

## Deceptive pollination of the Lady's Slipper *Cypripedium tibeticum* (Orchidaceae)

P. Li<sup>1,4</sup>, Y. B. Luo<sup>1</sup>, P. Bernhardt<sup>2</sup>, X. Q. Yang<sup>1,4</sup>, and Y. Kou<sup>3</sup>

<sup>1</sup>State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, China

<sup>2</sup>Department of Biology, St. Louis University, St. Louis, Missouri, U.S.A.

<sup>3</sup>Huanglong Administration of National Scenic Spots, Sichuan, China

<sup>4</sup>Graduate School of the Chinese Academy of Sciences, Beijing, China

Received November 15, 2005; accepted April 3, 2006

Published online: October 24, 2006

© Springer-Verlag 2006

**Abstract.** To test whether the nectarless flowers of *Cypripedium tibeticum* attract pollinators through mimicry like the allied species *C. macranthos* var. *rebunense*, pollination biology of *C. tibeticum* was investigated in western China. Although *C. tibeticum* was also pollinated by bumble bee queens, i.e. *Bombus lepidus*, *B. lucorum* and *B. hypnorum*, no special, rewarding model plants were found in the habitat. Field experimentation confirmed that the flowers were self-compatible but insects were required to transfer orchid pollen to the stigma. Both *Bombus* queens and workers were visitors, but queens were much more frequent than workers and only queens were effective pollinators. Floral functional morphology analysis showed that it was large queens rather than small workers that fitted well with the flowers of *C. tibeticum*. With the faint sweet-fruity scent, the minor floral fragrance compound, ethyl acetate, probably plays a role in attracting bumble bees by food deception. The dark flowers with the inflated, trap-like labellum are hypothesized to mimic the nest site of queens. Therefore, bumble bee queens tend to be attracted by *C. tibeticum* through nest site mimic combined with food deception. Considering that the co-blooming flowers of *C. flavum* are pollinated by the *Bombus* workers, and *C. smithii* pollinated by a

queen, we suggest that using the same bumblebees with different body sizes as the pollinators is the main reproductive isolation between interfertile *C. tibeticum* and *C. flavum*, while *C. tibeticum* and *C. smithii* tend to hybridize naturally.

**Key words:** *Bombus*, *Cypripedium tibeticum*, food deception, nest site mimic, functional morphology, reproductive isolation.

It is still an enigma in pollination biology that one-third of orchid species are estimated to be deceptive (Dafni 1984, Nilsson 1992, Schiestl 2005, Cozzolino and Widmer 2005). These orchids rely on two main kinds of deception to attract pollinators, including generalized resemblance to food-sources (Nilsson 1983a, 1992; Dafni 1984), and specific resemblance to other rewarding flowers (Dafni 1981, 1987; Nilsson 1983b; Johnson 1994, 2000). The former is assumed to be common among orchids (Dafni 1984), but the latter has been demonstrated rarely (Schiestl 2005).

The subfamily Cypripedioideae is a group of well-known deceptive orchids, and its

flowers are one-way trap flowers, with entrance into the labellum easy from the front, but exit much easier to the rear, where the insect must first pass beneath the stigma and then an anther (Dressler 1993). Most of the reported *Cypripedium* species attract pollinators through deception without special model plant (Stoutamire 1967, Nilsson 1979, Davis 1986, Catling and Catling 1991, Van der Cingel 2001, Bänziger et al. 2005). In *C. calceolus* L., the flowers attract pollinators through general food deception (odor components, color, false nectar guides), as well as nest site resemblance (odor and cavity) and with scent-mark resemblance (odor) (Nilsson 1979). In the recently studied *C. guttatum* Sw., the flowers evidently exploit innate susceptibilities of the pollinator *Halictid* bees (Bänziger et al. 2005). Up to now, however, only in *C. macranthos* Sw. var. *rebunense* (Kudo) Miyabe et Kudo, a specific model plant was identified. Sugiura et al. (2001, 2002) suggested it mimicked the co-blooming *Pedicularis schistostegia* Vved. because their flower color could be perceived similar within the range of a bumble bee's visual spectrum, and both species overlapped in spatial distribution and flowering time.

*Cypripedium tibeticum* King ex Rolfe is thought to be an ally of larger-flowered forms of *C. macranthos*, but can be distinguished from *C. macranthos* by its flower, which is usually "larger and darker maroon with boldly striped sepals and petals, broader 9–13 veined petals and labellum that has a characteristically corrugated surface" (Cribb 1997). In this study, we investigated the breeding system and pollination ecology of *C. tibeticum* and analyzed the floral size, color, and scent. The aim of the present contribution is to test whether a floral mimicry mechanism exists in other *Cypripedium* species allied to *C. macranthos* var. *rebunense*, and to highlight the sophisticated pollination mechanism of this group.

### Material and methods

Studies were conducted in the Huanglong valley, Huanglong Nature Reserve, Sichuan, China, 2004

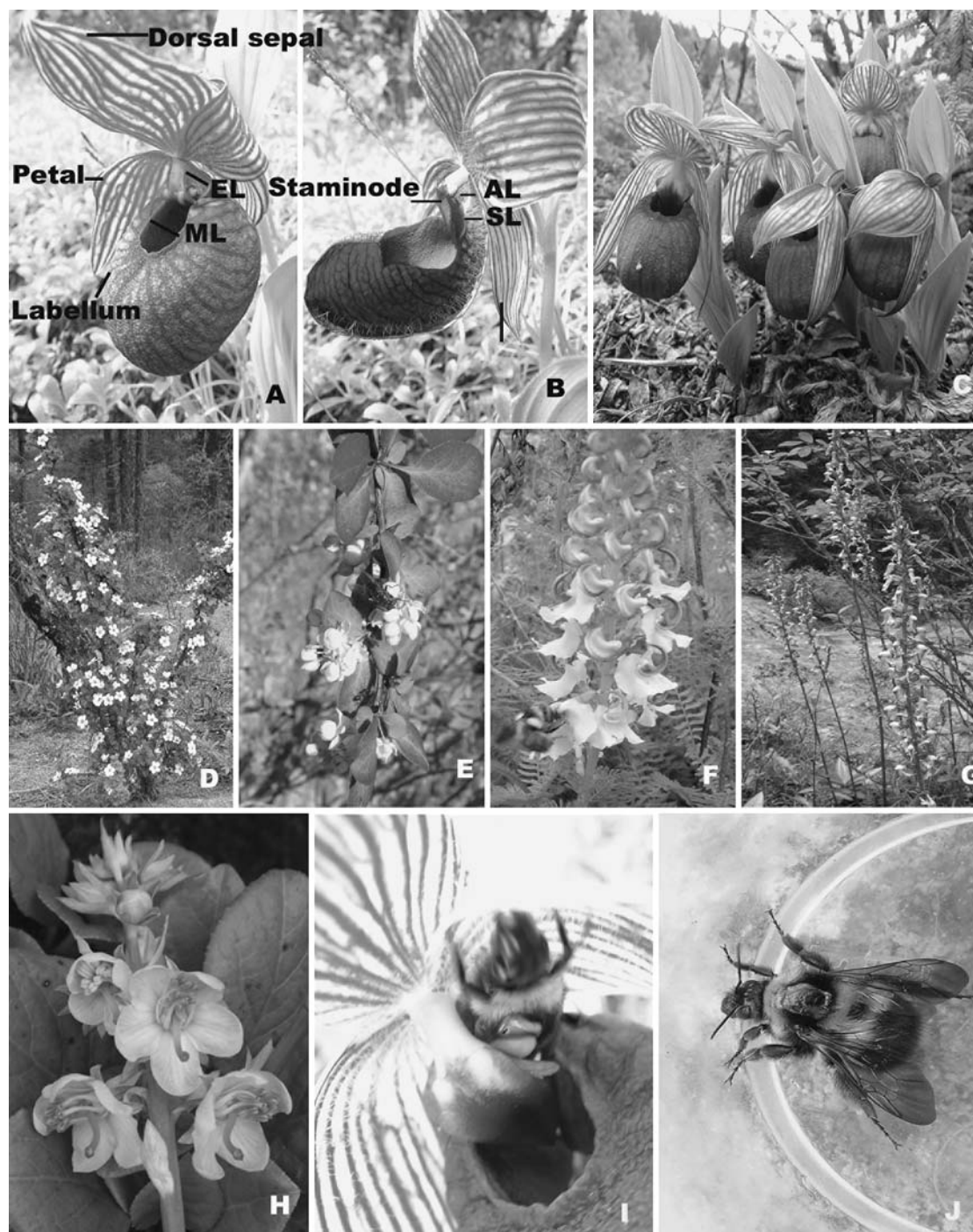
and 2005. Huanglong valley, 3.6 Km long, ranged from 3100 m to 3500 m elevation, contains the largest travertine formation of its kind in the world. It has a typical plateau temperate sub-frigid monsoon climate with a mean 758.9 mm rainfall per year, and the forest is mainly coniferous and mixed coniferous and broadleaf (Li et al. 2005a).

Thousands of *Cypripedium tibeticum* flowers thrive in the Huanglong valley (Li et al. 2005b). The plant has only one nodding flower with purple or dark maroon color (Fig. 1A, B and C). The sepals and petals have darker marked veins. The dorsal sepal and synsepal are ovate-elliptic. The petals are incurved and pubescent at the base of the inner surface. The labellum is usually darker with a white-margined rim to the mouth, and deeply ventricose with the outer surface corrugated. The column is short and the staminode is subsessile, somewhat concave at the blunt tip. The stigma is oblong and its surface is retusely grooved with obviously papillae. The pollen masses are yellow and sticky.

**Floral phenology.** Twenty flower buds of *C. tibeticum* were randomly chosen and marked. Each plant was visited daily and the date of flower opening and wilting was noted throughout the flowering season. A flower was judged as "opening" when the dorsal sepal rose, and a flower visitor could enter the pouched labellum, and as "wilting" when its color changed from maroon to scorch, or its labellum changed from hard to soft, thereby losing its role in the pollination process.

At population level, a quadrat (20 m × 16 m) was set on a gentle slope to examine the flowering patterns of *C. tibeticum* and several cohabiting plants. From May 28 to August 16, 2005, the flowers of the shrub species *Rosa omeiensis* Rolfe (Fig. 1D) and *Berberis polyantha* Hemsl. (Fig. 1E) were opening. The inflorescence of the herb species *C. tibeticum*, *C. flavum* P. F Hunt et Summerh, *Phaius delavayi* (Finet) P. J Cribb et Perner, *Pedicularis torta* Maxim. (Fig. 1F), *P. recurva* Maxim. (Fig. 1G), *Polygonum sphaerostachyum* Meisn. and *Pyrola* sp. (Fig. 1H) were recorded every two days from the first day of anthesis.

**Pollination observation.** Pollination observations were made for a total of 47.5 hours in 2004 and 2005. The observer was first located above 5 m from a group of 5–20 flowers, then came close to the flower after the insects were found having entered into the labellum. The behavior of visitors



**Fig. 1.** A–J Flowers, pollinators of *Cypripedium tibeticum* and its concurrent flowers in the Huanglong valley, Sichuan, China. **A** Close view of an individual flower of *C. tibeticum*; ML=entrance diameter of the labellum; EL=the exit width of the labellum. **B** Longitudinal section of the labellum of *C. tibeticum*; AL=distance between the anther and the bottom of the labellum, SL=distance between the stigma and bottom of the labellum. **C** One natural cluster of *C. tibeticum*; **D** *Rosa omeiensis*; **E** *Berberis polyantha*; **F** *Pedicularis torta*. Note worker *Bombus* sp. foraging the flower; **G** *Pedicularis recurva*. **H** *Pyrola* sp.; **I** Queen of *Bombus* sp. escaping from the exit of the labellum of *C. tibeticum*; Note the pollen mass of *C. tibeticum* being taken away by the bee; **J** Queen of *Bombus* sp.; Note the pollen mass of *C. tibeticum* on its thorax

was recorded from the moment they entered the vicinity of the flowers until they left for other activities. Every insect activity, especially pollinators, including approaching, alighting, entering, creeping and climbing, which were defined in detail by Nilsson (1979), was recorded as much detailed as possible with a tape recorder. Insects observed visiting *C. tibeticum* were captured and killed in a jar using ethyl acetate fumes. Insect specimens were identified by Dr. H. L. Xu, from Chinese Agriculture University. Voucher specimens are deposited at the Chinese National Herbarium (PE), Institute of Botany, Chinese Academy of Sciences.

**Floral functional morphology.** To assess the relationship between floral morphology and pollinators, twenty fresh flowers were randomly chosen. Floral traits, especially those which are considered to be related to the success of pollination, including the entrance diameter of the labellum (ML), the distance between the stigma and the bottom of the labellum (SL), the distance between the anther and the bottom of the labellum (AL) and the exit width of the labellum (EL) (Stoutamire 1967, Nilsson 1979) were measured with digital calipers to the closest 0.1 mm. ML and EL were measured in the whole flowers (Fig. 1A), while SL and AL in the longitudinal cross-section flowers (Fig. 1B). The length, width of the body and the thorax height of the collected insects were also measured with digital calipers to the closest 0.1 mm.

**Floral fragrance collection and analysis.** Floral fragrance collection was carried out on June 17 and June 18, 2004. Two floral fragrance samples and one air control were collected. For field sampling studies, one flower was enclosed in an inertia bag (Reynolds Company, USA) for about one hour prior to sampling. The fragrance-laden air was drawn from it through a sorbent tube with a portable battery-powered sampling pump and was collected by using Tenax TA packed in a glass tube. The air was led through the glass tube for an hour between 11:00–12:00 am with a flow rate of approximately 100 mL/min.

Volatiles were desorbed from the Tenax TA by heating in a CP-4010 TCT thermol desorption device (Chrompack, The Netherlands) at 250°C for 10 min, and then cryo-focused in a cold trap at –100°C. Then the cold trap was quickly heated to 200°C in one minute to transfer the volatile compounds into the GC-MS (Trace 2000-Voyager,

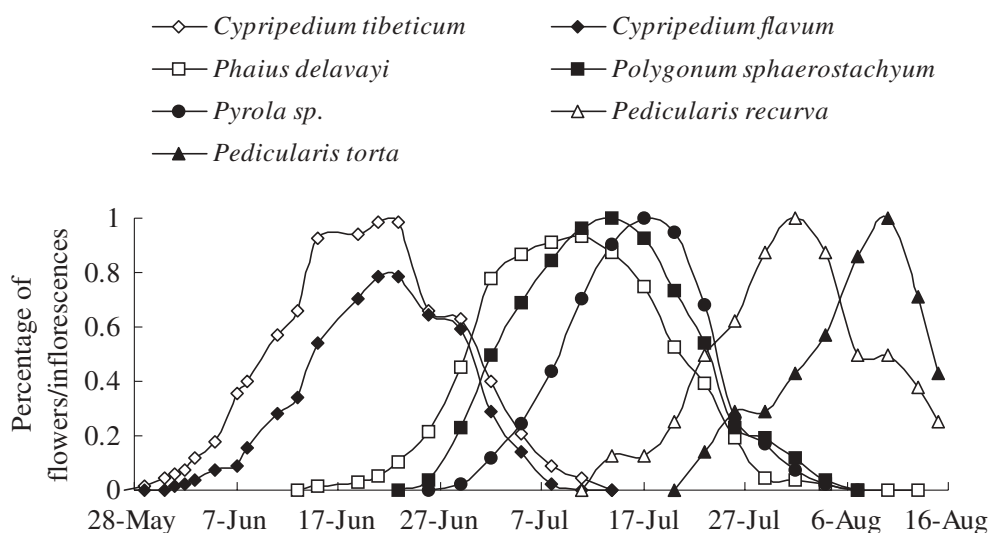
Finnigan, Thermo-Quest). Compounds were identified by searching the NIST library in the Xcalibur data system (Finnigan), and by comparing with the compositions in the air control. Compound relative abundance was grossly measured with normalization method of peak area.

**Breeding system.** Three experiments were conducted to test the breeding system of *C. tibeticum*. In each experiment, ten flowers were randomly chosen on the first day of anthesis, and the labellum was removed after they were treated as follows: (1) cross-pollinating flowers from the pollen mass of another flower a minimum of one meter away from the experimental plant; (2) self-pollinating flowers from the pollen mass of the same flower; (3) no pollination after labellum deleted. Fruit set of the treated flowers and the natural flowers were recorded in August every year.

## Results

**Flowering phenology of *Cypripedium tibeticum* and longevity of individual flower.** The life span of a single flower was varied from 19 to 30 days, average  $22.944 \pm 2.818$  days ( $n=20$ ). At population level, the flowers of *C. tibeticum* bloomed from May 28 to July 16 in 2005 (Fig. 2). During the flowering period of *C. tibeticum*, there were two rewarding shrub species in the quadrat, i.e. *Berberis polyantha* ( $n=2$ ) (Fig. 1E) which bloomed from June 1 to June 29, and *Rosa omeiensis* ( $n=2$ ) (Fig. 1D) which bloomed from June 13 to July 19. The flowering time of *C. tibeticum* was largely synchronized with another rewardless lady's slipper *C. flavum* (Fig. 2). Other herb species, including rewarding *Pyrola* sp. (Fig. 1H) *Polygonum sphaerostachyum*, and rewardless orchid species *Phaius delavayi* co-bloomed with *C. tibeticum* for a short time (Fig. 2). In addition, *Pedicularis torta* (Fig. 1F) and *P. recurva* (Fig. 1G) grew also near *C. tibeticum*, but *C. tibeticum* began to wither when the two *Pedicularis* species started blooming (Fig. 2).

**Insect visitors and their behavior.** Thirteen anthophilous species were recorded as flower visitors but many of them only alighted or rested on the flower (Table 1). Five species



**Fig. 2.** Flowering phenology of *Cypripedium tibeticum* and six other plants within a quadrat in 2005. *Cypripedium tibeticum*, n=68; *Cypripedium flavum*, n=309; *Phaius delavayi*, n=400; *Polygonum sphaerostachyum*, n=26; *Phrola sp.*, n=41; *Pedicularis recurva*, n=7; *Pedicularis torta*, n=8

including *Baccha elongae* Fabricius, *Formica* sp. and three bumble bee queens, i.e. *Bombus lepidus* Skorikov, *B. lucorum* L. and *B. hypnorum* L., were found entering into the labellum of the flowers. *Baccha elongae* flew away from the entrance of the labellum and a small ant (*Formica* sp.) climbed out along the crinkly labellum surface. Only queens of *Bombus lepidus*, *B. lucorum* and *B. hypnorum* were found escaping from the exit of the labellum and taking the pollen mass of *C. tibeticum* away (Fig. 1I, J).

During the total 47.5-hour observation, we recorded only eight times that the bumble bee queens landed on the labellum. They usually flew slowly when near the flower, and landed directly on the labellum. Three times we observed that the queens flew away immediately from the labellum. Five times they entered into the labellum voluntarily from the entrance. The queens usually stayed in the labellum from 1 min 40 sec to 2 min 10 sec. Only one queen spent 18min before escaping from the exit because it was apparently weak due to unknown reasons. After entering the labellum, the queens first walked around in the labellum for a few seconds, then they went

forward to pass the stigma and anther sometimes with buzzing noise. At last, they forced their way out of an anther opening (Fig. 1I) and flew away immediately. All of them took the pollen mass away (Fig. 1J).

Workers of *Bombus lepidus* and *B. rufofasciatus* were found on the labellum of *C. tibeticum* twice. Bewilderingly, both workers seemed rather weak because they did not fly away when being disturbed. They walked around the labellum, but did not enter into the labellum before leaving the flowers. One worker had a yellow pollen mass on its thorax, but the pollen maybe belonged to the co-blooming *C. flavum* whose pollen is also a yellow mass and whose pollinators are workers of *Bombus lepidus* (Li et al. unpublished data). In addition, no male bumblebees were found visiting the flowers of *C. tibeticum*.

When workers of *Bombus* (n=4) were artificially introduced into the labellum of *C. tibeticum*, they emerged through the exit, but only one took a little pollen on its thorax. However, when queens of *Bombus* (n=2) were artificially introduced into the labellum, both escaped from the exit of the labellum and took most of the pollen away.

**Table 1.** Insects recorded as flower visitors of *Cypripedium tibeticum*

Order and Family	Species
<b>HYMENOPTERA</b>	
Apidae	<i>Bombus hypnorum</i> L.* <i>Bombus lepidus</i> Skorikov* <i>Bombus lucorum</i> L.* <i>Bombus rufofasciatus</i> Smith
Halictidae	<i>Lasioglossum subopatum</i> Smith
Formicidae	<i>Formica</i> sp.*
<b>DIPTERA</b>	
Syrphidae	<i>Baccha elongae</i> Fabricius*
Culicidae	<i>Platosciara</i> sp.
Calliphoridae	<i>Calliphora vomitoria</i> L.
Anthomyiidae	<i>Delia unguetigris</i> Xue <i>Polietes orientalis</i> Pont.
<b>COLEOPTERA</b>	
Elateridae	<i>Agrypnus</i> sp.
Lepidoptera	
Pieridae	<i>Pieris rapae</i> L.
<b>ORTHOPTERA</b>	One species (unidentified)

\*Found in the labellum

Although not recorded in detail, *Berberis polyantha*, *Rosa omeiensis*, *Pedicularis recurva*, *P. torta* and *Polygonum sphaerostachyum* were noted being visited by bumble bees, regardless of queens and workers. Only *B. polyantha* was frequently visited by bumble bees, but no bumble bees were found directly flying towards *C. tibeticum* from the flowers of *B. polyantha*.

**Floral functional morphology.** The labellum of *C. tibeticum* is  $4.967 \pm 0.463$  cm long,  $3.701 \pm 0.602$  cm wide and  $3.037 \pm 0.275$  cm deep ( $n = 20$ ). The size of the entrance diameter of the labellum (ML), the distance between the stigma and the bottom of the labellum (SL), the distance between the anther and the bottom of the labellum (AL) and the exit width of the labellum (EL), and the body length (BL), body width (BW) and thorax height (TH) of the collected bumblebee queens and workers are listed in Table 2. The results showed the entrance diameter of the labellum (ML) ( $1.287 \pm 0.183$  cm,  $n = 20$ ) is larger than the body width (BW) of the bumblebees, which guarantees the entering of the pollinators. The depth ( $3.037 \pm 0.275$  cm,  $n = 20$ ) of the label-

lum is apparently larger than BL of bumble bees which may keep the pollinators from escaping through the entrance. The distance between the stigma and the bottom of the labellum (SL) ( $0.689 \pm 0.07$  cm,  $n = 20$ ) and that between the anther and the bottom of the labellum (AL) ( $0.549 \pm 0.043$  cm,  $n = 20$ ) are lower than the thorax height (TH) of the queens ( $0.71 \pm 0.061$  cm,  $n = 4$ ), so that queens can touch both stigma and anther when passing the column. However, AL and SL are larger than TH ( $0.509 \pm 0.077$  cm,  $n = 17$ ) of workers. Thus, bumble bee workers would generally not take the pollen away. Therefore, the size of bumble bee workers fits poorly with the floral functional morphology and bumble bee queens are the most suitable pollinators.

**Floral fragrance.** A typical gas chromatogram is presented in Fig. 3. By comparing to background air analysis, five chemical compounds were found to be the volatile constituents of floral fragrance of *C. tibeticum* (Table 3). The relative abundance of styrene (84.226%) is much higher than the rest of the compounds: ethyl acetate (3.181%), 1-methyl-

**Table 2.** Floral functional morphology of *Cypripedium tibeticum* and body size of bumble bees

Floral traits	<i>C. tibeticum</i>	Queens	Workers	Bumble bee
ML (cm)	1.287 ± 0.183	1.992 ± 0.233	1.434 ± 0.212	BL (cm)
SL (cm)	0.689 ± 0.07	0.895 ± 0.113	0.618 ± 0.077	BW (cm)
AL (cm)	0.549 ± 0.043	0.71 ± 0.061	0.509 ± 0.077	TH (cm)
EL (cm)	0.735 ± 0.1	-	-	-

Notes: (1) ML = the entrance diameter of the labellum, SL = the distance between the stigma and bottom of the labellum, AL = the distance between the anther and the bottom of the labellum, EL = the exit width of the labellum; BL = body length; BW = body width; TH = thorax height. (2) The results of floral traits are got from 20 individuals; the results of bumble bee queens are got from 4 individuals; bumble bee workers from 17 individuals.

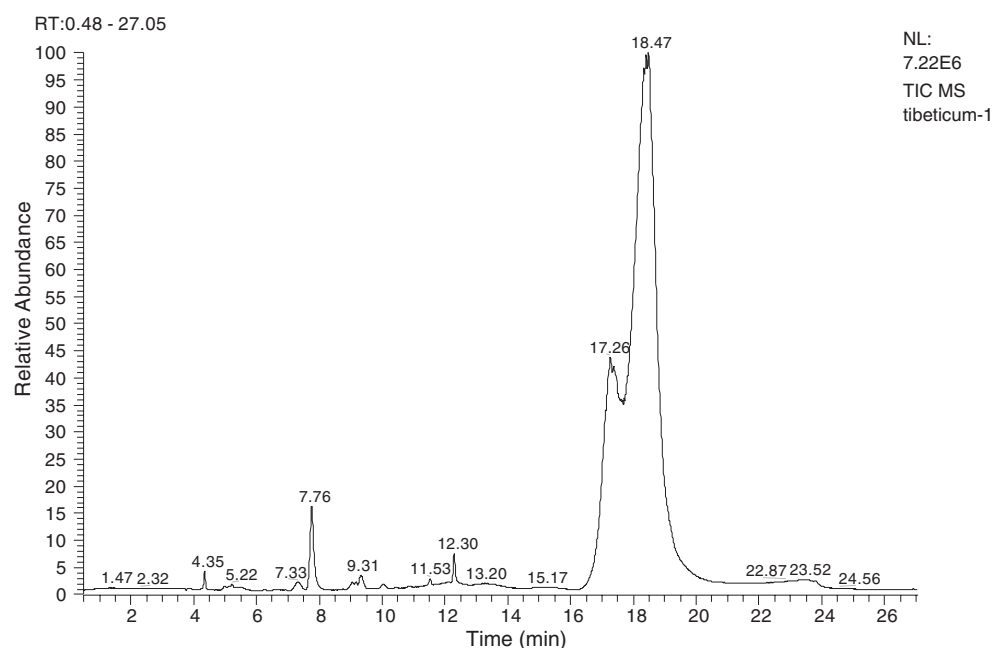
pentyl-hydroperoxide (1.076%), 1,5-hetadien-3-yne (0.712%) and ethylbenzene (10.442%).

**Breeding system.** The difference between the natural fruit set 9.57% (n = 564) in 2004 and 13.8% (n = 710) in 2005 was not significant ( $\chi^2$  test,  $P > 0.05$ ). Both hand self-pollination and cross-pollination produced 100% fruit set (Table 4). The difference between natural pollination and hand pollination was significant ( $\chi^2$  test,  $P < 0.01$ ). Control flowers that were labellum-removed and not pollinated set no fruit, which showed that spontaneous self-pollination was impossible and that the

insects would not affect pollination success after the labellum were removed because the insects lost the platform for landing.

### Discussion

The results of breeding system (Table 4) indicate that the flowers of *Cypripedium tibeticum* are self-compatible but need pollen vectors for fruit production. The finding of pollen-bearing queens of *Bombus lepidus*, *B. lucorum* and *B. hypnorum* suggests that *C. tibeticum* is bumble bee queen pollinated. The fruit set by artificial

**Fig. 3.** Gas chromatogram of the floral fragrance of *Cypripedium tibeticum* emitted from one flower

**Table 3.** Volatile compounds and their relative abundance from the flower fragrance of *Cypripedium tibeticum*

Retention time	Volatile compounds	Skeletal type	Relative abundance (%)
7.33 (9.33)	1-methylpentyl-Hydroperoxide	FA	1.076
7.76	Ethyl Acetate	FA	3.181
12.30	1,5-Heptadien-3-yne	FA	0.712
17.26	Ethylbenzene	B	10.442
18.47	Styrene	B	84.226

Note: compounds classified according to general skeletal type; B = benzenoid, FA = fatty acid

**Table 4.** Breeding system and fruit set of *Cypripedium tibeticum*

Treatment	No. of flowers	No. of fruits	Fruit set (%)
Cross-pollination	10	10	100
Self-pollination	10	10	100
Spontaneous self-pollination	10	0	0
Natural pollination (2004)	564	54	9.57
Natural pollination (2005)	710	98	13.8

pollination is much higher than that of natural pollination indicates the fruit set of *C. tibeticum* is pollinator limited.

Sugiura et al. (2002) proposed that *C. macranthos* var. *rebunense* mimicked *Pedicularis schistostegia* to attract bumble bees because (1) Both plants overlapped greatly in flowering time. (2) *Cypripedium* occurred with lower frequency relative to *Pedicularis*. (3) Bumble bees were sometimes confused between *Cypripedium* and *Pedicularis*. (4) Flower color of both plants would be similar within the range of a bumble bee's visual spectrum. (5) Both species largely overlapped in spatial distribution. In our study, the pink *Pedicularis recurva* (Fig. 1G) and the yellow *P. torta* (Fig. 1F) grew also in the same community with *C. tibeticum*, but they began to bloom when *C. tibeticum* started to wither (Fig. 2). So it is impossible that *C. tibeticum* mimics the co-habiting *Pedicularis* like its species ally *C. macranthos* var. *rebunense*.

The anthesis of the shrub species *Berberis polyantha* (n=2) and *Rosa omeiensis* (n=2)

overlapped for several days with *C. tibeticum*, but the flowers of the former are yellow and the latter are white. Moreover, both shrubs are different in flower size, shape and height of rachis from *C. tibeticum*. Although *B. polyantha*, *R. omeiensis* and *C. tibeticum* are all visited by bumble bees, it seems unreasonable to suggest that *C. tibeticum* mimics rewarding *B. polyantha* and *R. omeiensis*. The flowers of *C. flavum* (n = 309) were almost synchronized with *C. tibeticum* in flowering time, but it is also a rewardless lady's slipper orchid. Other herb species *Phaius delavayi*, *Polygonum sphaerostachyum* and *Pyrola* sp. had overlapped anthesis with *C. tibeticum* for a short time (Fig. 2). However, *P. delavayi* is another rewardless orchid and, *P. sphaerostachyum* and *Pyrola* sp. occurred with lower frequency relative to *Cypripedium* species (Fig. 2). Considering the low occurrence and the different flower color, we don't think that these rewarding plants should be regarded as the special mimic models in the deceptive system of *C. tibeticum*.



A more prevalent category of food deception does not involve models, but exploits instinctive behavior of the pollinators (Dafni 1984, Ackerman 1986, Schiestl 2005, Cozzolino and Widmer 2005). Flowers of lady's slippers are generally regarded as this type (cf. Van der Cingel 2001). In *C. calceolus*, Nilsson (1979) found pollinator bees (*Andrena*, *Lasioglossum* and *Halictus*) became optically attracted from afar by the yellow labellum and the patterns of the crimson spots on the staminode, while on the veins in the labellum are false nectar guides, and the floral fragrance dominated by acetates might interfere with pheromone controlled alighting reactions and marked nest tunnels on the ground, and thereby increases entering into the labellum. Thus *C. calceolus* probably attracts pollinators through general food deception (odor components, color, false nectar guides), as well as nest site resemblance (odor and cavity) and with scent-mark resemblance (odor). In *C. acaule* Ait., pollinators bumble bee queens are probably attracted to the lady's slipper flower by its pink color and by a sweet, sugary smell concentrated in the sepals and lateral petals (Stoutamire 1967, Davis 1986). In contrast, the red-maroon flowers of *C. tibeticum* seem not specialized to attract bumble bees through food mimic because most bees show a significant preference for yellow and blue over other colors (Simonds and Plowright 2004). The dominating floral scent of *C. tibeticum*, styrene (84.226%) and ethylbenzene (10.442%) (Table 3) with pungent scent, would not also indicate food resources and not effectively attract bumble bees because most bumble bees prefer sweet-scent flowers (Proctor et al. 1996). However, the minor compound ethyl acetate with sweet-fruity scent (73.181%) (Thien et al. 1985) probably indicates food-resources and plays an important role in the attraction of the bumble bees.

Within the genus *Cypripedium*, two species have been reported being pollinated only by *Bombus* queens, one is *C. acaule* and

another is *C. macranthos* var. *rebunense* (Stoutamire 1967; Davis 1986; Sugiura et al. 2001, 2002). Both of them bloom early in spring when only *Bombus* queens emerged from hibernation. However, *C. tibeticum* blooms later in spring when both queens and workers emerged in the area. The flowers of *C. tibeticum* are dark purple in colour and bend downward. The dorsal sepal appears to "half cover" the labellum orifice (Fig. 1C). When queen bumble bees nest in temperate zones, the fertilized females must find a nesting site such as the burrow of a rodent or a hole in a wooden branch after she emerges from hibernation in the spring season (Proctor et al. 1996). Thus, the queen bumble bee probably enters the labellum of *C. tibeticum* as if she were examining a mouse burrow or a hole in a piece of wood, and is trapped until she exits the flower by crawling under the stigma at the opposite end and carrying off the pollinia. By the time the worker reaches maturity the queen mother has her own established burrow and the worker is not interested in exploring "alien nesting sites." A worker bee, with an instinct to return to her home nest, will not be attracted to *C. tibeticum*. That is why most *Bombus* workers are not interested in the flowers of this species. *C. tibeticum* is probably pollinated only by "naive queens" who have not made their own nest site. Bee uses flowers as nest site has also been found in other plants. Some male anthophorine bees stay overnight in the dark floral tube of the orchid *Serapias vomeracea* as if it was a nesting cavity and served as pollinators when it exits the flower (Dafni et al. 1981). Similar behaviors of male bees were reported in *Oncocycclus* irises (Iridaceae) (Sapir et al. 2005). Whether *Bombus* queens stay overnight in the flowers of *C. tibeticum* needs further observation. Comparing with the male bees, however, the *Bombus* queens probably have different motivation to be attracted to the flowers of *C. tibeticum*.

The experiment of artificial introduction suggests bumblebee queens and workers have

different pollination efficiency in *C. tibeticum*. It is correspondent with the comparison between bumblebee's size and the floral morphology (Table 2). Our pollination observations have provided evidence that bumble bee queens were the effective pollinators of *C. tibeticum*. It is interesting to note that co-flowering *C. flavum* are pollinated by bumble bee workers and that, while *C. tibeticum* and *C. flavum* succeed in setting fruit by hand-hybridization, no natural hybrids were found in the field (Li et al., unpubl. data). Thus, *C. tibeticum* and *C. flavum* probably retain their species' boundaries by using different sized bees, which is their main reproductive isolation mechanism. In contrast, another co-blooming lady's slipper *C. smithii* (= *C. calcicolum*), being close similarity with *C. tibeticum* in floral morphology and floral color, grows in the same site and is found once pollinated by one bumble bee queen *B. lucorum*, same as the pollinator of *C. tibeticum* (Li et al., unpubl. data). It seems that no effective reproductive isolation exists between interfertile *C. tibeticum* and *C. smithii*, which is accordant with the view of Cribb (1997).

We would like to thank Dr. Holger Perner, the orchid specialist and the senior consultant for the Administration of the Huanglong Nature Reserve, for guiding us to the habitat of this lady's slipper orchid, Prof. Xu Huanli from Chinese Agriculture University for identification of insects, Prof. Chen Huajun from Beijing Forestry University for helping analyze the floral fragrance, and Mr. Tang Siyuan, Mr. Kou Yahui, Mr. Dong Li, Mr. Tai Yundong, Mr. An Dejun from Huanglong Administration of National Scenic Spot for their helping in the field work. We are greatly indebted to Prof. Amots Dafni, University of Haifa for his valuable suggestions. This study was funded by the Program for Key International S and T cooperation Project of the P. R. China (2001CB711103), and supported by the Co-researching Station of Institute of Botany, Chinese Academy of Sciences and Huanglong Administration of National Scenic Spots, Sichuan, China.

## References

- Ackerman J. D. (1986) Mechanisms and evolution of food-deceptive pollination systems in orchids. *Lindleyana* 1:108–113.
- Bänziger H., Sun H. Q., Luo Y. B. (2005) Pollination of a slippery lady slipper orchid in southwest China: *Cypripedium guttatum* (Orchidaceae). *Bot. J. Linn. Soc.* 148: 251–264.
- Catling P. M., Catling V. R. (1991) A synopsis of breeding systems and pollination in North American orchids. *Lindleyana* 6(4): 187–210.
- Cozzolino S., Widmer A. (2005) Orchid diversity: an evolutionary consequence of deception? *Trends Ecol. Evol.* 20(9): 487–494.
- Cribb P. (1997) The genus *Cypripedium*. Timber Press, Portland, Oregon, USA.
- Dafni A., Ivri Y. (1981) Floral mimicry between *Orchis israelitica* Baumann and Dafni (Orchidaceae) and *Bellevalia flexuosa* Boiss (Liliaceae). *Oecologia* 49: 229–232.
- Dafni A., Ivri Y., Brantjes N. B. M. (1981) Pollination of *Serapias vomeracea* Briq. (Orchidaceae) by imitation of holes for sleeping solitary male bees (Hymenoptera). *Acta. Bot. Neerl.* 30: 69–73.
- Dafni A. (1984) Mimicry and deception in pollination. *Annual Rev. Ecol. Syst.* 15: 259–278.
- Dafni A. (1987) Pollination in *Orchis* and related genera: evolution from reward to deception. In: Arditti J. (ed.) *Orchid Biology: reviews and perspectives, IV*. Cornell University Press, Ithaca and London, pp. 79–104.
- Davis R. W. (1986) The pollination biology of *Cypripedium acaule* (Orchidaceae). *Rhodora* 88: 445–450.
- Dressler R. L. (1993) Phylogeny and classification of the orchid family. Cambridge University Press, Cambridge, UK.
- Johnson S. D. (1994) Evidence for Batesian mimicry in a butterfly pollinated orchid. *Biol. J. Linn. Soc.* 53: 91–104.
- Johnson S. D. (2000) Batesian mimicry in the non-rewarding orchid *Disa pulchra*, and its consequences for pollinator behaviour. *Biol. J. Linn. Soc.* 71:119–132.
- Li P., Tang S. Y., Dong L., Kou Y., Perner H., Luo Y. B. (2005a) Temperate paradise: orchids in the Huanglong valley. *Orchid Review* 113: 154–159.
- Li P., Tang S. Y., Dong L., Luo Y. B., Kou Y., Yang X. Q., Perner H. (2005b) Species diversity and flowering phenology of Orchidaceae in

- Huanglong valley, Sichuan. Biodiversity Science 13(3): 255–261.
- Nilsson L. A. (1979) Anthecological studies of the lady's slipper, *Cypripedium calceolus* (Orchidaceae). Bot. Notiser 132: 329–347.
- Nilsson L. A. (1983a) Anthecology of *Orchis mascula* (Orchidaceae). Nord. J. Bot. 3: 157–179.
- Nilsson L. A. (1983b), Mimesis of bellflower (*Campanula*) by the red helleborine orchid *Cephalanthera rubra*. Nature 305: 799–800.
- Nilsson L. A. (1992) Orchid pollination biology. Trends Ecol. Evol. 7(8): 255–259.
- Proctor M., Yeo P., Lack A. (1996) The natural history of pollination. Timber Press, Portland, Oregon, USA.
- Sapir Y., Shmida A., Ne'eman G. (2005) Pollination of *Oncocyclus irises* (Iris: Iridaceae) by night-sheltering male bees. Pl. Biol. 7: 417–424.
- Schiestl F. P. (2005) On the success of a swindle: pollination by deception in orchids. Naturwissenschaften 92: 255–264.
- Simonds V., Plowright C. M. S. (2004) How do bumblebees first find flowers? Unlearned approach responses and habituation. Animal Behavior 67: 379–386.
- Stoutamire W. P. (1967) Flower biology of the lady's slippers (Orchidaceae: *Cypripedium*). Michigan Botanist 3: 107–119.
- Sugiura N., Taketoshi F., Ken I., Kenji K. (2001) Flowering phenology, pollination, and fruit set of *Cypripedium macranthos* var. *rebunense*, a threatened lady's slipper (Orchidaceae). J. Pl. Res. 114: 171–178.
- Sugiura N., Goubara M., Kitamura K., Inoue K. (2002) Bumblebee pollination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae): a possible case of floral mimicry of *Pedicularis schistostegia* (Orobanchiaceae). Pl. Syst. Evol. 235: 189–195.
- Thien L. B., Bernhardt P., Gibbs G. W., Pellmyr O. M., Bergstrom G., Groth I., McPherson G. (1985) The pollination of *Zygogynum* (Winteraceae) by a moth, *Sabatinca* (Micropterigidae): an ancient association? Science 227: 540–542.
- Van der Cingel N. A. (2001) An atlas of orchid pollination. Balkema A. A., Rotterdam, Netherland.

Addresses of the authors: Peng Li (e-mail: lipeng@ibcas.ac.cn), Yibo Luo (correspondence; e-mail: luoyb@ibcas.ac.cn) and Xiaoqin Yang, State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Beijing 100093, China. Yong Kou, Huanglong Administration of National Scenic Spots, Huanglong 623300, Sichuan, China.