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RESEARCH PAPER

Cypripedium lichiangense (Orchidaceae) mimics a humus-rich oviposition site to attract its female pollinator, Ferdinandea cuprea (Syrphidae)

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ABSTRACT

- Most species in the genus *Cypripedium* (Cypripedioideae) produce trap flowers, making it a model lineage to study deceptive pollination. Floral attractants in most species studied appear to target bee species of different sizes. However, more recent publications report fly pollination in some subalpine species, suggesting novel suites of adaptive floral traits.
- *Cypripedium lichiangense* (section *Trigonopedia*) is an endangered subalpine species endemic to the Hengduan Mountains, China. We observed and analysed its floral traits, pollinators and breeding systems over 2 years *in situ* and in the lab.
- *Cypripedium lichiangense* was visited by females of *Ferdinandea cuprea* (Syrphidae). The pollinia were carried dorsally on the fly thoraces. The eggs of this fly were frequently found in the saccate labellum and on other floral organs, suggesting brood-site mimesis. The orchid is self-compatible, but cross-pollination produces more viable embryos.
- We propose a new mode of floral mimesis, humus-rich oviposition site mimicry, for *C. lichiangense*. Compared with the mimesis of aphid colonies attracting syrphid pollinators (subfamily Syrphinae), whose larvae are entomophagic, as reported in some *Paphiopedilum* species (Cypripedioideae), pollination by deceit in *C. lichiangense* represents a distinct and separate mode of exploitation of another saprophagic (or phytophagic) larvae syrphid lineage in the subfamily Eristalinae and appears to indicate diversity of pollination strategies in Section *Trigonopedia* of *Cypripedium*. However, this new brood-site mimesis seems to be less attractive to pollinators. As a possible adaptation to the weak attracted pollination strategy, this plant species has a long flowering period and extended lifespan of individual flowers to ensure reproductive success.

INTRODUCTION

Most flowering plants are pollinated by animals and the flowers provide edible rewards, including nectar, pollen, oils and starch bodies (Renner, 2006; Johnson & Schiestl, 2016). However, an estimated 3.7–6.0% of animal-pollinated plants (see Renner, 2006 *versus* Vogel, 1993) offer 'empty' flowers that lack rewards. These deceptive flowers exploit various behaviours of their floral visitors, including nectar drinking, pollen collection, mating, oviposition and sheltering, to achieve reproductive success (Johnson & Schiestl, 2016). Within the species-rich family Orchidaceae, deceptive pollination has evolved independently within four subfamilies (Jersáková *et al.* 2006; Tang *et al.* 2014; Shrestha *et al.* 2020) and is regarded as a major driving force in orchid speciation (Cozzolino & Widmer, 2005; Givnish *et al.* 2015; Johnson & Schiestl, 2016).

However, brood-site mimesis (BSM) has been studied less frequently in orchids. This exploits insect oviposition and has been described in 20 plant families (Sakai, 2002; Jürgens *et al.* 2006, 2013; Ollerton & Raguso 2006; Pemberton, 2013; Johnson & Schiestl, 2016; Policha *et al.* 2016). This mode of deception is especially common in the Araceae, Rafflesiaceae and Aristolochiaceae (Urru *et al.* 2011; Jürgens *et al.* 2013; Chen *et al.* 2015). Plants employing BSM have evolved novel attraction patterns, *i.e.* visual (*e.g.* pigmentation patterns and floral shapes; Urru *et al.* 2011; Chen *et al.* 2015), olfactory (Jürgens *et al.* 2013) and sensory (*e.g.* temperature change and tactile cues; Urru *et al.* 2011; Zhang *et al.* 2020) signals. Olfactory signalling remains the most well-studied factor in BSM and plays an important role in substrate imitation, including mimesis of carrion, decaying plant material, animal dung, fungi (see reviews in Jürgens *et al.* 2013 and Urru *et al.* 2011) and aphid colonies (Jiang *et al.* 2020). Visual cues exploit aphid-like 'decoys' in *Paphiopedilum* (Orchidaceae) (Pemberton, 2013; Ma, 2015). Flowers employing BSM appear to be pollinated primarily by species that belong to families in the Orders Diptera (*e.g.*

Calliphoridae, Muscidae, Scatophagidae, Sphaeroceridae and Syrphidae) and Coleoptera (*e.g.* Staphylinidae and Scarabaeidae; see reviews in Urru *et al.* 2011; Pemberton, 2013).

Within the subfamily Cypripedioideae of the Orchidaceae, BSM appears to be most common in the genus *Paphiopedilum*. The staminodium has pigmented structures that egg-laying flies mistake for aphid colonies before they slip and fall into the labellum trap. Eggs of the pollinator have been found on the floral organs of four *Paphiopedilum* species (Table 1; Atwood, 1985; Shi *et al.* 2009; Pemberton, 2013; Edens-Meier *et al.* 2014; Tang *et al.* 2014; Ma, 2015).

In contrast, the pollination of species in the closely related genus *Cypripedium* (Cypripedioideae; Guo, 2012) have been studied more frequently and are now regarded as a model lineage for trap blossoms that typically do not reward their pollen vectors. The majority of species studied are bee-pollinated and are usually interpreted as generalist pollen/nectar mimics (Bernhardt & Edens-Meier, 2010; Pemberton, 2013). More recent studies indicate that at least six *Cypripedium* species are pollinated by flies that exploit different suites of floral attractants (Liu *et al.* 2008; Ren *et al.* 2011; Li *et al.* 2012; Jiang *et al.* 2020). Specifically, within section *Trigonopedia* (*sensu* Li *et al.* 2011), fly-pollination occurs consistently in *C. farges*ii Franch., *C. sichuanense* Perner and *C. lentiginosum* P.J. Cribb & S.C. Chen (Liu *et al.* 2008; Ren *et al.* 2011; Li *et al.* 2012). Within section *Trigonopedia*, *C. farges*ii is pollinated by male and female fungus-eating (mycophagic) syrphid flies (Ren *et al.* 2011). The blackish, hairy spotted leaves may mimic fungus-infected foliage to lure flies that feed on fungal exudates and/or on infected vegetation. BSM was proposed for *C. lentiginosum* since it is pollinated by a fly species in the genus *Ferdinandea* (Syrphidae; Liu *et al.* 2008), but fly eggs have not been found on or in the flowers. Li (2006) speculated that BSM occurred in *C. sichuanense*, following 18 h of field observations. Species in *Trigonopedia* have similar pigmentation patterns on the leaves and flowers (Chen & Cribb, 2009), however, little is known about the pollination relationship among these species.

In this study, we focused on *Cypripedium lichiangense* S. C. Chen & P. J. Cribb. This species is closely related to *C. lentiginosum* and produces the largest flowers in section *Trigonopedia*. It is endangered and endemic to the Hengduan Mountains of China (Chen & Cribb, 2009). We observed some white egg-like structures on the flowers in our preliminary field observation in southwest China, suggesting that BSM may be employed by *C. lichiangense*. We speculate that *C. lichiangense* might attract mainly female flies as pollinators and that the larvae of the fly (or flies) would live in habitats with decaying, fungus-infected vegetation, rich in humus. Furthermore, we discuss the connections between pollination mechanisms among species in *Trigonopedia*.

MATERIAL AND METHODS

Plant population and study site

Cypripedium lichiangense is a perennial herb 7–14-cm tall, with a short rhizome that usually produces only one shoot per year, similar to the closely related species, *C. lentiginosum* (Liu *et al.* 2008). Leaf blades are dark green and marked with purplish black spots; a solitary flower bud opens between two flat and prostrate leaves. The open flower lacks an elongated scape so it lies close to the ground. Lateral petals show abaxial pubescence (Fig. 1; see Chen & Cribb, 2009).

Field observations and experiments were conducted near Heba Village, Kangding, Sichuan Province, southwest China in 2019 (from 27 April to 7 July) and 2020 (from 10 May to 16 May). A large population of *C. lichiangense* consisting of more than 1000 flowering plants was located within a 15,540 m^2 area on a limestone mountain with secondary deciduous broad-leaved and coniferous mixed forest at an elevation of 2200–2300 m. Co-blooming species included *Berberis wilsoniae* Hemsl., *Calanthe arcuate* Rolfe, *C. davidii* Franch., *Campylotropis polyantha* (Franch.) Schindl. and *Cotoneaster horizontalis* Decne. Pressed specimens of these co-blooming plants

Table 1. Species in the Cypripedioideae employing some mode of brood-site mimesis with '+' = eggs found on or in floral organs; '−' = no eggs; '*' = eggs observed but unpublished (laboratory of Prof. Y. B. Luo).

species	Eggs	pollinator	reference
Cypripedium lentiginosum	−	*Ferdinandea formosana* (Eristalinae)	Liu *et al.* (2008)
C. fasciculatum	−	*Cinetus* spp. (Vespidae)	Ferguson & Donham (1999) Pemberton (2013)
Paphiopedilum barbigerum	+*	*Allograpta* sp. (Syrphinae) *Erisyrphus* sp. (Syrphinae)	Shi *et al.* (2008) Tang *et al.* (2014)
P. dianthum	+	*Episyrphus* sp. (Syrphinae)	Shi *et al.* (2007)
P. hirsutissimum	+*	*Erisyrphus* sp. (Syrphinae) *Allobaccha* sp. (Syrphinae)	Shi *et al.* (2009) Pemberton (2013)
P. rothschildianum	+	*Dideopsis* sp. (Syrphinae)	Atwood (1985)
P. purpuratum	−	*Ischiodon* sp. (Syrphinae)	Liu *et al.* (2004)
P. villosum	−	*Betasyrphus* sp. (Syrphinae) *Episyrphus* sp. (Syrphinae) *Syrphus* sp. (Syrphinae)	Bänziger (1996)
Phragmipedium caudatum	−	*Syrphus* sp. (Syrphinae)	McCook (1989) Pemberton (2011) Pemberton (2013)
Phragmipedium pearcei	−	*Ocyptamus* sp. (Syrphinae)	Pemberton (2011) Pemberton (2013)

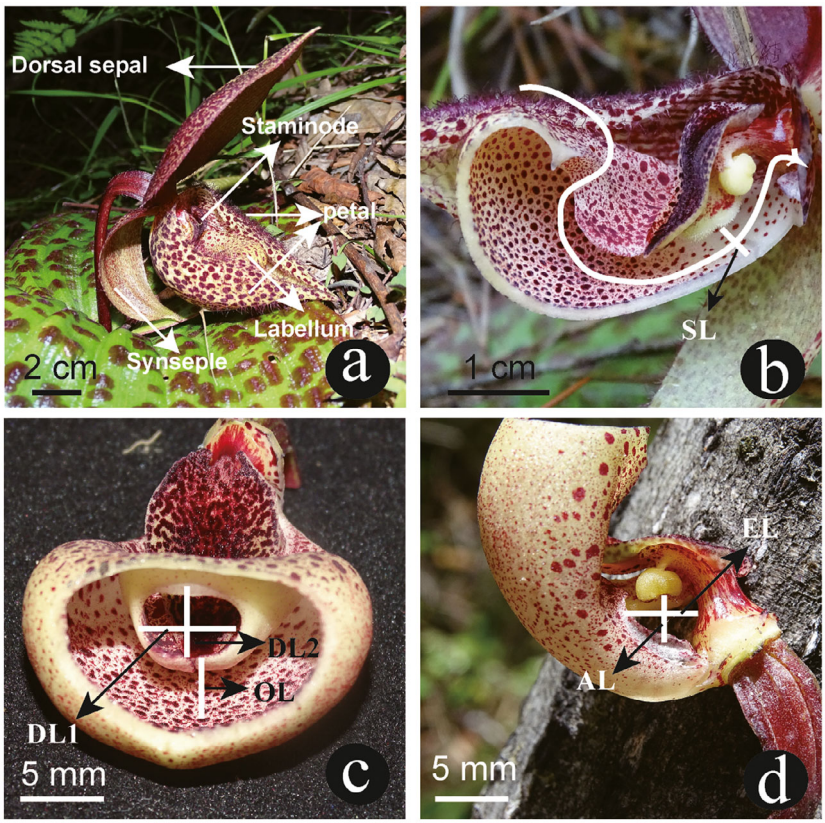

Fig. 1. Flower morphology and measurements. (a) Flower in profile. (b) Pollinator pathway through flower (arrow), SL = distance between receptive stigmatic surface and labellum floor. (c) DL (DL1 = labellum rim length; DL2 = labellum rim width; OL = distance between labellum rim and labellum floor). (d) EL = rear exit width and AL = distance between anther and labellum floor.

were deposited in the Herbarium of Chengdu Institute of Biology, Chinese Academy of Science (CDBI), Chengdu.

Floral phenology and lifespan

In 2019, we observed and recorded flowering of the above population and the individual lifespan of *C. lichiangense* flowers. To confirm the individual lifespan of a flower, we marked more than 200 flowers and recorded the floral lifespan from opening to wilting. None of the recorded flowers received pollinia on their stigmas. We also recorded the flowering period of the population from first flower opening to final flower wilting. Here, we mainly followed the criterion provided by Sugiura *et al.* (2001) to judge floral opening and wilting: a flower was judged as 'opening' when the dorsal sepal rose, and any visitor could enter the pouched labellum; a flower was regarded as 'wilting' when no longer visually attractive to human observers (*i.e.* perianth and labellum discoloured, collapsed and withered), thereby losing its role in the pollination process.

Field observations of prospective pollinators

Field observations were performed during two flowering seasons: from 2 May to 7 June 2019 (09:00–18:00 h daylight and 21:00–01:00 h at night) and from 10 May to 16 May 2020 (10:00–16:00 h for daylight). Field observations performed in 2020 determined whether the identities of prospective pollinators matched those observed and collected in 2019 and 2020. In total, there were 342 h of observation with 330 h in daylight and 12 h at night (including three nights during the flowering season in 2019). We recorded the behaviours of floral visitors (see Nilsson, 1979) entering and exiting flowers. They were caught with nets, euthanized with ethyl acetate, examined for deposition of orchid pollinia (here and below we use the terms pollinia or pollinium because the pollinator will generally carry the whole pollen mass of an anther when escaping from the rear exit), pinned, measured, labelled and deposited in the Herbarium of the Chengdu Institute of Biology, Chinese Academy of Science (CDBI), Chengdu. We sent pinned specimens to an entomologist for identification. Additionally, we attempted to track insects after they left the orchid flower to record whether these pollinators visited other co-blooming species and check whether *C. lichiangense* benefited from its co-flowering species, that is, sharing same floral visitors or pollinators with similar floral attractants.

Brood-site mimesis

To test the BSM hypothesis, we examined the *C. lichiangense* flowers to determine whether eggs had been laid on or in floral organs. In order to better determine whether pollinators laid eggs in flowers, we waited until an insect entered the labellum then closed the floral sinus and rear escape apertures with cotton balls for some time. After removing the cotton balls, we examined the flower interior for eggs.

Floral signalling, microstructure of plants and pollinators

To determine when and where the flowers secrete scents detectable to the human nose, we tested individual floral organs for odour sites following Ren (2010). We collected three fresh flowers of *C. lichiangense*, in which we could detect scent. We

dissected these flowers, placed each dorsal sepal, synsepal, both lateral petals, labellum and staminodium in separate clean jars. The jars were capped for 3 min then the content of each jar was smelled.

We also examined additional flowers for the presence of nectar and glandular epidermis. To explore the microstructures of flowers and leaves, we collected and fixed whole fresh flowers and leaves in a solution of 70% alcohol:acetic acid:formaldehyde (8:1:1). The floral and leaf epidermis was first observed under a light microscope (Olympus BX43F, Olympus, Japan). Then pieces of floral organs, *i.e.* parts of leaves, labella, petals and sepals, were dehydrated in a graded ethanol–isoamyl acetate series. We plated the dried specimens with gold palladium before observing and photographing them at an accelerating voltage of 10 kV (Ren *et al.* 2011) in a scanning electron microscope (SEM; Phenom Pro, the Netherlands). We observed segments of four dried pollinators found in the flowers using the same gold palladium plating treatment and SEM.

Floral morphometrics and pollinator traits

We measured the functional morphological traits of the flowers using Vernier calipers to a resolution of 0.001 mm: length and width (Fig. 1c) of the dorsal opening (large dorsal sinus) of the labellum; depth between the sinus rim and the floor of the labellum; depth between the receptive ventral portion of the stigma and the floor of the labellum (Fig. 1b); depth between one anther and the floor of the labellum (Fig. 1d); and width of one of the two rear exit apertures (Fig. 1d).

We used the same vernier calipers to measure the insects that were observed entering the labellum and/or escaping *via* the rear exit apertures: insect length from frons to terminus of the abdomen, thorax depth and thorax width as this was the widest part of all fly bodies we collected. We used t-tests to compare traits of flowers *versus* those of pollinators. Analyses and comparisons of floral and insect traits followed Li *et al.* (2008). We also measured the traits of other visitors using the same method.

Breeding systems

To determine whether pollinators were necessary for *C. lichiangense* to produce fruits and seeds, we conducted hand-pollination experiments following Zheng & Li (2009). We divided marked plants (n = 671 flowers in 2019 and 558 flowers in 2020) into four treatment categories before buds opened: (i) self-pollinated (n2019 = 110, n2020 = 20); (ii) cross-pollinated (n2019 = 22, n2020 = 14); (iii) Open- (Insect-) pollinated (n2019 = 527, n2020 = 524); and (iv) control (n2019 = 12, n2020 = 13). For self-pollination, we removed each labellum with a razor blade so that insects could not enter and interfere with the pollination process (see Bernhardt *et al.* 2014). Stigmas were hand-pollinated with pollinia derived from anthers in the same flower. For cross-pollination, we also removed the labellum but then hand-pollinated the stigma with pollinia from flowers located >5 m away, avoiding geitonogamous crosses. As we excavated several plants to determine the length of the rhizome, we found that each plant produced only one flower and that individual rhizomes were much shorter 5 m (see above). Open flowers retained their labellum, which allowed us to estimate the natural rate of successful insect pollination and compare it with hand-pollination. For a control, we removed the labellum, thus exposing flowers to insects but did not apply pollinia to stigmas. We collected fruits from plants marked for these experimental procedures in mid-October 2019.

Fruit set and seed viability test

Fruit set in 2019 was based on collection of whole dehiscent capsules and fruit set in 2020 was based on counts of swollen ovaries on plants. In 2019, we removed and mixed seeds from three capsules that had been self-pollinated; the same treatment was also applied to cross-pollinated, open- (insect-)pollinated and control samples. Seeds selected randomly ($n \geq 400$ seeds from each treatment) and their embryos were categorized and recorded as big, small, aborted or absent (Jersáková & Johnson, 2006) using a stereoscope (Stemi DV4, Carl Zeiss, China). Big embryos were obviously larger because they contained more cells than small embryos. Seed viability was tested through pretreatment by soaking in 5% sodium hypochlorite (w/v) for 2 h and 1% tetrazolium (w/v) (Van Waes & Debergh, 1986; He, 2010). We then observed seeds under a stereoscope (Stemi DV4; Carl Zeiss, China) and only counted seeds in which the embryos (big or small) were stained pink–red. Seeds in which embryos failed to stain were recorded as non-viable.

RESULTS

Floral phenology and lifespan

The flowering period of the population of *C. lichiangense* in 2019 was 67 days (2 May to 7 July). The individual lifespan of flowers that did not receive pollinia on their stigmas was 25.02 ± 8.30 days (mean ± SD, n = 247).

Field observations of pollinators

No insects visited *C. lichiangense* at night, but many insects (Table 2) visited flowers during daylight hours. However, only one hoverfly species (*Ferdinandea cuprea* Scopoli, Syrphidae) carried the orchid's pollinia. Most visits by *F. cuprea* occurred from 12:00–15:00 h (Fig. 2), at which time we also detected floral scents (for more information on floral scents, see below). We recorded 18 specimens of *F. cuprea* visiting flowers of *C. lichiangense*. Four were observed to enter the flower *via* the large dorsal opening and exit via one of the rear apertures carrying pollinia on their thoraces (*e.g.* Fig. 4a,b). One of these flies already carried a pollinium (or pollinia) before entering the flower. Of the remaining 14 flies, three flew around the flowers but did not land; six landed on floral organs but did not enter the dorsal openings on the labellum; two entered the flower through the dorsal openings but crawled out the same way; two entered *via* the dorsal openings and died in the labellum as they attempted to escape *via* the rear exits; and one entered the flower *via* the dorsal opening and escaped *via* one of the rear exits but did not carry a pollinium.

Field observations showed that successful deposition of the pollinium on a specimen of *F. cuprea* required six sequential aspects of fly behaviour: (i) the fly approached the flower with an aerial zig-zag pattern; (ii) it landed on the staminodium, the labellum, dorsal sepal or one of the lateral petals; (iii) after a brief pause it crawled into the labellum through the dorsal

Table 2. Insect visitors recorded on and in Cypripedium lichiangense in situ.

insect taxon	visiting type	length/mm	width/mm	height/mm
Syrphidae				
Ferdinandea cuprea	1/2/3/4	11.96	4.4	3.97
Calliphoridae				
Lucilia bufonivora	1/2	9.53	3.89	3.77
Anthomyiidae				
Scathophaga sp.	1/2	15.07	4.76	4.24
Unidentified 1	1/2	6.86	2.30	2.46
Unidentified 2	1/2	6.94	2.55	2.80
Unidentified 3	1/2	7.17	2.84	2.69
Unidentified 4	1/2	7.03	2.08	2.31
Stratiomyidae				
Ptectitus aurifer	1/2	17.59	4.33	5.38
Stratiomyia apicalis	1/2	10.73	3.49	3.06
Tabanidae				
Unidentified	1/2	17.65	5.12	5.53
Tachinidae				
Dexia ventralis	1/2	9.57	3.63	3.66
Unidentified 1	1/2	16.81	5.99	5.93
Unidentified 2	1/2	11.34	4.62	3.90
Unidentified 3	1/2	8.17	2.88	3.05
Tephritidae				
Unidentified	1/2	6.56	1.97	2.24
Solvidae				
Xylomia sp.	1/2	12.33	2.93	3.82
Sarcophagidae				
Unidentified	1/2/3	8.60	3.11	3.38
Drosophilidae				
Unidentified 1	1/2/3	3.37	1.01	1.45
Unidentified 2	1/2			
Pentatomidae				
Eysacoris guttiger	1/2	5.30	4.20	2.51
Coreidae				
Riptortus pedestris	1/2	15.04	3.46	3.87
Reduviidae				
Haematoloecha nigrorufa	1/2	11.69	3.12	2.31
Cantharidae				
Unidentified	1/2	10.48	3.02	3.21
Unidentified	1/2	7.99	3.92	1.83
Nitidulidae				
Hoptoncus luteolus	1/2/3	2.16	1.78	1.4
Theridiidae				
Unidentified	1/2	–	–	–

Visiting type, '1' insects fly close to *C. lichiangense* but do not contact plants; '2' insects contact *C. lichiangense* but do not enter labellum; '3' insects enter and exit labellum without pollinia; '4' insects enter and exit flower with pollinia.

opening; (iv) it then moved back and forth on the labellum floor; (v) it crawled under the stigma and struggled to crawl upwards and squeeze through one canal of the rear exit apertures; and (vi) upon squeezing out and emerging from a rear aperture, the dorsum of the fly thorax contacted a dehiscent anther and the freed insect flew away immediately, bearing a dorsal deposition of pollinia. The combined procedure lasted a maximum of 1 h 46 min. Flies, once freed, left the study site, so we did not observe any escapee visiting co-blooming flowers *in situ*. We also did not observe a fly directly moving from co-blooming flowers to *C. lichiangense* during the 342-h field observation period.

Brood-site mimesis

According to the morphological characteristics of *F. cuprea* (Huang & Chen, 2012), all eight flies collected, including the two that died in the labellum canals, were female. Fly eggs were found on the rim of the dorsal opening (Fig. 4i), the inside of the labellum (Fig. 4j), on the stigma (Fig. 4k) and on the lower portion of the staminodium where it contacts the labellum (Fig. 4l). We found 29 eggs of the same colour and shape on seven flowers. Among these flowers, we directly witnessed fly visitations to four flowers. In order to better determine whether pollinators laid eggs in flowers, one of the four flowers was chosen for further study. We observed one fly entering a labellum already with an attached pollinium (or pollinia) on its mesonotum. We then sealed the dorsal and rear openings of this flower with a cotton ball and kept the insect in the labellum sac for 5 h. Upon removing the cotton ball, we found six eggs in the labellum (Fig. 4j) with two additional eggs (white arrow in Fig. 4k) and insect hairs (red arrow in Fig. 4k) on the stigma surface. These eggs are morphologically similar to the eggs found in the other flowers examined. Thus, we think all the eggs found in the flowers were from this pollinating fly species.

Floral signalling, microstructure of plants and pollinators

In this *C. lichiangense* population, flowers had liver-coloured sepals and staminodia, whereas corolla segments were yellow with maroon spots (Fig. 1). Based on human vision, the liver-coloured pigmentation of the flowers that were close to the ground appeared to overlap with the woodland detritus, composed primarily of dead leaves of *Pinus armandii* Franch., *P. densata* Mast., *Quercus dolicholepis* A. and *Q. monimotricha* Hand. On the dark green leaves of *C. lichiangense,* randomly distributed liver-coloured glandular hairs, each consisting of 2–5 cells, were observed on the purplish black spots (Fig. 3c,d). On the upper side of the petals, the liver-coloured abaxial pubescence consisted of multicellular trichomes (Fig. 3a), each of which was composed of four to 11 cells. Papillae on lateral petals and labella were maroon in colour (Fig. 3a,b). Whole flowers of *C. lichiangense* produced a strong unpleasant scent, reminiscent of that of decaying plant material. The air in the bottles containing the dorsal sepals for 3 min smelled like rotten fruit. However, lateral petals kept in bottles for 3 min smelled like ungulate dung, indicating the differences in scent between dorsal sepals and lateral petals. We did not find any nectar-like secretion during the floral lifespan.

The SEM images of four *F. cuprea* specimens showed additional, unidentified debris attached to their wings and legs (Fig. 4h). We also found pollen grains of at least three unknown plant species on two fly bodies (Fig. 4d–f) and in one fly's gut (Fig. 4g).

Floral morphometrics and pollinator traits

Floral width of *C. lichiangense* was 45.71 ± 7.55 mm (mean $\pm$ SD, n = 13) and floral height was 83.06 ± 18.64 mm (mean $\pm$ SD, n = 12). Additional morphometrics of the

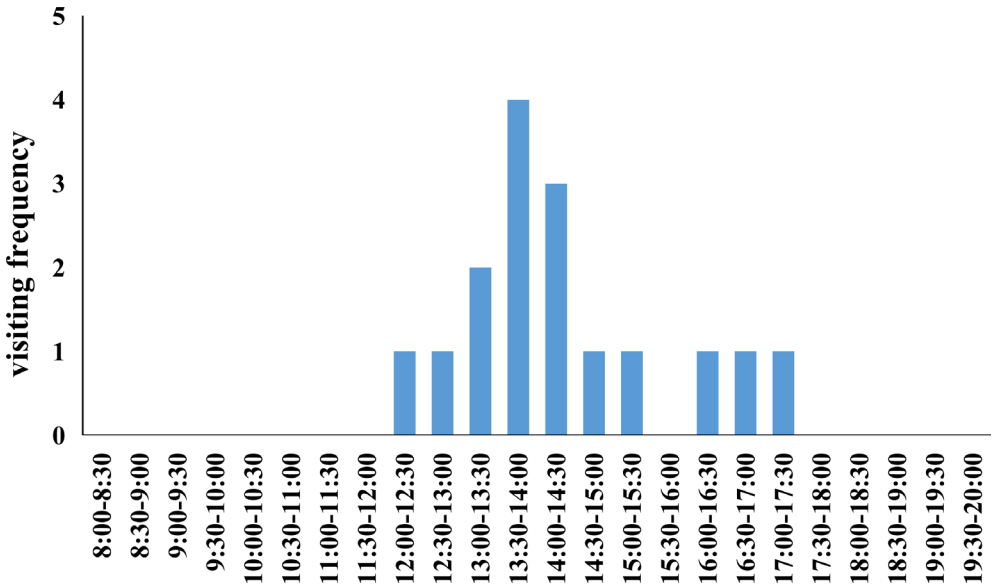

Fig. 2. Visiting frequencies of *Ferdinandea cuprea* to flowers of *Cypripedium lichiangense*.

flowers and the flies are available in Table 3. Comparative analyses of both flowers and flies indicated that pollinator dimensions (n = 7) showed an expected overlap with the floral architecture of *C. lichiangense* (n = 14). The female fly of *F. cuprea* entered the labellum with ease because its physical length and thorax were far smaller than the minimum circumference of the flower dorsal opening (Fig. 1c). The depth between the sinus rim and the floor of the labellum was significantly smaller than the fly length ($t = 6.200$, df = 18, $P < 0.001$), implying that once the fly entered the labellum, it was less likely to escape *via* the same route as it was too long to freely adjust itself to escape from the dorsal opening. There was

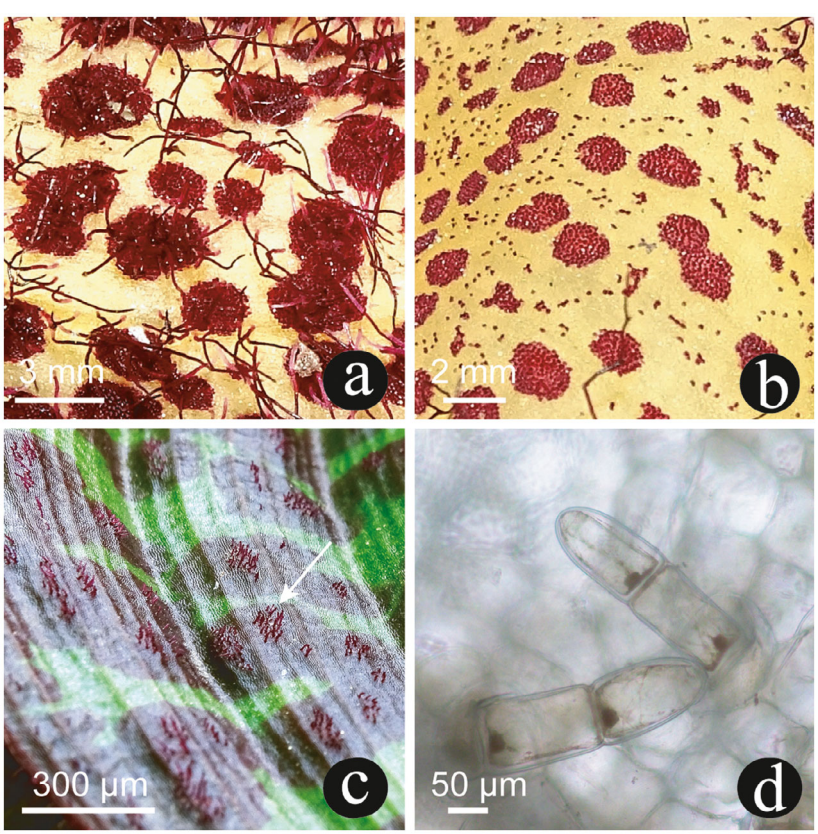

Fig. 3. Flower and leaf microstructure of *Cypripedium lichiangense*. (a) Petal with adaxial pubescence and maroon-coloured papillae. (b) Dorsal surface of labellum with liver-coloured papillae. (c) Section of foliage leaf with purplish black spots and glandular hairs (arrow). d. light microscopy image of glandular hairs on foliage leaf.

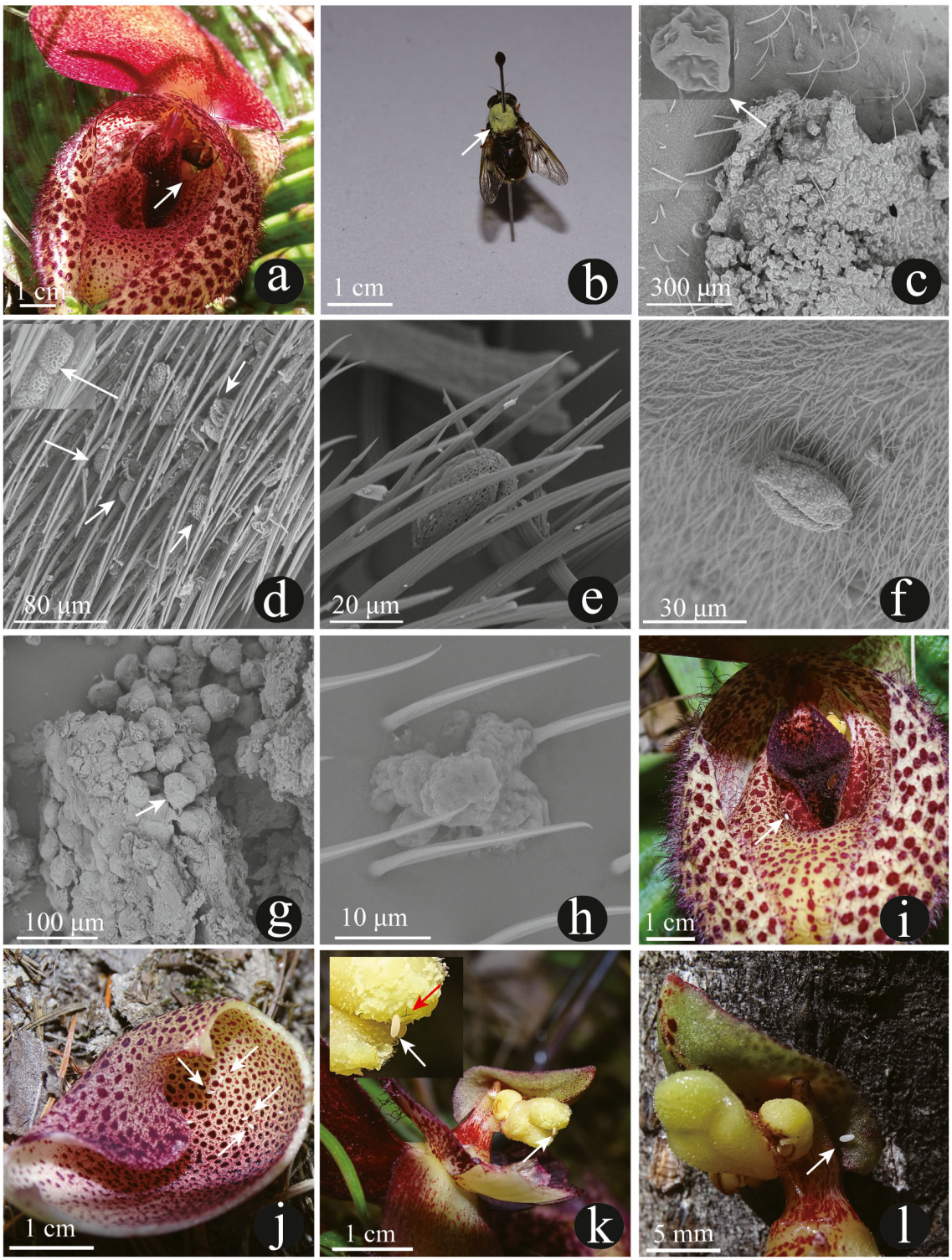

Fig. 4. Pollinators of *Cypripedium lichiangense* and eggs on the flower. (a) Female *Ferdinandea cuprea* squeezing out of rear exit (arrow). (b) Pollinium (arrow) on dorsum of *F. cuprea*. (c) SEM images of pollinium on thorax of *F. cuprea* (cf Fig. 4b. (d, e) SEM of two, unidentified angiosperm (non-orchid) pollen grains on leg of *F. cuprea*. (f) SEM image of third unidentified pollen grain on the thorax of *F. cuprea*. (g) SEM of unidentified pollen in the gut of *F. cuprea*. h. SEM of unidentified debris on wing of *F. cuprea*. (i) Egg on labellum rim (arrow). (j) Eggs in the labellum sac (arrow). (k) Eggs (white arrow) and insect hairs (red arrow) on stigma surface. (l) Basal location of egg on the staminodium of (arrow).

also no significant difference between the thorax and the distance between the receptive surface of the stigma and the labellum floor ($t = -0.550$, $df = 18$, $P > 0.05$). This should maximize contact between pollinia on the pollinator's dorsum and the stigmatic surface. Fly thorax depth was larger than the flower anther and labellum floor ($t = 2.295$, $df = 17$, $P < 0.05$),

suggesting that a fly would contact the dehiscent anther as it attempted to escape from one of the rear floral exits. This exit width did not completely restrict most female syrphids, as its minimum width proved larger than the maximum width of the thorax. However, when only minor variations in fly behaviour and dimensions conflicted with variations in floral dimensions, they were sufficient to decouple pollinia removal and deposition (Table 3). We note that while the dimensions of other visiting insect species matched floral dimensions, they did not enter the labellum or carry the orchid's pollinium (Table 1). Hence, based on comparative analyses of floral morphometrics, visitor body sizes and visiting behaviours, the female fly *F. cuprea* was the only effective pollinator species.

Breeding systems

All flowers of the controls with excised labella failed to set fruit. Fruit set rates of cross-pollinated and self-pollinated plants were significantly higher than for insect-pollinated plants ($P < 0.05$ each, χ^2 test; Table 4).

Table 3. Morphological dimensions (mm) of *Cypripedium lichiangense* and *Ferdinandea cuprea*.

	number	minimum	maximum	mean	±SD
DL1	14	7.77	10.42	8.79	1.04
DL2	14	6.32	9.40	7.88	0.90
OL	13	5.29	10.34	7.28	1.54
SL	12	2.39	6.29	4.17	0.90
AL	12	2.59	5.04	3.41	0.71
EL	12	5.67	10.00	7.58	1.34
Thorax width	7	3.77	4.91	4.40	0.40
Thorax height	7	3.03	4.71	3.97	0.51
Length	7	8.78	14.18	11.96	1.75

DL = Central dorsal opening (DL1 = Length of labellum rim; DL2 = Width of labellum rim); OL = Distance between labellum rim and labellum floor; SL = Height between stigma and base of labellum; AL = Height between anther and labellum floor; EL = Width of rear exit. Thorax and length pertain to the fly.

Table 4. Results of breeding system experiments with *Cypripedium lichiangense*. Seeds containing embryos are large + small embryos.

treatment	number of flowers	number of capsules	fruit set (%)	seeds containing embryos (%)	embryo viability (%)
2019					
Control	12	0	0	0	0
Self-pollinated	110	65	59	59	57
Cross-pollinated	22	16	73	77	86
Naturally pollinated	527	38	7	84	90
2020					
Control	13	0	0	–	–
Self-pollinated	20	15	75	–	–
Cross-pollinated	14	8	57	–	–
Naturally pollinated	524	30	6	–	–

Embryo viability refers to percentage of positive responses to the tetrazolium test.

Fruit set and seed viability

There were no significant differences between fruit set ratios in 2019 and 2020 ($P > 0.05$, χ^2 test) in natural, insect-pollinated flowers. The fruit set ratios in both years for the same population (Table 4) were only 7% in 2019 (n = 527 flowers) and 6% for 2020 (n = 524).

The seed sizes and viability test in breeding systems experiments are given in Table 4. Seeds containing large or small embryos (*i.e.* seeds containing embryos) were dominant in self-pollinated, cross-pollinated and naturally pollinated plants. However, in self-pollinated plants, the number of seeds with aborted/no embryos was significantly higher than in cross- and insect-pollination plants ($P < 0.05$ each, χ^2 test). The seed viability test showed that more seeds tested positive for embryo viability in the cross-pollinated and insect-pollinated plants compared to self-pollinated plants ($P < 0.05$ each, χ^2 test).

DISCUSSION

As control flowers (labellum removed) produced no fruits, we conclude that mechanical self-pollination (autogamy) does not occur in *C. lichiangense* and that pollinators are needed for sexual reproduction. Hand-pollination experiments indicate that *C. lichiangense* lacks obvious pre-zygotic self-incompatibility, as in some other *Cypripedium* species tested previously by Edens-Meier *et al.* (2010). However, our comparative analyses of seed development of hand-mediated pollinations showed that self-pollinations produced more empty seeds and/or abortive embryos. This indicates some degree of inbreeding depression. The tetrazolium test further supports a decrease in reproductive fitness after self-pollination. However, the no-rewarding floral trait and pollinator behaviours (*i.e.* low visit frequency and escape behaviour once squeezed out from a rear aperture) show that the pollination strategy of *C. lichiangense* can effectively avoid entomophilous self-pollination, thus avoiding inbreeding depression. Therefore, this species reduces self-pollination and inbreeding depression during the pollination process. Increased frequencies of non-viable seed following experimental self-pollination has also been shown in other Chinese orchid species, including members of the genera *Calanthe* (Ren *et al.* 2014), *Habenaria* (Tao *et al.* 2018b) and *Spiranthes* (Tao *et al.* 2018a).

The rate of fruit set following natural, insect-mediated pollination in *C. lichiangense* was rather low in both seasons (6–7%), compared to hand-manipulated treatments in cross- (57–73%) and self-pollinated flowers (59–75%). This indicates that the natural fruit set of *C. lichiangense* is pollinator limited. Furthermore, the pollinator limitation is perhaps related to the pollination pattern, as only one species of fly with low visit frequency (see above) was found to be the primary pollinator of *C. lichiangense*. Similarly, low rates of natural fruit set (2.5–7.3%) were reported in the allied species, for example, *C. fargesii* (Ren *et al.* 2011). Actually, low rates of fruit set continue to be documented in many deceptive species in subfamily Cypripedioideae, regardless of genus or section. Seasonal fruit sets may show even lower conversion ratios in other *Cypripedium* species, regardless of pollinator species or taxonomic subsection (0.45–1.30%; see Edens-Meier *et al.* 2014). Orchids mimicking food sources or brood sites are often considered as classic examples of pollinator-limited species (Tremblay *et al.* 2005).

The pollination mechanism: humus-rich oviposition site mimesis

Beyond all doubt, the no-rewarding *C. lichiangense* employs BSM as its pollination strategy, as eggs of female syrphid *F. cuprea* (Fig. 4b), the only pollinator, are frequently found on the flower (see above). Compared with previous studies, the syrphid pollination system in *C. lichiangense* differs from that of *C. subtropicum* (Jiang *et al.* 2020) and *C. fargesii* (Ren *et al.* 2011). Jiang *et al.* (2020) showed that *C. subtropicum* mimics aphid colonies to attract syrphids with entomophagous larvae. *Cypripedium subtropicum* also appears to be the only *Cypripedium* species known to offer an edible reward. In section *Trigonopedia*, *C. fargesii, C. lentiginosum* and *C. lichiangense* do not offer an edible reward, with *C. fargesii* pollinated by syrphids in the genus *Cheilosia*. Adults of *Cheilosia* species appear to feed on fungal spores and oviposit in fungus-infected foliage. Ren *et al.* (2011) suggested that leaf trichome morphology and pigmentation patterns in *C. fargesii* were also part of the presentation pattern attracting flies to the flowers. In fact, mottled, hairy leaves may offer cues in other Asian *Cypripedium* species that are pollinated by flies of several different families, which usually lay their eggs in diseased and/or decaying plant material, *i.e.* fungus-infected, humus-rich vegetation (*e.g.* Zhang *et al.* 2020). The floral attractants (including flowers and leaves), especially the pigmentation patterns on leaves (Fig. 3) of *C. lichiangense* are similar to those of *C. fargesii* (Ren *et al.* 2011, see above) and establish a connection between the living plant and fungus-infected vegetation; however, we propose a different pollination mechanism, *i.e.* humus-rich oviposition site mimesis, employed by *C. lichiangense*. Besides the floral attractants, the lifestyle of the pollinator also supports to this hypothesis.

In the family Syrphidae, many species consume nectar and pollen as winged adults (Huang & Chen 2012), contributing to pollination on a global scale (Proctor *et al.* 1996). This includes syrphid pollination in some members of the Orchidaceae. Besides the feeding habits of adults, other life habits of syrphids may also be utilized by plant species. For example, different species in the Syrphidae oviposit in different environments following the eating patterns of their larvae. Some maggots are exclusively mycophagic, while others are phytophagic, saprophagic or entomophagic (Rotheray & Gilbert, 1999). Therefore, there are diverse rewards and deception in orchid species pollinated exclusively by syrphids. Some of these orchids offer only nectar (*e.g. Prasophyllum* see Bernhardt & Burns-Balogh, 1986; Kuiter, 2016), some attract pollen-foraging syrphids by mimicking pollen (*e.g.* Pansarin, 2008), others combine edible rewards with BSM, *e.g. Cypripedium subtropicum* S.C. Chen & K.Y. Lang (Jiang *et al.* 2020) and some *Epipactis* species (Jin *et al.* 2014). Among Cypripedioideae, BSM is far more common in *Paphiopedilum* than in other genera (Table 1). In these orchids, ornamentation of staminodia mimics colonies of the prey insects, aphids (Atwood, 1985; Shi *et al.* 2009; Pemberton, 2013; Edens-Meier *et al.* 2014; Tang *et al.* 2014; Ma, 2015). Moreover, maggots in subfamily Syrphinae mainly feed on aphids (Rotheray & Gilbert, 1999; Skevington & Yeates, 2000; Stahls *et al.* 2010; Huang & Chen, 2012; Young *et al.* 2016). These syrphids respond to a specialized suite of visual (*i.e.* colour and shape) and/or olfactory signals, often resulting in oviposition on ornamented staminodia (Shi *et al.* 2009; Bänziger *et al.* 2012; Pemberton, 2013; Jin *et al.* 2014; Ma, 2015). In contrast, our *F. cuprea* is in subfamily Eristalinae; their larvae are more likely to consume decaying vegetable matter. Rotheray & Gilbert (1999) found that *F. cuprea* looks for humus-rich oviposition sites, such as diseased trees exuding sap and wet fungal decay in infected plant roots. We speculate that the unidentified debris attached to the wings and legs of the pollinators (Fig. 4h) is humus fragments derived from real oviposition sites. In short, based on the behavioural traits for choosing oviposition sites of the *Ferdinandea* hoverfly, we proposed a new BSM, humus-rich oviposition site mimesis responding to the floral attractants found in *C. lichiangense*.

Although additional pollen morphotypes were found on the primary pollinators (Fig. 4d–f) and in their digestive systems (Fig. 4g), we do not believe that *C. lichiangense* benefits from sympatric, co-blooming species. One reason is that *C. lichiangense* does not mimic the pigmentation patterns and scents of co-flowering plants. Second, we have not directly observed that *F. cuprea* interrupts the egg-laying bouts to visit co-flowers in the vicinity. Finally, all or nearly all adult syrphid feeding habits and their larvae feeding habits differ (Rotheray & Gilbert, 1999), thus the pollen-eating habit and oviposition site choice for winged *F. cuprea* adults are not correlated.

The discovery of this BSM related to an environment with decaying material employed by *C. lichiangense* to attract *F. cuprea* (Syrphidae) as pollinators indicates that similar strategies may also be utilized by other flowering plant species to attract other flies as pollinators. Theses flies, *e.g.* Calliphoridae, Sarcophagidae and Muscidae, are generally associated with sapromyophilous plant species mimicking their humus-rich brood sites (Pansarin & Pansarin, 2013).

Characteristics of humus-rich oviposition site mimesis

Floral phenology and individual floral lifespan among *Cypripedium* species in different sections are highly variable according to the studies published to date (see Table 5). Within section *Trigonopedia*, the long flowering period of a population of *C. lichiangense* (67 days) and the long floral lifespan of individual flowers (25.02 ± 8.30 days) may be selectively advantageous when pollinators are infrequent and/or fail to respond consistently to floral attractants, adapting to unfavourable pollination conditions (Primack, 1985). The floral phenology and individual floral lifespan of *C. lichiangense* is obviously longer compared to other species in the same genus (Table 5). In contrast, *Cypripedium* species with food deception, mimicking the generalized colours (*e.g.* yellow and white) and scents of nectar and/or pollen producers, usually have shorter flowering periods in their populations and/or in their individual flowers. For example, the food deceptive *Cypripedium* species include *C. henryi* Rolfe (Li *et al.* 2008a), *C. macranthos* Swartz (Sugiura *et al.* 2001; Zhang *et al.* 2014) and *C. plectrochilum* Franch. (Li *et al.* 2008b). On the one hand, all of them have relatively short blooming periods; on the other hand, the fruit set of these slipper orchids is generally higher than that of *C. lichiangense*. Considering the similar breeding systems among these, we believe that the lower fruit set of *C. lichiangense* indicates weak attraction to pollinators. Therefore, we suggest that the long flowering period of *C. lichiangense*, with the generalized humus-rich oviposition site mimicry, could be considered as an adaptation to the weak attraction of the flower.

Table 5. Comparative floral phenology and individual flower lifespan among *Cypripedium* species.

species	individual lifespan	flowering period	reference
Section *Trigonopedia*			
Cypripedium lichiangense	25 ± 8.3 (n = 247)	67 days	This study
C. lentiginosum	~1 month	–	Liu *et al*. (2008)
C. fargesii	16.1 ± 4.53 (n = 20)	25–30 days	Ren (2010)
Section *Sinopedilum*			
C. bardolphianum	23.923 ± 0.828 (n = 10)	45 days	Zheng *et al*. (2010)
Section *Subtropica*			
C. wardii	6.86 ± 3.26 (n = 158)	–	Zheng *et al*. (2021)
Section *Arietinum*			
C. plectrochilum	10.9 ± 4.518 (n = 10)	4–5 weeks	Li (2006)
C. arietinum (=*C. plectrochilum)*	1 week	mid-May to early June	Wolfe *et al*. (2009)
Section *Bifolia*			
C. guttatum	~2 weeks	–	Bänziger *et al*. (2005)
Section *Acaulia*			
C. acaule	3 weeks	Late May to mid-June	O'Connell & Johnston (1998)
Section *Enantiopedium*			
C. fasciculatum	Several weeks	–	Lipow *et al*. (2002)
Section *Obtusipetala*			
C. flavum	21.96 ± 2.736 (n = 10)	–	Zheng *et al*. (2010)
	~3 weeks	–	Bänziger *et al*. (2008)
Section *Flabellinervia*			
C. japonicum	14.12 (12–16)	25 days	Liu *et al*. (2013)
	3 weeks	3–4 weeks	Sun *et al*. (2009)
Section *Cypripedium*			
C. parviflorum	5–23	–	Light & MacConaill (2002)
C. candidum	10–14	–	Pearn (2013)
C. henryi	12.1 ± 2.688 (n = 10)	4–5 weeks	Li (2006)
C. tibeticum	22. 944 ± 2. 818 (n = 10)	50 days	Li (2006)
C. macranthos	7.92 (n = 434)	–	Sugiura *et al*. (2001)
	9.42 ± 1.81 (n = 36)	~20 days	Zhang *et al*. (2014)
C. yunnanense	~3 weeks	–	Bänziger *et al*. (2008)

Allied pollination systems in section Trigonopedia and Paphiopedilum

In section *Trigonopedia*, all species have similar pigmentation patterns on leaves and flowers (Chen & Cribb, 2009) and the larvae of pollinators for studied species are saprophagic and/or mycophagic rather than entomophagic. BSM in *C. lichiangense* overlaps best with floral presentation in *C. lentiginosum*. As mentioned above, neither species offers edible rewards. Both species share the mottled leaf characteristic of section *Trigonopedia*, with dark spotted papillae on floral organs and emission of unpleasant odours. *Cypripedium lentiginosum* is also pollinated by a single species of female fly, *F. formosana,* in the same genus *Ferdinandea*, which lays its eggs in rotting wood. Both orchid species should be interpreted as brood-site mimics, attracting pollinators searching for sites where larvae can feed on decaying vegetation. The pollination system of *C. fargesii* also attracts a syrphid, *Cheilosia lucida* Barkalov et Cheng (Ren *et al*. 2011), in the same sub-family as *F.* cuprea, but adults of *C. lucida* species appears to be mycophagous. Germinating spores were found attached to pollinator specimens (Fig. 1F in Ren *et al*. 2011), and the leaves and floral organs of *C. fargesii* may also mimic fungus-infected tissues. The scents produced by *C. fargesii* included some compounds associated with fungi; however, the feeding habits of some larvae in genus *Cheilosia* are mycophagous, either feeding on fungi and their breakdown products in pockets of decay in live or dead plants or feeding on the fruiting bodies of macro-fungi (Rotheray & Gilbert, 1999), and the pollinator photographed by Ren *et al*. (2011) was female. In contrast, Li (2006) described pollination of *C. sichuanense* by a flesh fly (*Scathophaga*, Scathophagidae). This fly was also female (Fig. 1D in Li *et al*. 2012), and maggots of most *Scathophaga* spp. are saprophagous (Xue & Zhao, 1996). Floral scents secreted by *C. sichuanense* are associated with decaying tissue (Li, 2006). Li (2006) also observed an ant carrying out a white egg-like structure from the labellum, implying possible BSM. Therefore, members of section *Trigonopedia* may all favour pollination by BSM attracting flies, whose larvae are associated with saprophagy and/or mycophagy. Further work is needed to confirm this hypothesis and to compare the floral presentation of allied species. Compared with aphid colony mimesis, as reported in *Paphiopedilum* (see above), the discovery of humus-rich oviposition site mimicry in *C. lichiangense* and the previous description in *C. lentiginosum* indicate a new BSM pollination strategy to exploit another subfamily within the Syrphidae. However, flies with saprophagous larvae in the genus *Eumerus* (Eristalinae, subfamily of Syrphidae) are also pollinators of *Paphiopedilum bellatulum* (Rchb.f) Stein, *P. concolor* (Lindle. Ex Bateman) Pfitzer and *P. godefroyae* (God.-Leb.) Stein (Bänziger *et al*. 2012). Therefore, the

humus-rich oviposition site mimicry strategy may have evolved twice in subfamily Cypripedioideae.

CONCLUSION

Based on field observations, floral *versus* pollinator traits and breeding systems, we propose a new BSM strategy – humus-rich oviposition sites mimesis – for *C. lichiangense*. Egg laying females of *F. cuprea* (Eristalinae; Syrphidae) were the only dispersal agents of pollinia. Moreover. the long flowering period and floral lifespan of this orchid are infrequent in *Cypripedium*, which may be an adaptive floral trait to weak floral attraction. *Cypripedium lichiangense* becomes the third species within section *Trigonopedia* pollinated by flies typically associated with decaying, often fungus-infected, plant tissues. As in the majority of *Cypripedium* species studied to date, *C. lichiangense* is self-compatible but reproductive fitness is reduced with hand-manipulated self-pollination.

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