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Pollination by fungus gnats in four species of the genus *Mitella* (Saxifragaceae)

YUDAI OKUYAMA[1]*, MAKOTO KATO[1] and NORIAKI MURAKAMI[2]

[1]*Graduate School of Human and Environmental Studies, Kyoto University, Sakyo, Kyoto 606–8501, Japan*
[2]*Department of Botany, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606–8502, Japan*

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The first example of pollination by fungus gnats in the eudicots is reported. The genus *Mitella* (Saxifragales) is characteristically produces minute, inconspicuous, mostly dull-coloured flowers with linear, sometimes pinnately branched, petals. To understand the function of these characteristic flowers, we studied the pollination biology of four *Mitella* species with different floral traits and different sexual expression: dioecious *M. acerina*, gynodioecious *M. furusei* var. *subramosa*, and hermaphroditic *M. stylosa* var. *makinoi* and *M. integripetala.* Flower-bagging experiments showed that wind pollination did not occur in the dioecious and gynodioecious species. Two years of observations of flower visitors at six study sites in Japan revealed that the principal pollinators of all four *Mitella* were specific species of fungus gnats (Mycetophilidae), which landed on the flowers with their long spiny legs settling on the petals. Characteristically, numerous pollen grains were attached to the fungus gnats in specific locations on the body. Although, on average, 1.3–2.6 fungus gnats visited each inflorescence per day, the fruit set of both bisexual and female flowers exceeded 63%. These results suggest that fungus gnats are highly efficient pollinators of *Mitella* spp., and that *Mitella* flowers are morphologically adapted to pollination by fungus gnats.

ADDITIONAL KEYWORDS: bishop's cap – dioecy – gynodioecy – *Heuchera* group – Nematocera – specialized pollination.

INTRODUCTION

It is thought that the ancient radiation of angiosperms from the mid Cretaceous was facilitated by partnerships between plants and insect pollinators (Proctor, Yeo & Lack, 1996). Various pollination systems involving a variety of flower-visiting insects may reflect different floral traits, and pollinator-mediated selection mechanisms should cause floral diversification. Bees, which are highly specialized and highly effective pollen vectors, pollinate the largest number of angiosperm species (e.g. Bawa, 1990; Kato *et al*., 1990; Momose *et al*., 1998). However, some plants bear less conspicuous flowers that are pollinated by other minor and minute insects, such as the basal dipterans. In general, basal dipterans, such as fungus gnats, have been considered inefficient pollinators (Faegri & van der Pijl, 1979), although they are the principal visitors to the flowers of some plants (e.g. Olesen & Warncke, 1989; Kato *et al*., 1990; Proctor *et al*., 1996). Pollination by fungus gnats has been only reported in the Aristolochiaceae (Vogel, 1978a; Sugawara, 1988), Araceae (Vogel, 1978b; Vogel & Martens, 2000), Liliaceae (Mesler, Ackerman & Lu, 1980) and Orchidaceae (Sargent, 1934; Ackerman & Mesler, 1979; Mesler *et al*., 1980; Proctor *et al*., 1996), but not in eudicot families (*sensu* Soltis, Soltis & Chase, 1999; Soltis *et al*., 2000). Here we describe another system of pollination by fungus gnats in the Saxifragaceae (Saxifragales; eudicots).

Saxifragaceae *s.s.* (550 spp.) is a moderate-size family comprising 30 genera (Soltis *et al*., 2001) that show great variation in their floral characters. Their geographical distribution ranges throughout the temperate regions of the Northern Hemisphere, and some are disjunctly distributed in South America (Soltis *et al*., 2001). Soltis *et al*. (2001) divided the family into ten subclades, according to a molecular phylogenetic tree based on DNA sequence data. They noted one well-supported subclade, the *Heuchera* group (comprising nine genera; 80 spp.; see also Soltis *et al*., 1993), which

*Corresponding author. E-mail: okuyama@bio.h.kyoto-u.ac.jp

generally bears minute flowers and is variable in its floral morphology.

In the *Heuchera* group, the floral uniqueness of the genus *Mitella* is noteworthy. Unlike other angiosperms, its petals are dull-coloured (but there are some exceptions; see Discussion), linear, filamentous and usually pinnately branched. In addition, floral morphology varies among species in this genus. *Mitella* species are also characterized by their habitats: the plants grow along stream banks where they are frequently sprayed with water. The uniqueness of the floral morphology and the streamside habitat of the genus may reflect the specialized pollination systems of the species. However, little information is available regarding the pollinators of the dull-coloured *Mitella* flowers, although Savile (1975) suggested without direct observation that primitive dipterans such as mosquitoes might pollinate them.

In order to clarify whether this unusual floral morphology is related to their respective pollination systems, we studied the reproductive systems of four native *Mitella* species that differed in floral traits and sexual expression (Table 1).

In this paper we: (1) provide a report of the pollination systems of four *Mitella* species native to Japan, (2) examine the relationship between pollinator specificity and the unique floral characters of *Mitella*, (3) discuss the pollination efficiency of fungus gnats in *Mitella* and (4) discuss the floral modifications in the *Heuchera* group that may be affected by pollinators.

MATERIAL AND METHODS

Study sites and species

Mitella is a genus of perennials composed of over 20 species found in North America and East Asia. Most of the *Mitella* species are hermaphroditic, but three gynodioecious and one dioecious species are known from Japan (Wakabayashi, 1987). Eleven species of *Mitella* have been described from Japan, of which ten are endemic (Wakabayashi, 2001). We studied the pollination biology of the following four *Mitella* species, each of which has different floral characters (Table 1): *M. acerina* Makino, *M. furusei* Ohwi var. *subramosa* Wakabayashi, *M. stylosa* Boissieu var. *makinoi* (Hara) Wakabayashi and *M. integripetala* H. Boissieu.

M. acerina has a very limited geographical distribution and is found only in the prefectures of Kyoto, Shiga and Fukui. This is the only dioecious species reported in *Mitella* (Wakabayashi, 1987, 2001). It bears 16–52 flowers, which are compactly arranged on a single inflorescence. The flowers are small (4–5 mm in diameter), saucer-shaped, with flat calyx lobes, and each petal is 3–5-pinnately branched.

M. furusei var. *subramosa* (hereafter referred to as *M. furusei*) has a wider distribution throughout western Japan. This species is reported to be gynodioecious (Wakabayashi, 1987). It bears 12–40 flowers on a single inflorescence. The flowers are small (4–5 mm in diameter) and tubular, with erect calyx lobes. Petals are 3–5-pinnately branched.

M. stylosa var. *makinoi* (hereafter referred to as *M. stylosa*) is distributed in Shikoku. This species produces 3–27 bisexual flowers on a single inflorescence. The flowers are similar to those of *M. furusei* but differ in the shape of the petals (usually 5-pinnately branched) and the depth of the calyx tube (*M. stylosa* is the deeper).

M. integripetala is a fairly rare species that is distributed in western Hokkaido and northern Honshu. It bears 7–21 bisexual flowers on a single inflorescence. The cup-shaped flowers are different from those of the other Japanese *Mitella* species in their relatively large size (9–10 mm in diameter), subsuperior ovaries (the others have completely inferior ovaries), stamens opposite the calyx lobes (the others are alter-

Table 1. Sexual expression and floral characters of four *Mitella* species we studied (from Wakabayashi, 1987, 2001). Study site and study date for each species are also shown

Species	Sexual expression	Ovary	Floral shape	No. of petal lobes	Study site	Dates of pollination study
M. acerina	dioecy	inferior	saucer-shaped	3–5	Site 1	6–28.v.2002 16.iv – 6.v.2003
M. furusei	gynodioecy	inferior	tubular	3–5	Site 1 Site 2 Site 3	6–28.iv.2002 16.iv. – 6.v.2003 11.iii – 20.iv.2002 13–23.iv.2003 27.iv.2003
M. stylosa	hermaphrodite	inferior	tubular	5	Site 4 Site 5	11–12.iv.2002 22–24.iv.2003
M. integripetala	hermaphrodite	subsuperior	cup-shaped	1	Site 6	19–21.vi.2002

Table 2. Locations and dominant tree species of four study sites in Japan

	Location	Latitude	Longitude	Altitude	Dominant tree species
Site 1	Ashu; Kyoto University forest, Miyama-cho, Kyoto Prefecture	35°18′10″N	135°44′20″E	450 m	*Aesculus turbinata, Cryptomeria japonica, Fagus crenata*, *Quercus crispula*
Site 2	Mt. Daimonji, Sakyo, Kyoto Pref.	35°1′40″N	135°47′60″E	150 m	*Castanopsis cuspidata*, *Quercus glauca*, *Quercus serrata*
Site 3	Akame 48 falls, Nabari-shi, Mie Pref.	34°33′40″N	136°5′15″E	350 m	*Cryptomeria japonica, Quercus glauca*
Site 4	Yanaze, Umaji-mura, Kochi Pref.	33°39′0″N	134°5′40″E	600 m	*Abies firma*, *Pterocarya rhoifolia, Quercus glauca*
Site 5	Iya, Ikeda-cho, Tokushima Pref.	33°56′5″N	133°49′10″E	450 m	*Cryptomeria japonica, Litsea acuminata*
Site 6	Mt. Shokanbetsu, Uryu–cho, Sorachi, Hokkaido Pref.	43°41′50″N	141°38′15″E	650 m	*Betula ermanii, Quercus crispula*, *Ulmus laciniata*

nate) and unbranched linear petals. This species is the only member of sect. *Spuriomitella* H. B., whereas all the other species investigated in this study belong to sect. *Asimitellaria* Wakab. (Wakabayashi, 2001).

Field studies of the four species were carried out at six locations in 2002 and 2003 (Tables 1, 2). All studies at Sites 1 and 2 were conducted during the full range of flowering of *Mitella* whereas those at the other sites were only at the flowering peak. At each study site, *Mitella* plants grew on stream banks in moist riparian forests.

Voucher specimens of the plants were deposited in the Herbarium of Kyoto University (KYO).

Observation and collection of flower visitors

In our preliminary study at Site 1, no insects visited the flowers of *M. acerina* or *M. furusei* at night. Thus, our observations of insects visiting *Mitella* flowers at the six sites were made during the daytime. After observation of their behaviour on the flowers, some insect visitors were easily collected by directly using a bottle and killed with sodium cyanide. We did not use insect nets because the flowers are very delicate and easily crushed. Collection of insect visitors was conducted throughout the period of pollination study (Table 1). Most of the collected insects were preserved separately under dry conditions, and others were kept in 70% ethanol for investigation of their stomach contents.

Dried insect specimens were used for binocular microscopic observation of pollen loads on their bodies. In order to evaluate the pollination effectiveness of each flower-visiting species, we counted the number of pollen grains on each insect's body and classified them into seven categories, based on pollen count: 0, 1–10, 10–50, 50–100, 100–250, 250–500 and >500.

Nectar secretion by flowers enclosed in polyethylene bags was monitored using filter paper wicks (see McKenna & Thompson, 1988) for *M. acerina* on 5.iii.2003 at Site 1, *M. furusei* on 6.iv.2003 at Site 2 and *M. stylosa* on 22.iv.2003 at Site 5. Approximately 1 μL nectar saturates a wick so it enables a rough estimate of nectar quantity per flower.

Intensive observations of the diurnal patterns of insect visits to *Mitella* flowers without insect collection were made for male *M. acerina* (38 fully blooming inflorescences) on 19.iv.2002, 5–6.v.2003, female *M. acerina* (19 fully blooming inflorescences) on 20.iv.2002, and *M. furusei* (12 fully blooming inflorescences) on 26.iv.2002, from 06:00 to 20:00 h (28 h, 14 h, 14 h, respectively), at Site 2. We monitored insect visits over the entire day, keeping more than 1 m distant from the plants in order not to disturb insect behaviour.

Bagging experiments and fruit set

We investigated fruit set of two *Mitella* species (one dioecious, the other gynodioecious) at Sites 1 and 2 in 2002. We tagged 62 inflorescences (51 individuals) of *M. furusei* and 34 (11) of *M. acerina* at Site 1, and 47 (23) of *M. furusei* at Site 2. Ten inflorescences of *M. acerina* and 26 of *M. furusei* (nine at Site 1, 17 at Site 2), which are some part of the inflorescences of each plant studied, were enclosed in nylon bags (0.2-mm mesh, which do not impede the flow of air, and allow for pollen transfer; see Dafni & Dukas, 1986; Gómez & Zamora, 1996) before anthesis. Bagging was conducted on 11.iii at Site 2, and on 6.iv at Site 1. Thus, our experiments involved a bagged treatment group and an open-pollinated control group. The bagged treatment group was kept enclosed during the entire flowering season.

We counted the number of flowers on each tagged inflorescence and identified the sexual expression of

all individuals. Sexual expression of the flowers of both species was easily recognized by the presence or absence of fertile, conspicuous anthers. After fruiting, we counted the number of fruits on each inflorescence and then calculated the percentage fruit set of each inflorescence.

RESULTS

DIOECIOUS *MITELLA ACERINA*

Various insects visited both male and female flowers (Table 3), but only during the daytime. Fungus gnats (four species) visited the flowers most frequently (74.7% of collected insects), and the most frequent (49.4%) visitors were *Coelosia fuscicauda* Okada. Fungus gnats visited the flowers mainly during the morning (07:00–10:00 h) and in the late afternoon (14:00–18:00 h; Fig. 1). On average the total number of fungus gnat visits per inflorescence per day was 2.0 ± 2.9 in male and 2.6 ± 1.9 in female flowers (± SD; N = 38 and 19, respectively). *Coelosia* gnats landed on the flowers with the spurs of their long legs settling on the pinnately branched petals; they then inserted their mouthparts into the bottom of the flowers (Fig. 2). We

Table 3. A list of flower visitors collected on the four species of *Mitella*. Total numbers of both sexes of the dipterans are also shown. The numbers in parentheses are those of individuals with >10 pollen grains on their body. The numbers following '+' are those of individuals in which pollen attachment was undetectable because they were preserved in 70% ethanol

		M. acerina							
Order		M		F		*M. furusei*		*M. stylosa*	*M. integripetala*
Family	Total								
Species	M/F	2002	2003	2002	2003	2002	2003	2003	2002
Coleoptera									
Cerambycidae									
Gaurotes doris	–	1(0)	–	–	–	–	–	–	–
Diptera									
Chironomidae									
Chironomidae sp.	0/1	–	1(1)	–	–	–	–	–	–
Empididae									
Rhamphomyia brunnostriata	12/6	3(1)+4	6(4)	1(0)	–	4(2)	–	–	–
Oedalea sp.	1/0	–	–	–	–	–	1(1)	–	–
Mycetophilidae									
Boletina sp.1*	1/19	–	3(3)	2(1)	–	–	–	–	15(15)
Boletina sp.2*	1/9	0(0)+2	1(1)	4(4)	–	0(0)+1	–	–	2(2)
*Coelosia fuscicauda**	2/37	13(12)+3	13(12)+1	4(3)	5(1)	–	–	–	–
*Gnoriste mikado**	21/14	2(2)	1(1)	4(3)	–	4(4)+2	9(9)	13(13)	–
Mycetophila lineora	0/1	–	–	1(0)	–	–	–	–	–
Syrphidae									
Melanostoma scalare	1/0	–	–	1(0)	–	–	–	–	–
Sphegina japonica	1/0	1(1)	–	–	–	–	–	–	–
Syrphidae sp.	0/1	–	–	–	–	–	–	1(1)	–
Tipulidae									
Erioptera sp.	0/1	1(1)	–	–	–	–	–	–	–
Tipulidae sp.1	1/0	–	–	–	–	1(0)	–	–	–
Tipulidae sp.2	1/0	1(0)	–	–	–	–	–	–	–
Hymenoptera									
Formicidae									
Camponotus japonicus	–	1(0)	–	–	–	–	–	–	–
Trichoptera									
Philopotamidae									
Dolophilodes sp.	–	–	–	–	–	–	–	–	1(1)
Total	42/89	23(17)+9	24(21)+1	17(12)	5(1)	9(6)+3	10(10)	14(14)	18(18)

*Tribe Gnoristini.

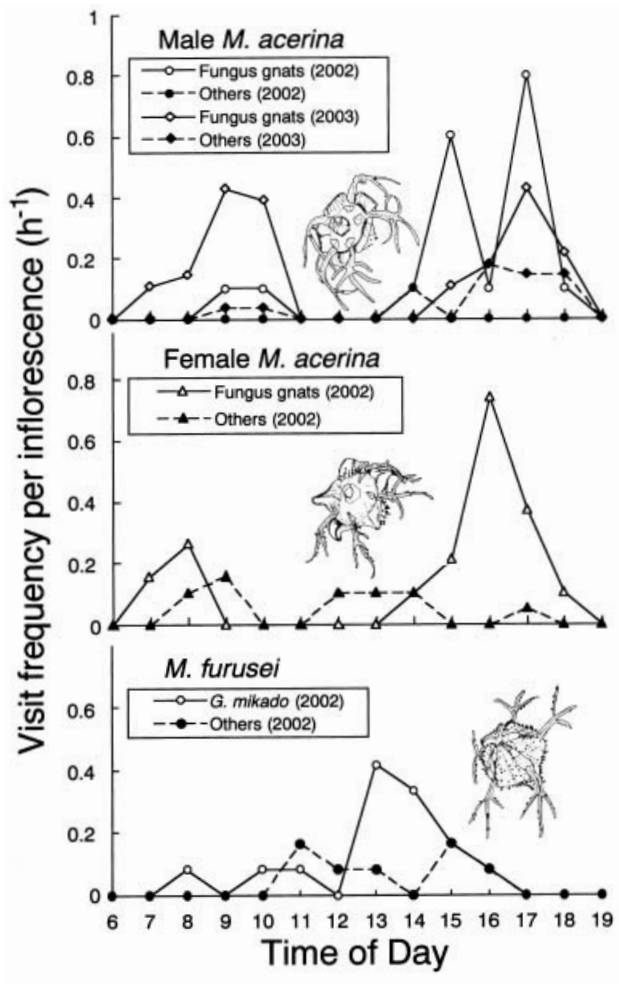


Figure 1. Diurnal patterns of insect visits to *Mitella acerina* flowers and *M. furusei* flowers at Site 1. We observed ten male and 19 female *M. acerina* inflorescences on 19 and 20.iv.2002, respectively, 28 male *M. acerina* inflorescences on 5.v.2003, and 12 *M. furusei* inflorescences on 26.iv.2002. The data points on the *x*-axis are pooled counts for the subsequent hour.

collected a small quantity of nectar (<1 μL per flower per hour) from bagged flowers, and could not find any pollen grains in the stomachs of fungus gnats, whereas all the females of *Rhamphomyia* (Empididae) had numerous pollen grains in their stomachs. When a gnat visits a flower, the frontal surface of its head touched the anthers, and pollen grains became attached to its head, often forming a characteristic cluster (Fig. 6). The gnats often rotated at the flower using the petals as a footing. After inspecting a flower, they moved to other flowers on the same inflorescence. Typically, they visited 1–6 flowers for one visit. After remaining on an inflorescence for about 5 min (mean = 5.2, SD = 7.4, $N = 95$), they flew to another inflorescence. The majority of collected fungus gnats had >50 pollen grains on their heads, whereas *Rhamphomyia* (Empididae) had fewer than 100 pollen grains on their body (Fig. 10A).

Bagged inflorescences bore no fruit, whereas some open-pollinated control inflorescences did (one-sided *t*-test; $t = 13.4$, $P < 0.001$; Table 4), which indicates that wind pollination does not occur in this species. The fruit set of open-pollinated controls was 69 ± 25%, suggesting effective pollen transfer from male to female inflorescences by the fungus gnats.

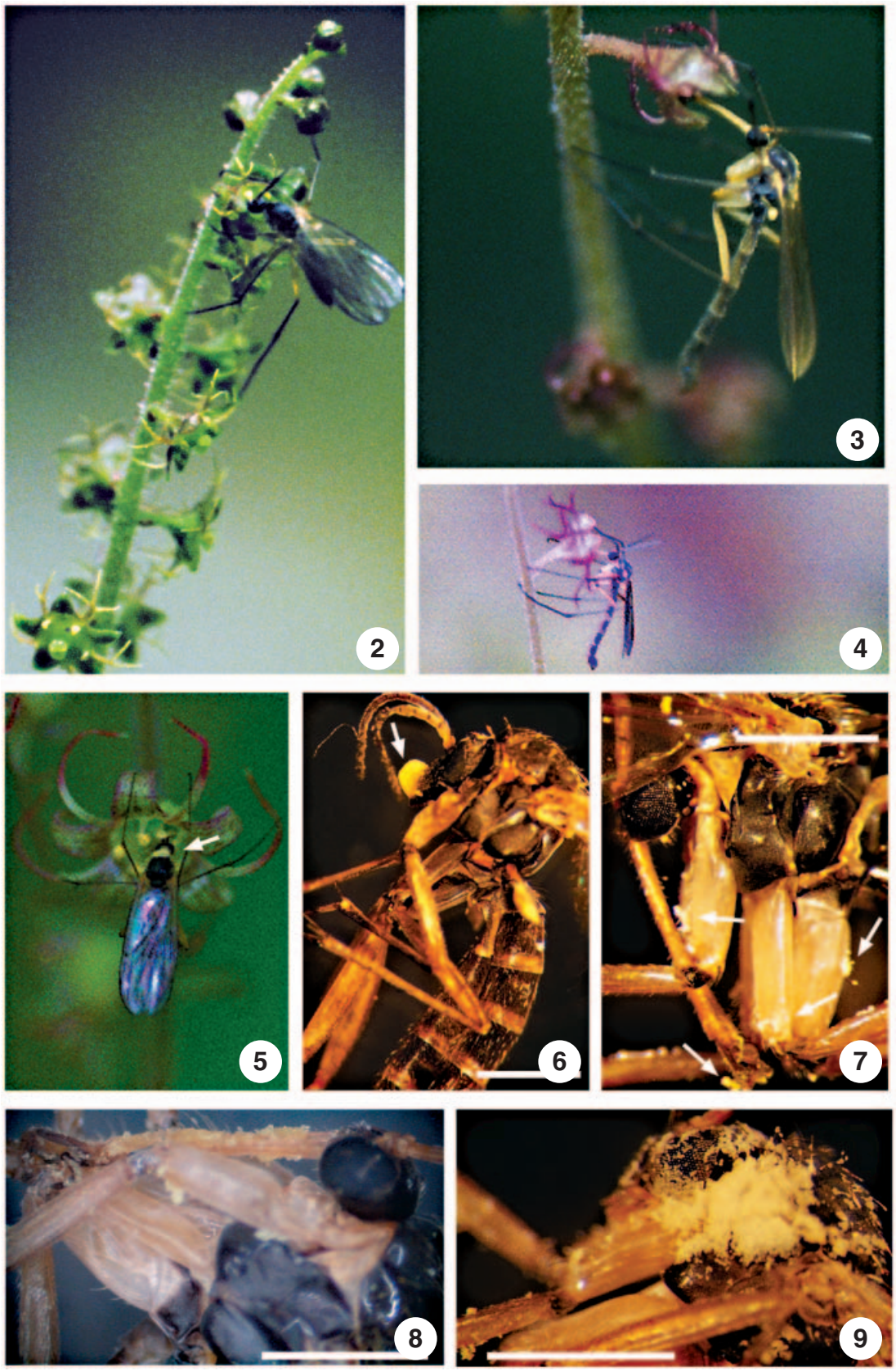
GYNODIOECIOUS *MITELLA FURUSEI* VAR. *SUBRAMOSA*

Five dipteran species infrequently visited both bisexual and female flowers (Table 3) during the daytime only at every site. The most frequent visitor (68%) was *Gnoriste mikado* Okada, a mycetophilid with a long, stout proboscis. These fungus gnats visited the flowers most frequently in the afternoon (13:00–16:00 h; Fig. 1). On average, the total number of insect visits per inflorescence per day was 1.3 ± 1.1 (± SD; $N = 12$). After landing on a flower, a gnat usually inserted its proboscis into the calyx tube as though it were sucking nectar (Fig. 3), and we collected nectar (<1 μL per flower per hour) in bagged flowers. After an average stay of 2.8 min (SD = 2.7, $N = 15$), the gnats flew to other flowers. All collected fungus gnats had >50 pollen grains (Fig. 10B) on their proboscises and legs (Fig. 7). Another dipteran species, *Rhamphomyia brunnostriata* Frey (Empididae), visited the flowers less frequently (18%) and 50% had no pollen grains on their body (Fig. 10B).

Although bagged female inflorescences did not bear fruit, the percentage fruit set of bagged bisexual inflorescences was 31 ± 22% and 54 ± 21% at sites 1 and 2, respectively (Table 4). These results show that bisexual inflorescences can bear fruit by autonomous self-pollination, and that wind pollination did not occur on female inflorescences. The fruit set of open-pollinated bisexual inflorescences was 71 ± 19% at Site 1 and 64 ± 23% at Site 2, which was higher than the fruit set of bagged bisexual inflorescences, although the differences were only significant at Site 1 (one-sided *t*-tests, Site 1: $t = 3.81$, $P < 0.01$; Site 2: $t = 1.01$, $P = 0.16$; Table 4). The fruit set of open-pollinated female inflorescences did not differ from that of open-pollinated bisexual inflorescences, at both Site 1 and Site 2 (two-sided *t*-tests, Site 1: $t = 0.39$, $P = 0.70$; Site 2: $t = 0.12$, $P = 0.90$).

HERMAPHRODITIC *MITELLA STYLOSA* VAR. *MAKINOI*

We recorded over ten insect visits to the flowers during our observation from 08:00 to 11:00 h at Site 4 in 2002. All but two (Dermaptera and Trichoptera) of the visi-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Figures 2–9. *Mitella* flowers visited by fungus gnats and pollen attachment on their bodies. Fig. 2. *Coelosia fuscicauda* visiting a *M. acerina* flower. Note that the head of the insect is touching an anther. Fig. 3. *Gnoriste mikado* visiting an *M. furusei* flower with its long proboscis inserted in the calyx tube. Fig. 4. *G. mikado* visiting a *M. stylosa* flower. Fig. 5. *Boletina* sp.1 visiting a *M. integripetala* flower. The white arrow indicates the pollen load on the lateral side of the thorax and head. Fig. 6. *C. fuscicauda,* collected on a *M. acerina* flower. The white arrow indicates a characteristic cluster of pollen grains on the head of the insect. Fig. 7. *G. mikado* collected on a *M. furusei* flower. White arrows indicate the pollen load on its long proboscis and legs. Fig. 8. *G. mikado* collected on a *M. stylosa* flower. Note that numerous pollen grains (>1000) cover the proboscises and some also attach to the legs. Fig. 9. *Boletina* sp.1 collected on a *M. integripetala* flower. Numerous pollen grains (>1000) cover the lateral side of its head, thorax and legs. Figs 6–9, scale bars = 1 mm.

tors were mycetophilids, *Gnoriste mikado* (Fig. 4). We again recorded over 20 insect visits to the flowers during our diurnal observation at Site 5 in 2003. At Site 5, all but one of the visitors were *Gnoriste mikado* also. The behaviour of *Gnoriste* gnats was similar to their observed behaviour on *M. furusei* flowers, and they visited the flowers most frequently in the afternoon (13:00– 17:00 h). We collected small quantities (<1 μL per flower per hour) of nectar from bagged flowers. Although we failed to collect the flower visitors at Site 4, we collected 14 dipterans at Site 5 in 2003 (Table 3). All *Gnoriste* gnats collected on flowers had >50 pollen grains on their proboscises and legs (Figs 8, 10C), whereas the other visitor, *Baccha maculata* Walker (Syrphidae), had only 15 pollen grains.

Hermaphroditic *Mitella integripetala*

Female fungus gnats of two mycetophilid species, *Boletina* sp.1 (83%) and *Boletina* sp.2 (11%), visited the bisexual flowers almost exclusively during the early morning (06:30–10:00 h) and late afternoon (16:30– 18:00 h). The fungus gnats flew to the flowers and usually hovered for a while before landing. They typically landed on the flowers with their legs elongated on the linear petals, and inserted their mouthparts into the cup-shaped calyx (Fig. 5). The gnats stayed on a flower for 1–5 min, and then either flew to the next flowers (often in another inflorescence) or flew away. All fungus gnats collected on flowers (15 *Boletina* sp.1 and two *Boletina* sp.2) had >90 attached pollen grains (Fig. 10D), especially on the lateral sides of the head and thorax (Fig. 9).

DISCUSSION

Pollination systems of *Mitella* spp.

Fungus gnats were the most frequent (68–94%) visitors to the four *Mitella* species that we studied. They visited the flowers only in the daytime (Fig. 1), and their heads or proboscises consistently touched the anthers and stigmas during their visits. A large proportion of collected fungus gnats had numerous pollen grains (Fig. 10) on the head, thorax and legs (Figs 6–9). Because the plants usually produce fewer than 70 seeds per fruit, the number of pollen grains on the insect bodies is large enough to explain the observed proportions of seed sets. These results indicate that fungus gnats are the principal pollen vectors of the four *Mitella* spp.

Bagged female flowers of dioecious *M. acerina* and gynodioecious *M. furusei* did not bear fruit, indicating that wind pollination did not occur. The fruit set of open-pollinated female flowers of these two *Mitella* species (63–69% and 69%, respectively) was as high as that of bisexual flowers of *M. furusei* (64–71%). This suggests that fungus gnats make a large contribution to cross-pollination among *Mitella* species. Bagged bisexual flowers of *M. furusei* set fruit as well, suggesting partial autogamy, although the percentage fruit set was lower than that of open-pollinated controls.

Characteristics of the pollination systems in *Mitella*

What is the role of the linear, pinnately branched petals in *Mitella* flowers? This was one of the primary questions of our study. We now believe that one of the roles of the petals is to provide a footing for the fungus gnats on the flowers. The Mycetophilidae have characteristically slender legs with many long spurs. The linear, pinnately branched petals of *Mitella* function as a workable footing for the spiny legs of fungus gnats. In addition, the pinnately branched petals orientate the gnats to specific directions on the flowers. Accordingly, the insects always touch the anthers or stigmas in a stereotypic way (Figs 2–5).

Although fungus gnats were the primary pollinators of all four *Mitella* species, there were some differences among the fungus gnat species that pollinated each *Mitella* sp. The species with tubular flowers, *M. furusei* and *M. stylosa*, were visited and pollinated almost exclusively by *Gnoriste mikado*, a gnat with a long (up to 2.4 mm) proboscis (Table 3; Figs 3, 4). The tubular flowers of *M. furusei* and *M. stylosa* seemed to be well adapted and specialized to the long proboscis of *Gnoriste*. By contrast, the other *Mitella* species (*M. acerina* and *M. integripetala*), which have saucer-

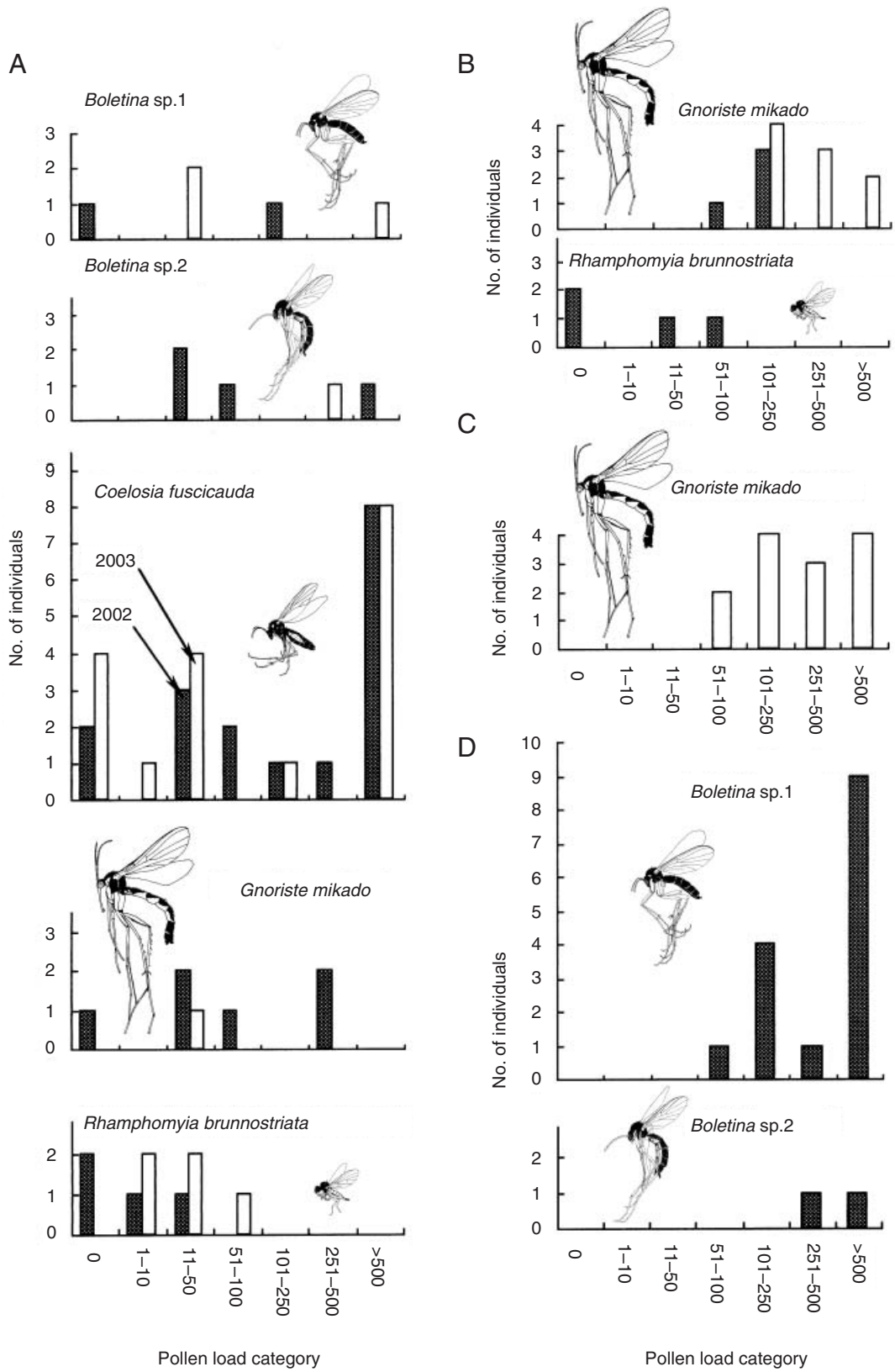

Figure 10. Frequency distributions of pollen loads on each insect species collected on *Mitella* flowers in 2002 and 2003. A, *M. acerina*; B, *M. furusei*; C, *M. stylosa*; D, *M. integripetala*.

Table 4. Percentage fruit set of *M. acerina* and *M. furusei* at Site 1 and Site 2. We show here the average proportions (% ± SD) of fruited flowers among inflorescences (*N* = number of inflorescences; total numbers of flowers are shown in parentheses)

	M. acerina		*M. furusei*			
	Female flower		Bisexual flower		Female flower	
	Bagged	Control	Bagged	Control	Bagged	Control
Site 1	0 ± 0%*	69 ± 25%	31 ± 22%*	71 ± 19%	0 ± 0%*	69 ± 24%
	N = 10(332)	*N* = 24(808)	*N* = 5(100)	*N* = 29(711)	*N* = 4(89)	*N* = 24(534)
Site 2	–	–	54 ± 21%†	64 ± 23%	0 ± 0%*	63 ± 16%
	N = 11(273)	*N* = 22(579)	*N* = 6(163)	*N* = 8(223)		

*The value is significantly lower than that of control (one-sided *t*-test, $P < 0.01$).
†The value is not significantly lower than that of control (one-sided *t*-test, $P = 0.16$).

or cup-shaped flowers, were mostly visited and pollinated by fungus gnats with short (<0.3 mm) mouthparts (Table 3; Figs 2, 5). This difference in flower visitors cannot be attributed to differences in the insect fauna among sites, because *M. furusei* and *M. acerina* co-occurred at the same site, but rather to different pollination systems. We found a few natural hybrids between *M. acerina* and *M. furusei* at Site 1, as is typical in the *Heuchera* group (Soltis *et al*., 1991). Thus, some interspecific pollen transfer does occur (and the vector is probably *Gnoriste*). Nevertheless, the most effective pollinators were differentiated between *M. acerina* and *M. furusei* (Fig. 10 A, B).

We also found some differences between the pollination systems of *M. acerina* and *M. integripetala*. Pollen grains of *M. acerina* often formed a characteristic (pollinium-like) cluster on the frontal surface of the head of the principal visitor, *Coelosia fuscicauda* (Fig. 6). By contrast, pollen grains of *M. integripetala* became attached laterally on the sides of the head and thorax of the primary visitor, *Boletina* sp.1 (Fig. 9). In addition, the two *Mitella* species have different floral morphologies. The flowers of *M. acerina* are much smaller, with anthers alternate to the calyx lobes, and with 3–5-pinnately branched petals (Fig. 2). By contrast, those of *M. integripetala* are larger, with anthers opposite to the calyx lobes, and with simple linear petals (Fig. 5). The difference in the anther/petal arrangement between *M. acerina* and *M. integripetala* may be related to the difference in the position of pollen attachment to the pollinators, i.e. frontal vs. lateral.

Although pollination by fungus gnats is rare in angiosperms, some examples have been reported. Aroids of the genus *Arisaema* are well known for their lethal 'kettle trap' pollination mechanisms, and their most important pollinators are fungus gnats and related dark-winged fungus gnats (Sciaridae; Vogel, 1978b; Vogel & Martens, 2000). *Arisarum* aroids have a similar pollination system (Vogel, 1978b; Vogel & Martens, 2000). The genus *Asarum* and related *Heterotropa* (Aristolochiaceae) are pollinated by female fungus gnats (Vogel, 1978a; Sugawara, 1988). Females visiting the plants often oviposit on the inner surface of the calyx tube, which resembles the gills of a mushroom, i.e. host of fungus gnat larvae (Vogel, 1978a; Sugawara, 1988). Male fungus gnats pollinate the Australian orchid *Pterostylis*. The orchid attracts a male gnat with an insect-like lip; once the gnat touches the base of the orchid, its lip springs up and traps the insect with its back against the column (Coleman, 1934; Sargent, 1934; Proctor *et al*., 1996).

In every example described above, the flowers are thought to be mimicking oviposition sites (such as fungi) or mating counterparts of insects. However, the pollination mechanism in *Mitella* is apparently different from these examples; the flowers have no insect-trapping structure, and flower-visiting fungus gnats never attempt to oviposit on the *Mitella* flowers or mate with them.

Pollination by fungus gnats without trapping mechanisms has been observed in *Listera* (Orchidaceae) and *Scoliopsis* (Liliaceae) in the coastal redwood forests of California (Ackerman & Mesler, 1979; Mesler *et al*., 1980). These authors reported high rates of fruit set among *Listera* (61–78%) and *Scoliopsis* (94.3–98.5%) that are similar to those of *Mitella* (63–71%; Table 4). However, they suggested that the high reproductive success of the plants could not be attributed to a close morphological fit between the insects and the plants or to consistent foraging behaviour. They concluded that the inefficiency of fungus gnats as pollinators was compensated for by a larger number of visits. By contrast, the foraging behaviour of fungus gnats on *Mitella* flowers was complex, and there was a close morphological fit between the insects and the plants,

as discussed above. In addition, the observed frequency of visits was much lower than that for the two plant species in the redwood forests (fewer than three visits per inflorescence per day in *Mitella* vs. many more than five visits per flower per day in *Scoliopsis*; Fig. 1). Thus, fungus gnats are probably not only the principal but also the most efficient pollinators of the *Mitella* species.

How do the *Mitella* flowers attract the fungus gnats, and why do the insects visit them? The attractant is possibly the scent, because most flowers pollinated by fungus gnats are known to attract the insects with their foul odour (Ackerman & Mesler, 1979; Mesler *et al*., 1980; Sugawara, 1988; Vogel & Martens, 2000). We found, at least, *M. furusei* and *M. integripetala* emitted a faint, foul odour. The fact that only three genera of the tribe Gnoristini (Mycetophilidae) are involved in pollination is noteworthy (Table 3), because the other flowers pollinated by fungus gnats are known to attract various genera of Mycetophilidae and even sciarid gnats (Ackerman & Mesler, 1979; Mesler *et al*., 1980; Vogel & Martens, 2000). By sweeping riparian vegetation, we confirmed that diverse fungus gnats including the three genera exist at the same study sites and time periods (Y. Okuyama, unpubl. data). Thus, the floral odour may function as very specific cue for the pollinator gnats.

The floral rewards for the insects are, probably, not pollen grains but nectar. We found that the fungus gnats did not feed on pollen grains, and the fungus gnats observed at the flowers seemed to be sucking nectar. We also found that the flowers of *Mitella* species secreted a very small quantity of nectar, which was only detectable by using small filter paper wicks. Nevertheless, our knowledge of the biology of Mycetophilidae, e.g. brood sites, mating systems or adult diets, is very limited. Whether the specific relationship between the fungus gnats and *Mitella* species is wholly mutualistic is a subject for future studies.

In conclusion, the fungus gnat pollination system of *Mitella* is characteristic in that the pollinators and the flowers show a close morphological fit; pollinator species differ somewhat among *Mitella* spp.; the flowers have no insect-trapping mechanisms; the low frequency of the insect visits is compensated for by efficient cross-pollination; and almost only the specific genera of Mycetophilidae contribute to the pollination.

Evolution of pollination systems in the *Heuchera* group

The *Heuchera* group, i.e. the relatively large subclade in the Saxifragaceae to which *Mitella* belongs, comprises nine genera with diverse floral morphologies (Soltis *et al*., 2001), whereas the remaining eight genera usually bear more showy flowers than *Mitella*. Several studies have investigated the pollination systems in this subclade. For example, a prodoxid moth species, *Greya mitellae*, which feeds on *M. stauropetala* (which is a white, showy-flowered species related to the genus *Conimitella* rather than other *Mitella* spp., which bear dull-coloured flowers; see Soltis & Kuzoff, 1995), is the only pollinator of its host plants (Pellmyr *et al*., 1996). Staphylinid beetles pollinate the flowers of *Tellima*, whereas bumblebees pollinate the flowers of *Tolmiea* (Weiblen & Brehm, 1996). Bumblebees, solitary bees, bombylid flies and ovary-parasitic *Greya* moths are the principal pollinators of *Heuchera* and *Lithophragma* (Thompson & Pellmyr, 1992; Segraves & Thompson, 1999; Thompson, 1999). These pollination systems differ from those of *Mitella* spp. pollinated by fungus gnats. In addition, self-incompatibility has been reported in moth-pollinated *M. stauropetala* (Pellmyr *et al*., 1996), bee-pollinated *Heuchera*, *Lithophragma* and *Tolmiea* (Rabe & Soltis, 1999), but *M. furusei* is self-compatible. These fundamental differences in floral traits between gnat-pollinated *Mitella* and the other species probably reflect differences in their pollination systems. In turn, the differences among the pollination systems may reflect differences among their habitats. Fungus gnats are most abundant in moist riparian woodlands, and during the day emerged adults congregate around stream banks (Søli, Vockeroth & Matile, 1997), where *Mitella* usually grows. Savile (1953, 1975) described the seed-dispersal mechanisms of some species of the *Heuchera* group (including *Mitella*), and noted that the mechanisms are strongly related to their habitats. We believe that the pollination system of *Mitella* is also related to its riparian habitat (see Pellmyr *et al*., 1996; which suggests the paucity of anthophilous insects in the riparian habitats). The habitats of the species of the *Heuchera* group range from moist riparian woodlands to drier localities such as grasslands, prairies, rocky soils, cliffs and sagebrush deserts (Hitchcock & Cronquist, 1961). The differences in habitat among the species in the *Heuchera* group may have caused the diversification of their floral traits.

We believe that the unusual floral trait in the genus *Mitella* is an adaptation to pollination by fungus gnats. Because the pollination systems of other *Mitella* species in other clades with similar floral traits are still unknown, we cannot elucidate the origin of the gnat pollination system in the *Heuchera* group. Because of their ancient origins, Nematocera (a basal suborder of Diptera that includes fungus gnats) may be contenders for being early pollinators of primitive angiosperms in early geological times (Larson, Kevan & Inouye, 2001), although in recent times, fungus gnats have been found to be effective pollinators of some plants in moist places (Ackerman & Mesler, 1979; Mesler *et al*., 1980; Proctor *et al*., 1996; reviewed

in Larson *et al*., 2001). Further studies of the pollination systems within the *Heuchera* group will reveal the origin of the fungus gnat pollination system and the diversification process of plant clades pollinated by fungus gnats.

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