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【Article】

Do Nest-site Searching Bumblebee Queens Prefer Entering into the Labelloid Cavity of Bumblebee-pollinated Orchid, *Cypripedium japonicum*?

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Abstract

To assess the relationship between floral morphology and body sizes of bumblebees, we observed pollination of *Cypripedium japonicum* by queen and worker bumblebees of *Bombus ardens ardens*. The workers are a poor fit with the floral functional morphology and the queens are the effective pollinators. In an experiment using artificial flowers, mated queens of *B. a. ardens* entered into a cavity of artificial flowers significantly more than virgin queens and workers of *B. a. ardens*, suggesting *C. japonicum* may mimic nest sites to deceive bumblebees into entering.

Keywords: Hymenoptera, bumblebee, orchid, nest-site mimicry, pollination

INTRODUCTION

The Cypripedioideae are a group of well-known pollination-deceiving orchids; the flowers are one-way traps with easy entrance into the labellum from the front, and much easier exit to the rear, where insects must pass first beneath the stigma and then the anther (Dressler 1993). Many reported *Cypripedium* species attract pollinators by deception (Nillson, 1979, Davis 1986). In *C. calceolus* L., flowers attract pollinators by general food deception (odor, color, false nectar guides), as well as nest-site mimicry (odor and cavity) and scent-mark mimicry (odor) (Nilsson 1979). In the recently studied *C. guttatum* Sw., the flowers exploit innate susceptibilities of pollinator *Halictid* bees (Bänziger et al. 2005). So far, a specific plant model has only been identified in *C. macranthos* Sw. *var. rebunense* (Kudo) Miyabe et Kudo. Sugiura et al. (2001, 2002) suggests this species mimics the co-blooming *Pedicularis schistostegia* Vved because the flower color within the bumblebee's visual spectrum of *Bombus pseudobaicalensis* is similar and both species overlap in spatial distribution and flowering time.

Cypripedium japonicum Thunb is distributed from Japan to Korea and China. It starts flowering from May to June with one large flower that is usually white-purplish with bold sepals and petals, and a labellum (sac) with a characteristically corrugated surface. The pollination and pollinator attractant system of this orchid is unreported, but it is well known that bumblebees are the functional pollinators (Tanaka and Hirano, 2000).

In this study, we investigated the pollination system of *C. japonicum* and measured floral size to discuss the relationship between floral morphology of *C. japonicum* and body sizes of bumblebees. Furthermore, we investigated whether this orchid deceives bumblebees into visiting and pollinating them by mimicking the nest site.

MATERIALS AND METHODS

1. Pollination observation by forced experiment

Studies were conducted in artificial habitat of *C. japonicum*, Oshino, Yamanashi Prefecture, Japan, and in our

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laboratory in 2007 and 2008. On the observation of bumblebee behavior in the labellum of *C. japonicum*, queens (n=5) and workers (n=2) of *Bombus ardens ardens* collected in Oshino before the experiments were forcefully introduced to the labellum because bumblebees rarely visited *C. japonicum* flowers during the observation periods.

2. Floral functional morphology

Twenty fresh flowers were chosen at random to assess the relationship between floral morphology and body sizes of bumblebees. The floral traits, especially those considered related to pollination success, including entrance diameter of labellum (ML), distance between stigma and bottom of labellum (SL), distance between anther and bottom of labellum (AL), and exit width of labellum (EL) (Nilsson 1979), were measured with digital calipers (Shinwa) to the nearest 0.01 mm. ML and EL were measured in whole flowers (Fig. 1(A)), while SL and AL were measured in the longitudinal cross-section of flowers (Fig. 1(B)). The body length (BL), body width (BW), and thorax height (TH) of queen (n=10) and worker (n=7) bumblebees, *B. ardens ardens* collected from Oshino were also measured with digital calipers to the nearest 0.01 mm.

3. Investigation of bumblebee's preference for the cavity using artificial flowers

We done the experiment using artificial flowers made from eggshell, tissue, wire, green tape and cellophane to investigate whether this orchid deceives bumblebees into visiting and pollinating by nest-site mimicry. Nine artificial flowers were put into a mesh cage into which separately released mated queens who have still searched for their own nest sites (n=10) of *B. ardens ardens* collected in Oshino, virgin queens (n=15) who have not searched for their own nest sites, and workers (n=15) of *B. a. ardens*

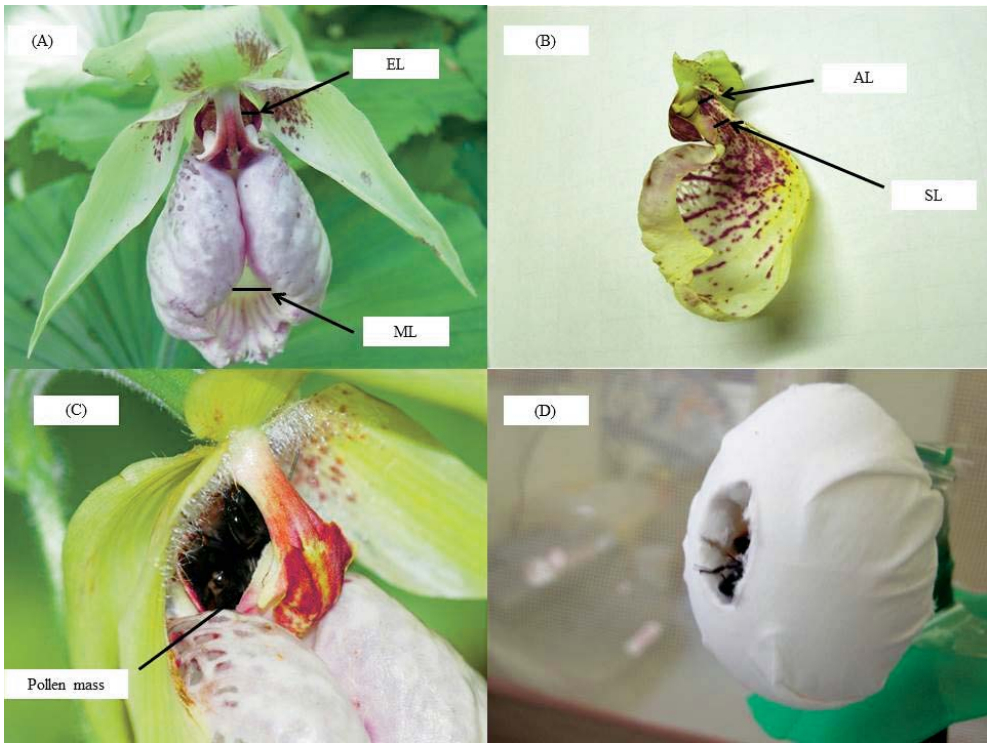

Figure 1

(A) Close up of individual *C. japonicum* flower. ML=entrance diameter of labellum; EL=exit width of labellum.
(B) Longitudinal section of labellum of *C. japonicum*. AL=distance between anther and bottom of labellum, SL=distance between stigma and bottom of labellum.
(C) Queen of *Bombus ardens ardens* escaping from exit of labellum of *C. japonicum*. Note *C. japonicum* pollen mass on bumblebee.
(D) Queen of *Bombus ardens ardens* entering artificial flower.

collected in Machida, Tokyo. We investigated the number of bumblebees who entered into a cavity of artificial flowers in each experiment. Each experiment was observed for 7 hours and repeated five times.

RESULTS

1. Pollination observation by forced experiment

The forced experiment suggests bumblebee queens and workers have different pollination efficiencies for *C. japonicum*. The bumblebees enter the labellum from the entrance (cavity of labellum) and usually stay inside from 1 to 5 minutes. Upon entering, they first walk around for a few seconds, then they go forward to pass the stigma and anther sometimes with buzzing for a few seconds. Finally, they force their way out of the anther opening and fly away. No workers carried pollen away. Four queens carried away pollen masses (Fig. 1(C)) and succeed the pollination. Only one queen did not receive a pollen mass, perhaps because she was older and her thorax was hairless. We think that thorax body hair is important in receiving the *C. japonicum* pollen mass.

2. Floral functional morphology

The labellum of *C. japonicum* is 47.91±2.47 mm long, 39.59 ± 2.29 mm wide and 38.04±1.34 mm deep (n=20). Table 1 lists the sizes of ML, SL, AL, and EL of the flowers, as well as those of BL, BW, and TH of the collected bumblebee queens and workers, *B. ardens ardens*. The results show that the ML (15.70±1.29 mm, n=20) is larger than the BW of the bumblebees, guaranteeing entry. The depth (38.04 ± 1.34 mm, n=20) of the labellum is apparently larger than the BL of bumblebees, which may stop them escaping through the entrance. The SL (7.28±0.20 mm, n=10) and AL (6.61 ± 0.27 mm, n=10) are less than the TH of queens (7.32 ± 0.31 mm, n=10), so queens can touch both the stigma and anther when passing the column. However, the AL and SL are larger than the TH (3.71±0.43 mm, n=7) of workers, explaining why bumblebee workers generally do not carry pollen away. The bumblebee workers are a poor fit to the floral functional morphology and the bumblebee queens are the more efficient pollinators.

Table 1 Floral functional morphology (mean±SD) of *C. japonicum* and body size of *B. ardens ardens*.

Floral traits	*C. japonicum*	Queens (n=10)	Workers (n=7)	Bumblebee
ML (mm) (n=20)	15.70 ± 1.29	19.85 ± 1.63	12.99 ± 1.53	BL (mm)
SL (mm) (n=10)	7.28 ± 0.20	7.36 ± 0.33	4.60 ± 0.45	BW (mm)
AL (mm) (n=10)	6.61 ± 0.27	7.32 ± 0.31	3.71 ± 0.43	TH (mm)
EL (mm) (n=20)	7.50 ± 0.58			

ML: entrance diameter of labellum
SL: distance between stigma and bottom of labellum
AL: distance between anther and bottom of labellum
EL: exit width of labellum

3. Investigation of bumblebee's preference for the cavity using artificial flowers

Mated queens of *B. ardens ardens* made significantly more enters into a cavity of the artificial flowers than virgin queens and workers (Fisher's exact test, $p<0.01$) (Table 2, Fig 1(D)). Only one virgin *B. a. ardens* queen entered into a cavity of the artificial flowers in trial 4 and three *B. a. ardens* worker entered in trial 1 and 3.

Table 2 Preferences of *B. ardens ardens* to a cavity of artificial *C. japonicum* flowers

Trial	Mated Queen (n=10)	Virgin Queen (n=15)	Worker (n=15)
1	3	0	1
2	2	0	0
3	2	0	2
4	7	1	0
5	1	0	0
Average	3.0*	0.2	0.6

* Fisher's exact test, $p<0.01$

DISCUSSION

A common food deception does not involve models, but exploits instinctive behavior of pollinators (Dafni 1984, Schiestl 2005). Flowers of lady's slippers (Cypripedioideae) are generally regarded as belonging to this type (Van der

Cingel 2001). In *C. calceolus*, Nilsson (1979) found pollinator bees are attracted from afar visually by the yellow labellum and the patterns of crimson spots on the staminode, while veins in the labellum are false nectar guides. The floral scent dominated by acetates might interfere with pheromone-controlled alighting and marked nest tunnels on the ground, thereby increasing labellum entry. Consequently, *C. calceolus* may attract pollinators by general food deception (odor, color and false nectar guides), as well as nest-site mimicry (odor and cavity) and with scent-mark resemblance (odor). On the other hand, Sugiura et al. (2002) excluded food deception and proposed that *C. macranthos* var. *rebunense* mimics *Pedicularis schistostegia* to attract bumblebees.

Two species in the genus *Cypripedium* (*C. acaule* and *C. macranthos* var. *rebunense* (Stoutamire 1967, Davis 1986, Sugiura et al., 2001, 2002)) are reported as being pollinated only by *Bombus* queens. Like *C. japonicum*, both species flower in early spring when only *Bombus* queens have emerged from hibernation. When queen bumblebees nest in temperate zones, the fertilized females must find a nesting site such as a rodent burrow or hole in a branch soon after emerging (Proctor et al. 1996). In *C. tibeticum*, Li et al. (2006) proposed that a queen bumblebee probably enters the labellum of *C. tibeticum* as if examining a mouse burrow, etc., and is trapped until exiting by crawling under the stigma at the opposite end and carrying off the pollen mass. Furthermore, the orchid is probably pollinated by "naive queens" who have not yet made their own nest. Our result shows mated queens who have still searched for their own nest sites of *B. ardens ardens* entered into a cavity of artificial flowers significantly more than virgin queens and workers of *B. a. ardens*, strongly suggesting that *C. japonicum* may mimic nest sites to deceive bumblebees into entering.

The match between *B. a. ardens* queen size and floral morphology found in the forced experiment suggests that *B. a. ardens* queens and workers have different pollination efficiencies for *C. japonicum* with queens being the effective pollinators.

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造巣場所探索マルハナバチ女王はクマガイソウの唇弁口に入ることを好む？

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要　約

マルハナバチ媒花ランであるクマガイソウ（*Cypripedium japonicum*）の花とマルハナバチの形態の関係を評価するために、人工条件下でコマルハナバチ（*Bombus ardens ardens*）の女王と働き蜂によるクマガイソウの受粉を観察し、花と蜂の各部位の比較計測をした。その結果、働き蜂は花の形態と体サイズが一致せず花粉を付着しない、一方で女王は体サイズが一致し花粉が付着する機能的なポリネーターであった。

また、なぜ女王がクマガイソウの唇弁内に入るのか？を検証するために、マルハナバチ女王の造巣場所探索行動に注目した。実験では網室内にクマガイソウを模した人工花を設置し、女王（交尾と未交尾）と働き蜂を別々に放ち、唇弁口に入る個体数を比較した。その結果、コマルハナバチの交尾女王（造巣場所探索女王）は、未交尾女王や働き蜂と比較して有意に人工花の唇弁口に入り込む習性があることが明らかになった。この習性により交尾女王は、クマガイソウの唇弁口を造巣場所と間違えて花の内部に入り込みトラップされる事で、無報酬花であるクマガイソウの送粉をさせられている可能性が考えられた。

キーワード：ハチ目、マルハナバチ、ラン、造巣場所擬態、受粉

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