Correction

EVOLUTION

Correction for "Flowers of *Cypripedium fargesii* (Orchidaceae) fool flat-footed flies (Platypezidae) by faking fungus-infected foliage," by Zong-Xin Ren, De-Zhu Li, Peter Bernhardt, and Hong Wang, which appeared in issue 18, May 3, 2011, of *Proc Natl Acad Sci USA* (108:7478–7480, first published April 18, 2011; 10.1073/pnas.1103384108).

The authors note that, due to misidentification by the entomologist, the flat-footed fly (*Agathomyia* sp.) is dropped and reidentified as *Cheilosia lucida* Barkalov et Cheng (Diptera, Syrphidae) by K. K. Huo of Shaanxi University of Technology. The genus *Cheilosia* includes some fungal feeders (1). Due to this error, we cannot prove that flat-footed flies (Platypezidae) are pollen vectors of an angiosperm species. Nevertheless, the specimens of *Cheilosia lucida* carried conidia of *Cladosporium*, thus this misidentification does not affect the main conclusions or interpretations in this article.

The authors thank Peter Chandler for uncovering this error, as well as K. K. Huo of Shaanxi University of Technology and C. D. Zhu of the Institute of Zoology, Chinese Academy of Sciences for reidentification of the insects.

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Flowers of *Cypripedium fargesii* (Orchidaceae) fool flat-footed flies (Platypezidae) by faking fungus-infected foliage

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Charles Darwin was fascinated by the orchid–pollinator interactions, but he did not realize that many orchid species are pollinated by deceit. *Cypripedium*, a model lineage of nonrewarding orchid flowers, is pollinated primarily by bees. Here we present both an example of floral mimesis of fungus-infected foliage in orchids and an example of flat-footed flies (*Agathomyia* sp.; Platypezidae) as pollen vectors for angiosperms. *Cypripedium fargesii* is a nectarless, terrestrial, endangered orchid from southwestern China that requires cross-pollination to produce the maximum number of viable embryos. All insects caught entering or leaving the labellum sac were *Agathomyia* sp. carrying conidia of *Cladosporium* sp. on their mouthparts and legs, suggesting mycophagy. Blackish hairy spots on the upper surface of foliage may imitate black mold spots, serving as short-term visual lures. Some odor molecules also associated with *Cladosporium* cultures were isolated in the floral scent. Mimesis of fungus-infected foliage probably represents an overlooked but important option in angiosperm diversification, because there are three to five more *Cypripedium* spp. in southwestern China with the same mode of floral presentation and black-spotted hairy leaves.

deceptive pollination | mimicry

Charles Darwin described and identified most of the functional floral morphology and biomechanics in orchid pollination without ever recognizing that many of the flowers that he examined lacked edible rewards (e.g., nectar, granular pollen) (1). In fact, floral evolution in the Orchidaceae appears to be dominated by modes of pollination by deceit (2), in which visual and/or olfactory cues mimic food sources, bodies of receptive females, and oviposition sites. Variation in floral mimesis appears to drive speciation in many lineages within the Orchidaceae (3–5).

The genus *Cypripedium* is regarded as a model lineage of food-deceptive orchids restricted to temperate regions of the Northern Hemisphere (6, 7). No *Cypripedium* flower studied to date has been found to secrete nectar or to offer accessible, granular pollen. However, observers continue to record insects entering their inflated labellum sacs (6), with the exception of three autonomously self-pollinating species (8, 9). The flower often produces a discernible and pleasant aroma, whereas the labellum is often a vivid and contrasting color from that of the broad, solitary staminode and the other five perianth segments. Insects that pollinate these flowers do not escape from the sac until they pass under the receptive stigma and then out through one of two rear apertures. This escape pathway forces the insect to contact one of two dehiscent anthers fixing either sticky, amorphous lumps of pollen or whole pollinia to the dorsum of the insect's thorax or head. With a few important exceptions (10, 11), bees of various sizes dominate the pollination of *Cypripedium* spp. (6, 7, 12, 13).

The biodiversity hotspot of the mountains of southwestern China is the center of diversity for this genus (8). It is here where we find most of the small, single-flowered, geoflorous, dark dull-colored, unpleasantly scented species bearing labellum 1sculptures that resemble the surfaces of fungal sporocarps (14, 15). Vogel suggested that these species are pollinated by small flies, particularly fungus gnats (Sciaridae and Mycetophilidae) (13–16), but he was unable to prove it during his lifetime. Pollination by fungus gnats has been shown in some genera of monandrous orchids (4, 17), although some of these flowers mimic the bodies of female gnats, not mushrooms (2).

Here we focus on *Cypripedium fargesii*, a critically endangered species endemic to southwestern China (18), to elucidate its mechanism of floral mimesis and to interpret the function of black spots on its leaves. In this species, each short flowering stem bears two leaves with rows of black spots on the upper surfaces (Fig. 1 *A* and *B*). The stem terminates in a small, solitary, dark-red to dull-yellow flower that produces a faint but unpleasant odor reminiscent of rotting leaves. We examined flower and leaf traits and conducted hand-pollination experiments to identify the breeding system. We tested embryo viability using a modified tetrazolium method (19). Pollinators were observed, captured by clogging floral apertures, and identified (20). Floral scent was collected outdoors by dynamic headspace adsorption methods and analyzed by GC-MS.

Results

The hand-pollination experiments showed that bagged controls never set fruit, but *C. fargesii* was self-compatible (Fig. 2), with fruit set rates for hand-mediated self- and cross-pollination of 80.0% and 77.3%, respectively, with no significant difference between treatments ($P > 0.05$, χ^2 test). The seed viability tests showed that cross-pollinated flowers produced significantly higher proportions of viable embryos compared with self-pollinated flowers ($t = 21.231$; df = 6; $P < 0.001$). Fruit set in flowers exposed to insects was low over four seasons (7.3%, 2.5%, 5.9%, and 3.3%, respectively; Fig. 2). This suggests that this population of *C. fargesii* is pollinator-limited, like most *Cypripedium* spp. (6).

As predicted by such low rates of insect-mediated fruit set, we saw few insects entering or escaping from these flowers despite 70 daytime and 15 nighttime h of observation at the field site. We collected and euthanized only three female and two male specimens in the orchids' labella or in the process of escaping from the interior of a labellum over a 4-y period. All specimens were flat-footed flies (*Agathomyia* sp., Platypezidae) (Fig. 1*E*). Specimen identification was based on morphology and confirmed by DNA barcoding. We observed that these flies entered and escaped from the labellum sac following the same

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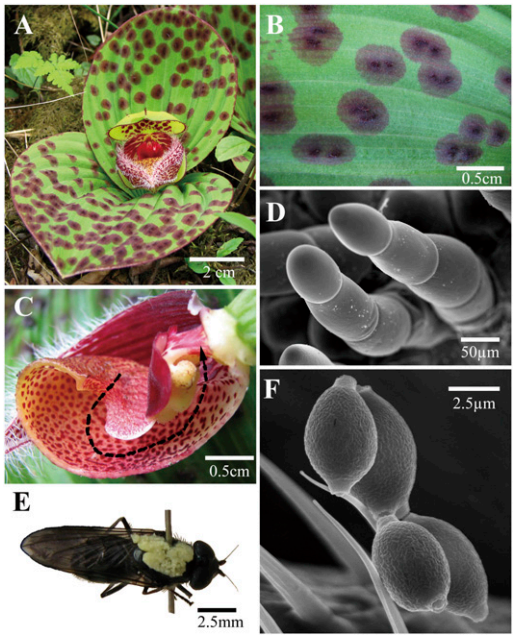

Fig. 1. A mimicry pollination system in *C. fargesii*. (*A*) Individual plant. (*B*) Upper surface of the leaf with black spots and trichomes. (*C*) Dissected flower of *C. fargesii* with half of the labellum removed. The dotted line indicates the pollination route and direction of fly movement. (*D*) SEM image of muticell trichomes at the center of the black spots. (*E*) A female *Agathomyia* sp. adult caught in the site with a pollinium on its thorax. (*F*) SEM image of conidia of *Cladosporium* sp. found on the bodies of *Agathomyia* sp.

interior one-way path (Fig. 1*C*) as described for the flowers of all other *Cypripedium* spp. When a fly escaped from a rear aperture of a *C. fargesii* flower, it contacted a dehiscent anther depositing the entire contents of the sacs on the dorsum of the fly's thorax as a soft but entire pollinium (Fig. 1*E*), as in *C. plectrochilum* (21).

The feeding habits of Platypezidae flies are poorly understood, but the females of some species congregate on the basidiocarps of polypores to lay eggs, and their larvae develop in the basidiocarp (22). To determine whether flat-footed flies visited flowers and/or fungi before they entered the orchid, we examined four specimens by SEM. Pollen grains resembling *Ilex* and three other unidentified eudicots were found on only one wing, one foreleg, and the abdomen of only one fly, but all flies carried germinating spores on their heads, mouthparts, tarsi, and pulvilli. Fungi found on the mouthparts and tarsi were identified as mycelia and germinating conidia of *Cladosporium* spp. (Davidiellaceae, Ascomycota; Fig. 1*F*). We presume that these flies feed on spores and/or exudates of *Cladosporium* spp. and/or the deliquescent exudates of plants infected with this fungus. When *Cladosporium* infects leaves and fruits, it produces black mold spots on the surface of its hosts (23, 24). It has been suggested that some Platypezidae flies forage at such infection sites, consuming spores and/or honeydew (22, 25).

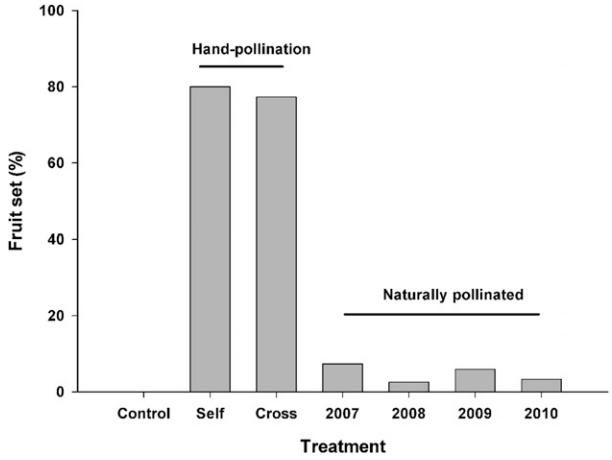

Fig. 2. Effects of pollination treatments on fruit set of *C. fargesii*.

Consequently, if the orchid flower mimics a black mold, it should smell like the fungus and/or the infected plant tissue, because some infectious ascomycetes "aromatize" their hosts (24). The flowers produced 50 scent molecules associated with a wide variety of flowers, leaves, and fruits, but 3-methyl-1-butanol (2.8%), 2-ethyl-1-hexanol (5.73–7.0%), and 1-hexanol (1.1%) were previously detected in *Cladosporium* cultures (26, 27). Previous attempts to analyze the floral odors in other *Cypripedium* spp. failed to detect 3-methyl-1-butanol and 2-ethyl-1-hexanol (28).

Discussion

We propose a previously uncharacterized deceptive pollination system for *C. fargessi*. The flowers mimic fungus-infected plant organs instead of discrete, fleshy sporocarps, which explains why the flowers were visited by both male and female *Agathomyia* spp. To our knowledge, this is the first recorded example of any member of the Platypezidae pollinating an orchid or any other angiosperm species (29). Floral mimesis of infected plant tissue provides a deceptive mode as distinct as the recently discovered rendezvous attraction (17) and alarm pheromone mimesis (30), which also are based in part on modified scent components.

When the fly shown in Fig. 1*E* escaped from the flower, it rested on one of the spotted leaves until it was caught. We examined these regimented spots and found dark pigmentation in both the epidermis and the dense clusters of multicellular trichomes in the center of each epidermal spot. These trichomes did not form swollen, multicellular heads (hammer glands) found in most *Cypripedium* spp. (31). Under SEM, these trichome tufts were somewhat reminiscent of conidia (Fig. 1*D*). We suspect that these leaf spots mimic black mold spots and serve as a broader but short-term visual cue as the floral scent trail lures the fly to the flower. So far, *C. fargesii* is one of only three to five Chinese species identified with such spots (e.g., *C. lichiangense* S. C. Chen & Cribb and *C. margaritaceum* Franch.). Floral size, presentation, and pigmentation patterns in these two species parallel those seen in *C. fargesii* (8), suggesting that flat-footed flies may pollinate more than one orchid species in China.

The Kingdoms Plantae and Fungi show convergent evolution. *Monilinia* spp. (Ascomycota) are plant pathogens that disperse their conidia by attracting pollinating insects to infection sites with flower-like patterns, pleasant scents, and a sugary reward (32). Conversely, *C. fargessi* recruits mycophagous insects to disperse its pollen with fungus-like patterns, unpleasant scents, and no reward.

Materials and Methods

All field observations and manipulative experiments were carried out over four consecutive summers (2007–2010) on Yaoshan Mountain, Qiaojia, in the province of Yunnan in southwest China, on a population of ~100 flowering stems distributed across a limestone mountain dominated by secondary deciduous broad-leaved and bamboo mixed forest at elevations of 2800–2900 m. To determine whether flowers of *C. fargesii* required vector-mediated pollination to produce fruit and seeds, following Dafni et al. (33), we placed flowering stems under nylon bags before the buds opened and divided them into four categories: (*i*) controls, which remained under bags and were never manipulated further or exposed to insects; (*ii*) self-pollinated flowers, which remained under bags but were hand-pollinated with the

contents of their own anthers; (*iii*) cross-pollinated flowers, which remained under bags but received pollinia from another individual located >5 m away; and (*iv*) naturally pollinated flowers, which were exposed to insects throughout the floral lifespan. Dehiscent fruits were counted and collected in mid-October. To test seed viability, we removed the seeds in each capsule within the first three categories and tested embryos using a modified tetrazolium method. The seeds were placed in 5% $Ca(OCl)_2$ (W/V) + 1% Tween-80 (V/V) for 15 h before 2, 3, 5-triphenyltetrazolium chloride testing. The development of embryos of self- and cross-pollinated seeds was checked under a dissecting microscope.

Plant materials were fixed in formalin-aceto-alcohol, dehydrated through a graded ethanol–isoamyl acetate, and then critical- point dried. Dried plant specimens and the bodies of flies were coated with gold palladium and viewed with a Hitachi S-4800 scanning electron microscope at 10.0 kV.

Insect pollinators were collected and identified by morphological characters and confirmed with DNA barcoding with cytochrome *c* oxidase I. The aperture clogging method was used to catch trapped insects within the labellum (20). Vouchers were deposited in the Zoological Museum of Institute of Zoology, Chinese Academy of Sciences, Beijing, and Kunming Institute of Botany, Chinese Academy of Sciences, Kunming.

Floral scent was collected outdoors by dynamic headspace adsorption methods (34). A single newly opened flower was enclosed in a polyethylene terepthalate cooking bag (~25 × 38 cm; Sainsbury's Supermarkets). Two holes were cut at opposite ends of the bag; one hole was fitted with an activated carbon filter (Supelco) for air intake, and the other was fitted with a Super-Q volatile collection trap (Analytical Research Systems), containing 30 mg of Alltech Super-Q adsorbent material. Each flower was enclosed for ~2 h, after which flower headspace was sampled with two micropumps driven by a portable battery. Constant airflow was adjusted to ~100 mL/min using a flow meter. Sampling periods were 3–4 h. Empty cooking bags placed in close proximity to the flower were sampled as controls. After fragrance sampling, adsorbed volatiles were eluted from the Super-Q with 1.5 mL of dichloromethane (Uvasol; Merck). Samples were sealed in glass vials and stored at −20 °C.

The volatiles were analyzed on a Hewlett-Packard 6890 Series GC System coupled to a Hewlett-Packard 5973 Mass Selective Detector using an Agilent 7683 Series Automatic Liquid Sampler. An HP-5MS column (5% phenylmethylpolysiloxane; 60 m long, 0.32 mm inner diameter, 0.25 μm film thickness; Agilent) was used for analyses. Electronic flow control was used to maintain a constant helium gas flow of 1.4 mL/min. The GC oven temperature began at 50 °C and was increased by 5 °C/min to 100 °C and held for 10 min, then increased by 5 °C/min to 280 °C and held for 5 min. The MS interface was 280 °C, and the ion trap was activated at 150 °C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 per scan from *m/z* 35–550. Component identification was carried out using the Wiley NIST 05 mass spectral database and Wiley 7.

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