*)-β-farnesene was detected in *C. calceolus* flowers, but it is not a dominant component (Braunschmid *et al.*, 2017). The divergence of the volatile profile may enable *C. subtropicum* to adapt to hoverflies for pollination in dense subtropical forests. The dense subtropical forest habitat in southwestern China is not occupied by any other known *Cypripedium* species but is a common habitat for *Paphiopedilum* species (a closely related genus). In the same habitat, *Paphiopedilum* spp. are pollinated exclusively by hoverflies with visual cues mimicking food sources or oviposition sites, but no olfactory cues (Shi *et al.*, 2007; Shi *et al.*, 2008). In *C. subtropicum*, both male and female hoverflies were caught on flowers and found to carry the pollen smear. Besides, egg-laying behavior of female hoverflies was absent during the entire observation period, thus not suggesting an oviposition mimicry.

Reproduction in such deceptive orchids, such as most *Cypripedium* species, often is severely pollen-limited because insects can learn to avoid plants without rewards (Cozzolino & Widmer, 2005; Tremblay *et al.*, 2005; Case & Bradford, 2009). But, in *C. subtropicum*, the presence of an edible reward did not guarantee a high reproductive success in the conversion of ovaries

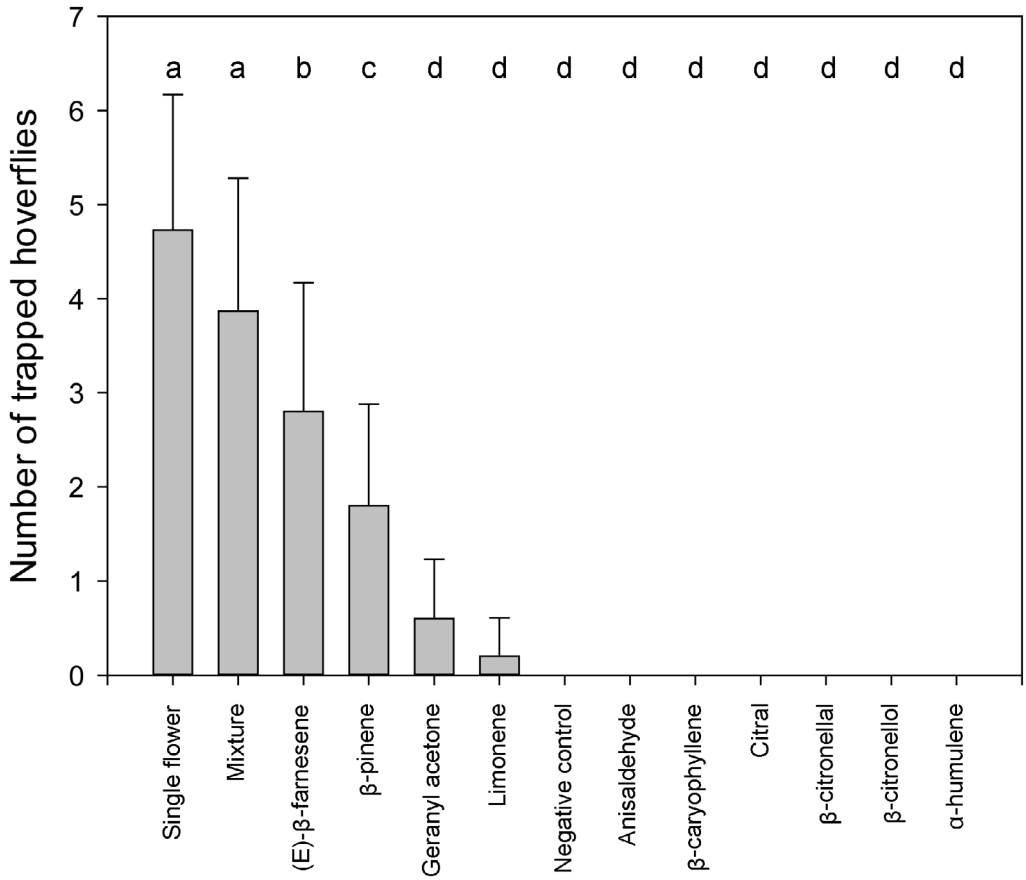

Fig. 4 Mean number of hoverflies trapped, including *Episyrphus balteatus*, *Eristalinus arvorum*, *Eupeodes confrater*, *Korinchia nova* and *Xanthandrus talamaui* for each volatile compound (mean number of hoverflies ± SD). Different letters indicate significant differences (one-way ANOVA with Tukey's honestly significant difference (HSD) test).

into fruits. In this study, a low conversion ratio of flowers into fruits (14.28–18.18%; Table S2) was recorded as compared to those in the other deceptive *Cypripedium* species (Bernhardt & Edens-Meier, 2010). The low fruit-set ratio in *C. subtropicum* may attribute to the predation of developing fruits by moth larvae. In our field observations, about half of the fruits were attacked. In North America, weevils have been reported to prey on developing fruits of *Cypripedium* species that resulted in the heavily reduced reproductive output (Light & MacConaill, 2011).

In conclusion, the rewarding mimicry system that we observed in *C. subtropicum* represents a new strategy of providing edible rewards combined with chemical mimicry to attract hoverflies that has not been reported previously in *Cypripedium*, a model lineage of food-deceptive orchids. *Cypripedium subtropicum* grows in dense subtropical forests with a shortage of typical *Cypripedium* pollinators (i.e. bees). In addition to its mimicry of aphid alarm pheromones, the evolution of its novel traits such as edible white hair tufts as a reward may be a successful way to guarantee the transfer of gametes in dense subtropical forests.

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Author contributions

HJ, J-JK and Y-IL planned and designed the research; H-CC, ZYX, WPZ and ZDH performed experiments; P-CL, J-JK and Y-IL analyzed the data; and J-JK and Y-IL wrote the manuscript. HJ and J-JK contributed equally to this work.

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References

Almohamad R, Verheggen FJ, Francis F, Lognay G, Haubruge EE. 2008. Emission of alarm pheromone by non-preyed aphid colonies. *Journal of Applied Entomology* **132**: 601–604.

Auclair JL. 1963. Aphid feeding and nutrition. *Annual Review of Entomology* **8**: 439–490.

Barkman TJ, Beaman JH, Douglas AG. 1997. Floral fragrance variation in *Cypripedium*: implications for evolutionary and ecological studies. *Phytochemistry* **44**: 875–882.

Bergstrom G, Birgersson G, Groth I, Anders Nilsson L. 1992. Floral fragrance disparity between three taxa of lady's slipper *Cypripedium calceolus* (Orchidaceae). *Phytochemistry* **31**: 2315–2319.

Bernhardt P, Edens-Meier R. 2010. What we think we know vs. what we need to know about orchid pollination and conservation: *Cypripedium* L. as a model lineage. *Botanical Review* **76**: 204–219.

Braunschmid H, Mükisch B, Rupp T, Schäffler I, Zito P, Birtele D, Dötterl S. 2017. Interpopulation variation in pollinators and floral scent of the lady's-slipper orchid *Cypripedium calceolus* L. *Arthropod-Plant Interactions* **11**: 363–379.

Budenberg WJ, Powell W. 1992. The role of honeydew as an ovipositional stimulant for two species of syrphids. *Entomologia Experimentalis et Applicata* **64**: 57–61.

Case MA, Bradford ZR. 2009. Enhancing the trap of lady's slippers: a new technique for discovering pollinators yields new data from *Cypripedium parviflorum* (Orchidaceae). *Botanical Journal of the Linnean Society* **160**: 1–10.

Chen SC, Lang K. 1986. *Cypripedium subtropicum*, a new species related to *Selenipedium*. *Acta Phytotaxonomica Sinica* **24**: 317–322.

Cozzolino S, Widmer A. 2005. Orchid diversity: an evolutionary consequence of deception? *Trends in Ecology & Evolution* **20**: 487–494.

Cribb P. 1997. *The genus* Cypripedium. Portland, OR, USA: Timber Press.

Dafni A, Bernhardt P. 1990. Pollination of terrestrial orchids in southern Australia and the Mediterranean region. Systematics, ecological, and evolutionary implications. In: Hecht MK, Wallace B, Macintyre RJ, eds. *Evolutionary biology*, vol **24**. New York, NY, USA: Plenum Publishing Corporation, 192–252.

Davies KL, Roberts DL, Turner MP. 2002. Pseudopollen and food-hair diversity in *Polystachya* Hook. (Orchidaceae). *Annals of Botany* **90**: 477–484.

Davies KL, Turner MP. 2004a. Pseudopollen in *Eria* Lindl. Section Mycaranthes Rchb.f. (Orchidaceae). *Annals of Botany* **94**: 707–715.

Davies KL, Turner MP. 2004b. Pseudopollen in *Dendrobium unicum* Seidenf. (Orchidaceae): reward or deception? *Annals of Botany* **94**: 129–132.

Davies KL, Turner MP, Gregg A. 2003. Atypical pseudopollen-forming hairs in *Maxillaria* (Orchidaceae). *Botanical Journal of the Linnean Society* **143**: 151–158.

Doi E, Shibata D, Matoba T. 1981. Modified colorimetric ninhydrin methods for peptidase assay. *Analytical Biochemistry* **118**: 173-184.

Francis F, Martin T, Lognay G, Haubruge E. 2005. Role of (*E*)-β-farnesene in systematic aphid prey location by *Episyrphus balteatus* larvae. *European Journal of Entomology* **102**: 431–436.

Hansen J, Moller I. 1975. Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. *Analytical Biochemistry* **68**: 87–94.

Jiang H, Liu E. 2009. The Quest for an elusive slipper orchid *Cypripedium subtropicum* rediscovered. *Orchid Digest* **73**: 240–245.

Jin XH, Ren ZX, Xu SZ, Wang H, Li DZ, Li ZY. 2014. The evolution of floral deception in *Epipactis veratrifolia* (Orchidaceae): from indirect defense to pollination. *BMC Plant Biology* **14**: 63.

Johnson SD, Ellis A, Dotterl S. 2007. Specialization for pollination by beetles and wasps: the role of lollipop hairs and fragrance in *Satyrium microrrhynchum* (Orchidaceae). *American Journal of Botany* **94**: 47–55.

Johnson SD, Schiestl FP. 2016. *Floral mimicry*. Oxford, UK: Oxford University Press.

Klecka J, Hadrava J, Biella P, Akter A. 2018. Flower visitation by hoverflies (Diptera: Syrphidae) in a temperate plant-pollinator network. *PeerJ* **6**: e6025.

Lee YI, Lee N, Yeung EC, Chung MC. 2005. Embryo development of *Cypripedium formosanum* in relation to seed germination in vitro. *Journal of American Society for Horticultural Science* **130**: 747–753.

Lee J, Xiao L, Zhang G, Ebeler SE, Mitchell AE. 2014. Influence of storage on volatile profiles in roasted almonds (*Prunus dulcis*). *Journal of Agricultural and Food Chemistry* **62**: 11236–11245.

Li P, Luo YB, Bernhardt P, Kou Y, Perner H. 2008b. The pollination of *Cypripedium plectrochilum* (Orchidaceae) by *Lasioglossum* spp. (Halictidae): the roles of generalist attractants vs. restrictive floral architecture. *Plant Biology* **10**: 220–230.

Li P, Luo YB, Bernhardt P, Yang XQ, Kou Y. 2006. Deceptive pollination of the lady's slipper *Cypripedium tibeticum* (Orchidaceae). *Plant Systematics and Evolution* **262**: 53–63.

Li P, Luo YB, Deng YX, Kou Y. 2008a. Pollination of the lady's slipper *Cypripedium henryi* (Orchidaceae). *Botanical Journal of the Linnean Society* **156**: 451–459.

Li P, Pemberton R, Zheng G, Luo Y. 2012. Fly pollination in *Cypripedium*: a case study of sympatric *C. sichuanense* and *C. micranthum*. *Botanical Journal of the Linnean Society* **170**: 50–58.

Light MHS, MacConaill MC. 2011. Potential impact of insect herbivores on orchid conservation. *European Journal of Environmental Sciences* **1**: 115–124.

Martos F, Cariou ML, Pailler T, Fournel J, Bytebier B, Johnson SD. 2015. Chemical and morphological filters in a specialized floral mimicry system. *New Phytologist* **207**: 225–234.

Nilsson LA. 1979. Anthecological studies on the lady's slipper, *Cypripedium calceolus* (Orchidaceae). *Botaniska Notiser* **132**: 329–347.

Nilsson LA. 1992. Orchid pollination biology. *Trends in Ecology & Evolution* **7**: 255–259.

Pansarin ER. 2008. Reproductive biology and pollination of *Govenia utriculata*: a syrphid fly orchid pollinated through a pollen-deceptive mechanism. *Plant Species Biology* **23**: 90–96.

Pansarin ER, Maciel AA. 2017. Evolution of pollination systems involving edible trichomes in orchids. *AoB Plants* **9**: plx033.

Pickett JA, Griffiths DC. 1980. Composition of aphid alarm pheromones. *Journal of Chemical Ecology* **6**: 349–360.

Rankou H, Averyanov L. 2014. Cypripedium subtropicum. In: *The IUCN Red List of Threatened Species 2014: e.T201848A2722593*. doi: 10.2305/IUCN.UK.2014-1.RLTS.T201848A2722593.en. [Accessed Downloaded on 23 May 2017].

Ren Z-X, Li D-Z, Bernhardt P, Wang H. 2011. Flowers of *Cypripedium fargesii* (Orchidaceae) fool flat-footed flies (Platypezidae) by faking fungus infected foliage. *Proceedings of the National Academy of Sciences, USA* **108**: 7478–7480.

Sabri A, Vandermoten S, Leroy PD, Haubruge E, Hance T, Thonart P, De Pauw E, Francis F. 2013. Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. *PLoS ONE* **8**: e74656.

Scholz D, Poehling HM. 2000. Oviposition site selection of *Episyrphus balteatus*. *Entomologia Experimentalis et Applicata* **94**: 149–158.

Shi J, Cheng J, Luo D, Shangguan FZ, Luo YB. 2007. Pollination syndromes predict brood-site deceptive pollination by female hoverflies in *Paphiopedilum dianthum* (Orchidaceae). *Acta Phytotaxonomica Sinica* **45**: 551–560.

Shi J, Luo YB, Bernhardt P, Ran JC, Liu ZJ, Zhou Q. 2008. Pollination by deceit in *Paphiopedilum barbigerum* (Orchidaceae): a staminode exploits the innate colour preferences of hoverflies (Syrphidae). *Plant Biology* **11**: 17–28.

Stökl J, Brodmann J, Dafni A, Ayasse M, Hansson BS. 2011. Smells like aphids: orchid flowers mimic aphid alarm pheromones to attract hoverflies for pollination. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **278**: 1216–1222.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN. 2005. Variation in sexual reproduction in orchids and its evolutionary consequence. A spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84**: 1–54.

Van der Pijl L, Dodson DH. 1966. *Orchid flowers: their pollination and evolution*. Coral Gables, FL, USA: University of Miami Press.

Verheggen F, Arnaud L, Bartram S, Gohy M, Haubruge E. 2008. Aphid and plant volatiles induce oviposition in an aphidophagous hoverfly. *Journal of Chemical Ecology* **34**: 301–307.

Zheng G, Li P, Pemberton R, Luo YB. 2011. Mixed bumblebee and blowfly pollination of *Cypripedium flavum* (Orchidaceae) in Sichuan, China. *Ecological Research* **26**: 453–459.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Trapped adult hoverflies with the pollen smear of *C. subtropicum* on the thorax.

Fig. S2 Total ion chromatogram of floral volatile compounds emitted from *C. subtropicum*, a local aphid colony, and baseline in GC-MS measurement.

Table S1 Insect visitors to *C. subtropicum* flowers.

Table S2 Effects of pollination treatments on fruit set and seed viability of *C. subtropicum*.

Table S3 Effects of different volatile compounds on the number of hoverfly species trapped over a 24-h period.

Video S1 A hoverfly eats the white hair tufts around the sidelobes of the labellum and is trapped by the labellum.

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