

Pollination by fungus gnats and associated floral characteristics in five families of the Japanese flora

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- **Background and aims** Pollination by fungus gnats (Mycetophilidae and Sciaridae) is uncommon, but is nevertheless known to occur in 20 genera among eight angiosperm families. Because many fungus gnat-pollinated plants possess a dark red floral display, we hypothesized that fungus gnat pollination is more widespread among plants with similar floral display than currently known. We thus studied the pollination biology of flowers with dark red pigmentation in five families, focusing particularly on plants having small, flat, actinomorphic flowers with exposed nectaries and short stamens, because these floral characteristics mirror those of a known fungus gnat-pollinated genus (*Mitella*).
- **Methods** We observed daytime and night-time floral visitors for a total of 194.5 h in *Aucuba japonica* (Garryaceae), *Euonymus* spp. (Celastraceae), *Disanthus cercidifolius* (Hamamelidaceae), *Micranthes fusca* (Saxifragaceae) and *Streptopus streptopoides* (Liliaceae). Visitors were categorized into functional groups, and a pollination importance index (PII) was calculated for each functional group based on visitation frequency, pollen load and behaviour on flowers.
- **Key results** Fungus gnats were dominant among the 1762 insects observed (36–92 % depending on the plant species) and were the most important pollinators among all plants studied (PII: 0.529–1). Fungus gnat visits occurred during the daytime and, more frequently, at dusk. Most often, pollen grains became clumped on the ventral side of the head and/or thorax as the short-proboscid fungus gnats foraged on nectar and came into contact with anthers located close to the flower base.
- **Conclusions** Pollination by fungus gnats is probably more common than previously thought, especially in habitats similar to those of the plants studied (moist forest understorey, streamside or subalpine meadow) where fungus gnats are abundant year-round. Our results further suggest that there may be a previously unnoticed association between fungus gnat pollination and dark red coloration, and a shared overall floral architecture among the plants studied.

Keywords: *Aucuba japonica*, dark red flower, Diptera, *Disanthus cercidifolius*, *Euonymus*, forest environment, fungus gnat, *Micranthes fusca*, Mycetophilidae, pollination syndrome, Sciaridae, *Streptopus streptopoides*.

INTRODUCTION

Dipteran insects are among the most common visitors to angiosperm flowers and often play key roles as pollinators in many plant communities (Kearns, 2001; Larson *et al.*, 2001; Szymank *et al.*, 2008; Lefebvre *et al.*, 2014; Inouye *et al.*, 2015; Orford *et al.*, 2015; Ollerton, 2017). However, the importance of dipteran pollination is possibly still underestimated, because research continues to uncover plants that depend strongly on dipterans for pollination, some of which attract dipteran pollinators by previously unsuspected mechanisms (Stöckl *et al.*, 2011; Ren *et al.*, 2012; Song *et al.*, 2014; Oelschlägel *et al.*, 2014; Heiduk *et al.*, 2016). Small dipteran insects, such as fungus gnats, gall midges and drosophilid flies, are particularly difficult to observe in the field, and thus their roles as pollinators are probably not fully appreciated (Larson *et al.*, 2001; Goldblatt *et al.*, 2004; Woodcock *et al.*, 2014).

Fungus gnats, or flies in Mycetophilidae, Sciaridae and related Sciaroidea families, are small, weak-flying insects common in moist forest and riparian habitats. Their larvae feed

on mushrooms, fungal mycelia in rotten wood, decaying plant material and, more rarely, bryophytes or other insects (Vockeroth, 1981; Steffan, 1981; Sjøli *et al.*, 2000; Jakovlev, 2011, 2012). Fungus gnats are usually considered as ineffective pollen carriers due to their small bodies, inconstancy and weak flight ability (Mesler *et al.*, 1980; Proctor *et al.*, 1996; Larson *et al.*, 2001; Willmer, 2011). Nevertheless, pollination by fungus gnats is known to occur in eight angiosperm families: Orchidaceae, Liliaceae, Asparagaceae, Araceae, Aristolochiaceae, Polygonaceae, Apocynaceae and Saxifragaceae (Vogel, 1973; Jones, 1974; Ackerman and Mesler, 1979; Mesler *et al.*, 1980; Sugawara, 1988; Fuller, 1994; Vogel and Martens, 2000; Goldblatt *et al.*, 2004; Okuyama *et al.*, 2008; Yamashiro *et al.*, 2008; Barriault *et al.*, 2010; Duque-Buitrago *et al.*, 2014; Ollerton *et al.*, 2017; Suetsugu and Sueyoshi, 2017). Some of these plants are believed to mimic fungal oviposition sites because pollinator eggs are often observed on the flowers (Vogel, 1978; Sugawara, 1988; Scanlen, 2006), whereas others employ sexual deception (Blanco and Barboza, 2005; Phillips *et al.*, 2014), offer nectar reward (Ackerman and Mesler, 1979;

Mesler *et al.*, 1980; Goldblatt *et al.*, 2004; Okuyama *et al.*, 2004) or even sacrifice developing ovules to seed-feeding pollinator larvae to accomplish the pollination (Song *et al.*, 2014).

Despite the diversity of mechanisms used by these plants to attract fungus gnats, several fungus gnat-pollinated plants share a characteristic floral display: petals or spathes with dark red pigmentation (Supplementary Data, Table S1). Dark red flowers are otherwise common in sapromyophilous plants (Fægri and van der Pijl, 1979; Willmer, 2011; Johnson and Schiestl, 2016; Shuttleworth *et al.*, 2017) and are also known in sexually deceptive orchids (Gaskett *et al.*, 2017) and some fly- and beetle-pollinated plants (Young, 1985; Sakai and Inoue, 1999; Teichert *et al.*, 2012; Policha *et al.*, 2016; Luo *et al.*, 2017); however, knowledge of an association between such a floral display and any particular pollination system remains fragmentary. This observation motivated us to examine the floral visitors of dark red-flowered *Aucuba japonica* (Garryaceae) in 2014. We found that *A. japonica* flowers were heavily visited by fungus gnats and noticed that the flowers of *Aucuba* resemble those of the known fungus gnat-pollinated genus *Mitella* (Okuyama *et al.*, 2004) in several ways: they are small (5–8 mm in diameter), flat and actinomorphic, with exposed nectaries, short stamens and dark red floral displays. We thus examined if plants in other families with floral characteristics similar to those of *Mitella* and *Aucuba* are also fungus gnat-pollinated.

We searched for plants that share the above set of floral characteristics in the Japanese flora. Consequently, we studied the pollinators of the following six shrubs and herbs: *Aucuba japonica* (Garryaceae), three *Euonymus* species (Celastraceae), *Disanthus cercidifolius* (Hamamelidaceae) and *Micranthes fusca* (Saxifragaceae) (Figs 1 and 2). Additionally, we studied *Streptopus streptopoides* (Liliaceae) because, although its flowers are mostly greenish and have only small areas of dark red pigmentation toward the tepal base, the overall floral morphology is very similar to those of *Mitella*, suggesting the possibility of fungus gnat pollination (Figs 1 and 2). Based on a 3-year field study, we report that these seven studied plants are predominantly pollinated by fungus gnats. Our findings point to a possibility that a certain set of floral characteristics is associated with fungus gnat pollination.

MATERIALS AND METHODS

Study materials and study sites

Aucuba (Garryaceae) is a genus of evergreen dioecious shrubs comprising ten species distributed from the Himalayas to temperate East Asia (The Plant List, 2013). *Aucuba* is one of three genera that constitutes the order Garryales, the other two being predominantly wind-pollinated: *Garrya* (Garryaceae) and *Eucommia* (Eucommiaceae) (Dahling, 1978; Stull *et al.*, 2015). Unlike those of the latter two genera, *Aucuba* flowers possess an open, prominent nectar disc, which is suggestive of an entomophilous pollination system (Abe, 2001; Liston, 2003). The Japanese laurel *A. japonica*, a shrub up to 2 m in height, is one of the most common components of the moist, shady floor of temperate deciduous, temperate and subtropical evergreen forests in Japan (Fig. 1A). The flowers of *A. japonica* are flat

and small, with dark red petals (Figs 1B and 2A, B). *Aucuba japonica* is one of the first woody plants to flower in early spring (March–May).

The spindle tree genus *Euonymus* comprises about 130 species with a broad distribution range, including North America, Europe, Asia, Australia and Madagascar (Ma, 2001). Most species are small trees or shrubs growing in a variety of habitats including the forest edge, forest floor and rocky coast. The flowers are small and typically greenish or yellowish; however, roughly 30 species have red, purple or chocolate-coloured flowers (Ma, 2001). To date, 18 *Euonymus* species have been recorded in Japan. These are typically small trees occurring on forest edges or within forests, whereas some are shrubs or shrublets on the forest floor. Four endemic species, *E. lanceolatus*, *E. melananthus*, *E. tricarplus* and *E. yakushimensis*, have red flowers and prefer the shaded forest floor or, occasionally, subalpine/boreal meadows. Inflorescences of *Euonymus* are cymes of up to ten flowers, developing on leaf axils. The flower possesses a prominent nectary disc that surrounds the pistils and stamens. Filament length varies among species; however, species with dark red flowers invariably have a very short to almost no filament.

Euonymus lanceolatus is an evergreen dwarf shrub that grows in shaded forests on the Sea of Japan side of the Japanese mainland, where there is heavy snow in winter. The plants are 0.5 m high and have flexible stems that are pressed flat against the ground under snow (Ma, 2001; Fig. 1C). During June and July (rainy season), each plant produces a few to dozens of flowers that open downwardly.

Euonymus melananthus is a small, deciduous shrub (up to 1 m) growing on the shaded riparian forest floor, having a wide distribution in cool-temperate areas of Japan, but occurring only infrequently. The plants resemble lianas, growing from chinks in the rocks along mountain streams. The flowers are dark red and 7–9 mm in diameter, blooming from June to July (Fig. 1D). In one of the study populations (Mt Haku-san), *E. melananthus* and *E. lanceolatus* grow in close proximity, and *E. melananthus* flowers earlier than *E. lanceolatus*, although their flowering periods partly overlap.

Euonymus tricarplus is a deciduous shrub up to 2–3 m in height and often found beneath the shade of other vegetation in subalpine or boreal meadows in northern Japan. The plants produce hundreds to thousands of flowers (8–10 mm in diameter) during June and July. Their flower colour is variable compared to those of *E. lanceolatus* and *E. melananthus*, ranging from pink to dark red.

Disanthus cercidifolius (Hamamelidaceae) is a deciduous shrub up to 2–3 m in height, often found growing along streams in temperate forests in very limited areas of Japan and China. *Disanthus cercidifolius* is one of two species in the genus *Disanthus*; the other is the recently described Vietnamese species *D. ovatifolius* (Averyanov *et al.*, 2017). *Disanthus cercidifolius* produces flowers from November to December when the leaves have already turned red (Fig. 1E). Its flowers are hermaphroditic and self-compatible, and produce nectar on the basal areas of the petals. Xiao *et al.* (2009) reported drosophilid and syrphid flies as pollinators of *D. cercidifolius*; however, their observations were conducted only during the daytime.

Micranthes fusca (Saxifragaceae) is a hermaphroditic perennial endemic to Japan, which grows on moist rocks along

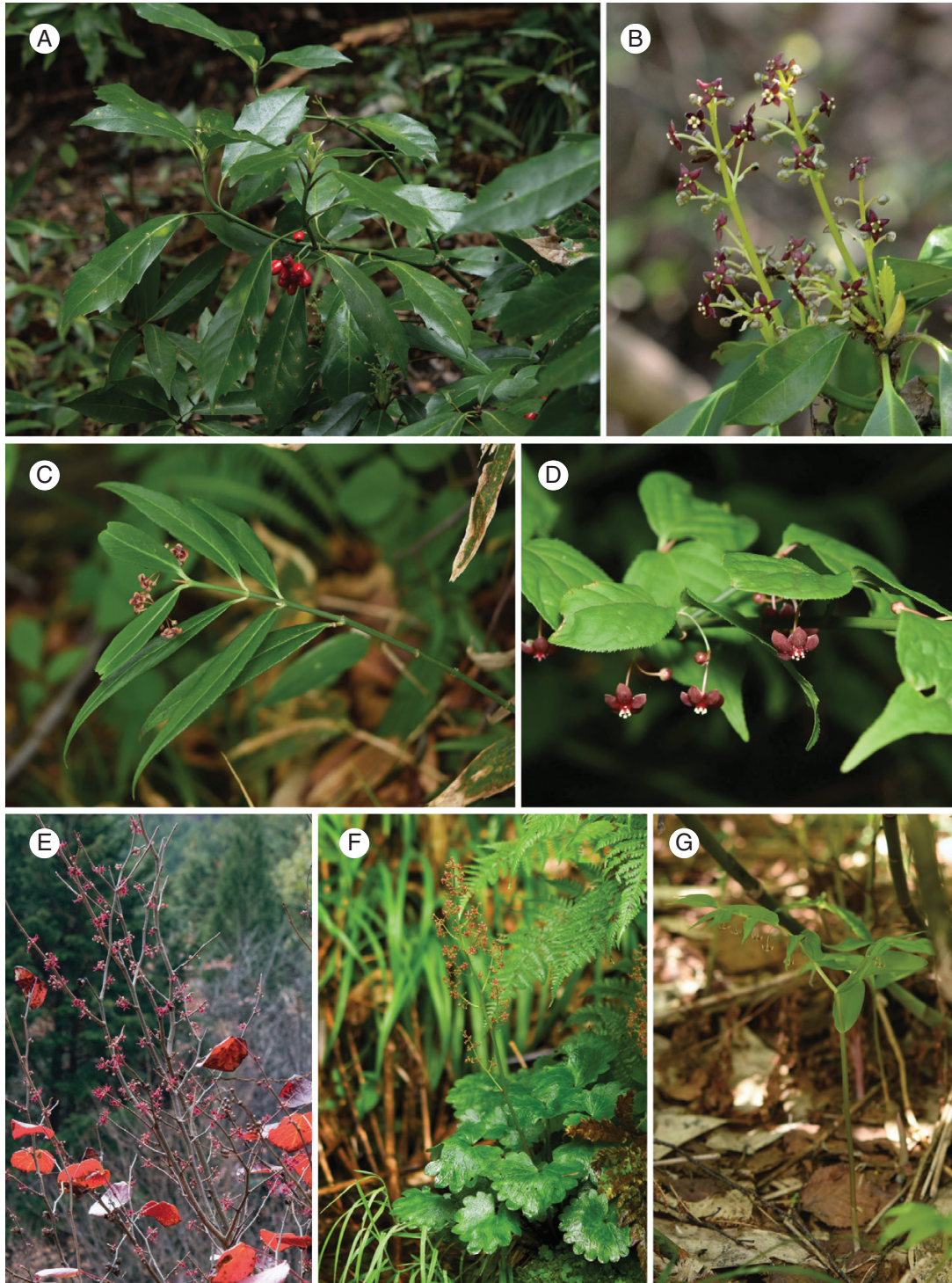


FIG. 1. Habitats and inflorescences of the studied plants: (A) *A. japonica* in forest understorey; (B) inflorescences of *A. japonica* (male plant); (C) *E. lanceolatus* on forest floor; (D) flowering branch of *E. melananthus*; (E) *D. cercidifolius* on streamside vegetation; (F) *M. fusca* on streamside rock in subalpine meadow; (G) *S. streptopoides* under shade of bamboo grass.

mountain streams or in alpine vegetation. The plants produce dark red flowers 5–8 mm in diameter on the approx. 30-cm thyse from July to September (Fig. 1F). The flower comprises a conspicuous, fleshy floral disc surrounded by ten stamens. The stamens are initially spread radially on the flat floral plane, which are bent upwardly as the anthers dehisce.

The Japanese twisted-stalk *Streptopus streptopoides* (Liliaceae) is a small perennial up to 50 cm in height, which grows in the shade of subalpine vegetation in central Japan (Fig. 1G). These plants produce single downward-facing flowers beneath the leaves from June to July. The tepals are generally greenish but have dark red markings toward the centre of the flower.

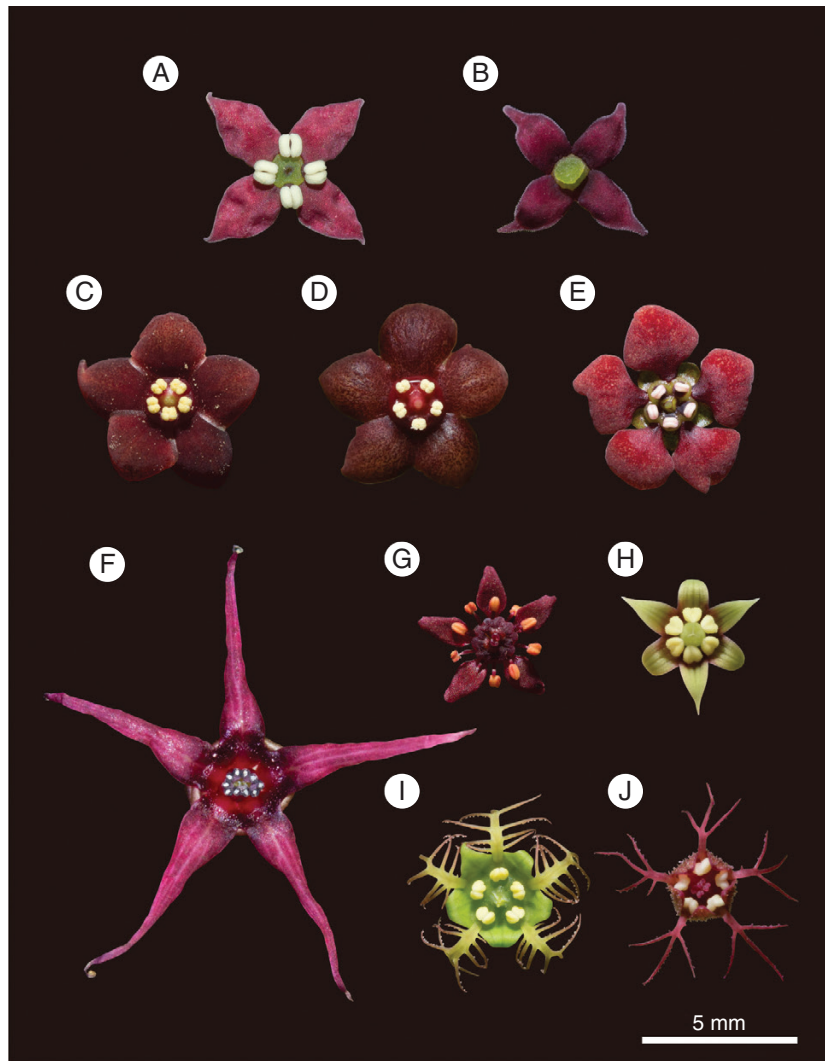


FIG. 2. Flowers of the studied plants: (A) *A. japonica* (male flower); (B) *A. japonica* (female flower); (C) *E. lanceolatus*; (D) *E. melananthus*; (E) *E. tricarpus*; (F) *D. cercidifolius*; (G) *M. fusca*; (H) *S. streptopoides*; (I) *Mitella koshiensis*, which has previously been reported to be pollinated by short-proboscid fungus gnats; (J) *Mitella furusei*, which has previously been reported to be pollinated by long-proboscid fungus gnats.

The nectary is located on the tepal base. Phylogenetic studies have revealed that *Streptopus* is closely related to bee-pollinated *Prosartes* and fungus gnat-pollinated *Scoliopus* (Mesler *et al.*, 1980, 2010).

Detailed information on the study sites and dates of data collection are provided in the Supplementary Data (Table S2).

Flower visitors and pollen load analysis

We conducted field observations of flower visitors from 2015 to 2017 in Japan. Flower visitors were observed during the daytime and night-time from 0500 to 2200 h at peak flowering for each species. For each observation, we selected three to ten flowering individuals and made 5–10 min observations of each, in turn. For night-time observations, a flashlight was used. Although there is a possibility that the lighting affected insect visitation, floral visitors were generally insensitive to

light when they were foraging on flowers. All flower visitors were captured; however, diurnal visitors of *E. lanceolatus*, *D. cercidifolius*, *M. fusca* and *S. streptopoides* were very sensitive to the presence of the observer, so some visitors to these species could not be captured. When visitors were not captured, their number and taxa were recorded. Visitors were captured mainly using a glass vial or aspirator, and some visitors of *E. lanceolatus* and *S. streptopoides* were captured using a small hand-made insect net for the reasons described above. Although the insect net facilitated the capture of sensitive fungus gnat visitors, their body pollen was lost when the fungus gnats struggled within the small net. Therefore, we did not include pollen load data for visitors captured using the insect net.

Flower visitors were identified to the family level, and fungus gnats belonging to Mycetophilidae, which were the most important pollinators among the studied species (see Results), were further identified to the genus level. Although

Sciaridae may contain several fungus gnat genera, including *Bradysia* and *Scatopsciara*, we assigned them all as Sciaridae spp. without separating them to morphospecies, due to the difficulty of distinguishing genera and species based on external morphology. Fungus gnats were also sexed. To facilitate interpretation of the results, flower visitors were classified into functional groups based on their taxonomy, morphology and behaviour on flowers (Fenster *et al.*, 2004). We established ten functional groups: fungus gnat, crane fly, other Nematocera, hover fly, non-syrphid Brachycera, Coleoptera, ant, non-ant Hymenoptera, Lepidoptera and the remaining short-proboscid insects, including Trichoptera, Neuroptera and Collembola. Hemipteran insects were not categorized into functional groups because they were rare (only two individuals were observed) and carried no pollen.

To assess the relative importance of floral visitors as pollinators, the pollinator importance index (PII) of each functional visitor group was calculated following Lindsey (1984), Hansen and Molau (1994), and Johnson *et al.* (2009). PII is based on four parameters: (1) relative abundance (A); (2) pollen-carrying capacity (PCC), expressed as the average number of pollen grains carried; (3) host fidelity (F), expressed as the average proportion of the pollen load containing host pollen; and (4) pollinator effectiveness (PE), the probability that the foraging behaviour of a given visitor results in pollination (Lindsey, 1984). Lindsey (1984) assigned each visitor group with 11 PE values ranging from 0 to 1 at 0.1 intervals based on insect size, behavioural and morphological fit with the flower, and movement among flowers. Because a detailed analysis of PE is beyond the scope of the present study, we assigned a PE value of 1 for visitors that contacted both the stigma and the anther, whereas insects that contacted neither anther nor stigma or contacted either anther or stigma only were given a PE value of 0. Because *A. japonica* is dioecious, some visitor species of *A. japonica* that were collected only a few times were observed on either male or female plants only. We assigned PE values of 1 for such visitor species if they contacted the anthers (on male plants) or stigma (on female plants), because all other *A. japonica* visitors that were collected on both male and female plants invariably touched the anthers and stigma.

As in Lindsey (1984), we calculated a pollination importance value (PIV) for each visitor group by multiplying the four values: $PIV = A \times PCC \times F \times PE$. We then gave each functional group a PII score by calculating the relative pollination importance among all visitor groups: $PII = PIV / \sum PII$ (Lindsey, 1984; Hansen and Molau, 1994; Johnson *et al.*, 2009). Separate PII scores were calculated for Mycetophilidae and Sciaridae. Although pollen load does not necessarily reflect true PE (Zych *et al.*, 2013), and pollination efficiency ideally should be evaluated by pollen removal, pollen deposition and seed set per visit (Spears, 1983; Mayfield *et al.*, 2001; Ne'eman *et al.*, 2010; King *et al.*, 2013), due to the logistical difficulties of obtaining these data for a broad assemblage of visiting insects for seven plant species, we decided that PII provides a basis for comparison that is better suited to the aim of our study.

Pollen grains carried on the bodies of visitors were counted under a stereo-microscope. When pollen grains formed a clump, they were removed using basic fuchsin jelly (Kearns

and Inouye, 1993), which was then spread on a glass slide to count the stained pollen grains. Pollen grains of the host plants were distinguished by comparing them to a pollen reference of the plants flowering at each study site, although in most cases the body pollen originated from the plants studied. We were not able to discriminate pollen grains of co-flowering *E. lanceolatus* and *E. melananthus* in the Mt Haku-san population. These two species shared *Neoempheria* sp. 1 as a pollinator, so heterospecific pollen may have been included in these counts.

Nectar secretion

Nectar secretion seems to be important for the pollination of the seven studied plant species, because nectar feeding by flower visitors was observed in all species. We cut branches and/or stems bearing flowers, and these cut plants were kept in water-filled vials. Entire plant cuttings were put into plastic bags to prevent nectar evaporation. After 24 h, nectar was collected using a 1- μ L capillary, and nectar volume was calculated based on the proportion of the length to which the capillary was filled with nectar to the entire capillary length.

RESULTS

Overall pollinator composition

In all seven plants studied, dipteran insects were the predominant visitors (71–96 % of the individuals observed), although taxonomic composition was variable among species (Tables 1 and S3). Among the dipteran insects, fungus gnats (Mycetophilidae and Sciaridae) were the most frequent floral visitors (36–92 %, Tables 1 and S3, Fig. 3). Based on the PII scores, fungus gnats were the most important pollinators for all studied species; the PII of fungus gnats ranged from 0.53 in *A. japonica* to 1 in *S. streptopoides* (Fig. 4). The major fungus gnat taxa present differed among plant species, although the genus *Boletina* was common (Table 2). Notably, the fungus gnats collected on flowers were strongly female-biased (proportion of females: 0.79 in *S. streptopoides* to 0.96 in *A. japonica*, Table 2). However, no fungus gnat visitors exhibited oviposition behaviour; instead, all fungus gnat visitors were observed feeding on nectar. All floral visitors, with the exception of moths, grass bugs and a camel cricket, contacted both the stigma and anthers (Table 1). Most pollen grains carried by flower visitors originated from the plants at which the visitors were captured; however, crane flies, an empidid fly, and dermestid beetles on *A. japonica* and various Brachycera flies and one mycetophilid on *M. fusca* carried some heterospecific pollen (Table 1).

Pollinators of *Aucuba japonica*

During the 33.5-h observation period, we recorded 465 insect individuals of 71 species belonging to 40 families of six orders: Diptera, Coleoptera, Lepidoptera, Hymenoptera, Trichoptera and Ephemeroptera (Tables 1 and S3). Dipteran insects represented 88.6 % of all visitors (Tables 1 and S3). Specifically,

TABLE 1. Importance of each functional group as a pollinator of the seven plant species studied.

Plant	Functional group	Individuals collected (individuals with pollen)*	Average pollen load (PCC)	Proportion of host pollen (F)	Relative abundance (A)	Pollinator effectiveness (PE)	Pollination importance value (PIV)	Pollination importance index (PII)	Pollen placement site	
<i>A. japonica</i>	Fungus gnat	319 (220)	17.8	1	0.686	1	12.211	0.529		
	Mycetophilidae	110 (98)	27.8	1	0.237	1	6.576	0.285	Mainly head, thorax and occasionally abdomen	
	Sciaridae	209 (122)	12.5	1	0.449	1	5.618	0.244	Head, thorax, abdomen	
	Crane fly	39 (35)	61.1	0.975	0.084	1	4.996	0.217	Mouthpart, ventral thorax	
	Other Nematocera	29 (21)	14.3	1	0.062	1	0.890	0.039	Entire body	
	Non-syrphid Brachycera	25 (18)	23.3	0.946	0.054	1	1.185	0.051	Legs, head	
	Coleoptera	12 (11)	13.1	0.692	0.026	1	0.233	0.010	Elytra	
	Ant	10 (10)	10	1	0.022	1	0.215	0.009	Thorax, abdomen	
	Non-ant Hymenoptera	26 (21)	57.2	1	0.056	1	3.198	0.139	Entire body	
	Lepidoptera	2 (2)	3	1	0.004	1	0.013	0.001	Legs	
	Others (caddisfly, mayfly)	3 (1)	19.3	1	0.006	1	0.125	0.005	Thorax, abdomen	
<i>E. lanceolatus</i>	Fungus gnat	80 (2/6)	29.5	1	0.615	1	18.154	0.991		
	Mycetophilidae	76 (2/2)	88.5	1	0.585	1	51.738	0.991	Foreleg femur	
	Sciaridae	4 (0)	0	–	0.031	1	0	0	–	
	Non-syrphid Brachycera	29 (8/18)	2.1	1	0.223	1	0.463	0.009	Head, abdomen	
	Ant	18 (3/4)	1.3	0	0.138	1	0	0	Head, abdomen	
	Others (camel cricket, grass bug)	3 (0)	0	–	0.023	0	0	0	–	
<i>E. melananthus</i>	Fungus gnat	21 (13/17)	67.2	1	0.553	1	37.137	0.924		
	Mycetophilidae	16 (10/12)	87.8	1	0.421	1	36.947	0.895	Foreleg femur	
	Sciaridae	5 (3)	9	1	0.132	1	1.184	0.029	Foreleg femur	
	Other Nematocera	5 (0)	0	–	0.132	1	0	0	–	
	Non-syrphid Brachycera	5 (2)	1.2	1	0.132	1	0.158	0.004	Legs	
	Coleoptera	3 (1)	16.7	1	0.079	1	1.316	0.032	Thorax, head	
	Ant	1 (0)	0	–	0.026	1	0	0	–	
	Lepidoptera	2 (0)	0	–	0.026	1	0	0	–	
	Others (green lacewing)	2 (2)	31.5	1	0.053	1	1.658	0.040	Head	
	<i>E. tricarpius</i>	Fungus gnat	697 (189)	4.3	1	0.895	1	3.843	0.548	
Mycetophilidae		31 (26)	39.0	1	0.040	1	1.564	0.223	Foreleg femur	
Sciaridae		661 (163)	2.7	1	0.855	1	2.279	0.325	Foreleg femur	
Crane fly		9 (6)	42.2	1	0.012	1	0.491	0.070	Legs (femur), head	
Other Nematocera		21 (0)	0	–	0.027	1	0	0	–	
Non-syrphid Brachycera		22 (12)	44.6	1	0.028	1	1.269	0.181	Head, thorax, abdomen	
Coleoptera		12 (9)	30.5	1	0.016	1	0.473	0.067	Head, thorax	
Ant		7 (4)	2.1	1	0.009	1	0.019	0.003	Thorax	
Non-ant Hymenoptera		8 (4)	88.1	1	0.010	1	0.912	0.130	Head, thorax	
Lepidoptera		2 (0)	0	–	0.003	0	0	0	–	
<i>D. cercidifolius</i>		Fungus gnat	31 (19/28)	156.8	1	0.388	1	60.760	0.863	
	Mycetophilidae	26 (16/23)	156.0	1	0.325	1	50.700	0.720	Legs (femur), head	
	Sciaridae	5 (3)	160.4	1	0.063	1	10.025	0.142	Legs (femur), head	
	Crane fly	14 (8)	16.5	1	0.175	1	2.888	0.041	Legs (femur), head	
	Hoverfly	11 (7)	2	1	0.138	1	0.330	0.005	Mouthpart	
	Non-syrphid Brachycera	19 (11/17)	26.5	1	0.238	1	6.294	0.089	Head, legs, abdomen	
	Lepidoptera	4 (0)	0	–	0.050	0	0	0	–	
	Others: caddisfly	1 (1)	13	1	0.013	1	0.163	0.002	Head	
	<i>M. fusca</i>	Fungus gnat	121 (65/67)	24.9	0.98	0.708	1	17.277	0.841	
		Mycetophilidae	105 (57/59)	29.1	0.98	0.614	1	17.510	0.802	Legs, head
Sciaridae		16 (8)	9.2	1	0.094	1	0.865	0.040	Head, thorax	
Other Nematocera		2 (0)	0	–	0.012	0	0	0	–	
Hoverfly		1 (1)	45	0.46	0.006	1	0.124	0.006	Head, thorax	
Non-syrphid Brachycera		37 (27)	17.5	0.82	0.216	1	3.100	0.142	Legs, head	
Coleoptera		2 (2)	17.5	1	0.012	1	0.210	0.010	Head, thorax	
Non-ant Hymenoptera		8 (1)	0.8	1	0.047	1	0.038	0.002	Head, thorax	
<i>S. streptopoides</i>	Fungus gnat	97 (13/21)	9	1	0.924	1	8.314	1		
	Mycetophilidae	93 (13/17)	10	1	0.886	1	8.857	1	Legs (femur)	
	Sciaridae	4 (0)	0	–	0.038	1	0	0	–	
	Other Nematocera	1 (0)	0	–	0.010	1	0	0	–	
	Non-syrphid Brachycera	2 (0)	0	–	0.019	1	0	0	–	
	Coleoptera	4 (0)	0	–	0.038	1	0	0	–	
	Others (grass bug)	1 (0)	0	–	0.010	0	0	0	–	

* The single numbers in parentheses indicate the number of individuals that carried pollen out of the number of individuals collected. The two numbers separated by a slash in parentheses denote the number of individuals that carried pollen (numerator) out of the number of individuals examined (denominator).

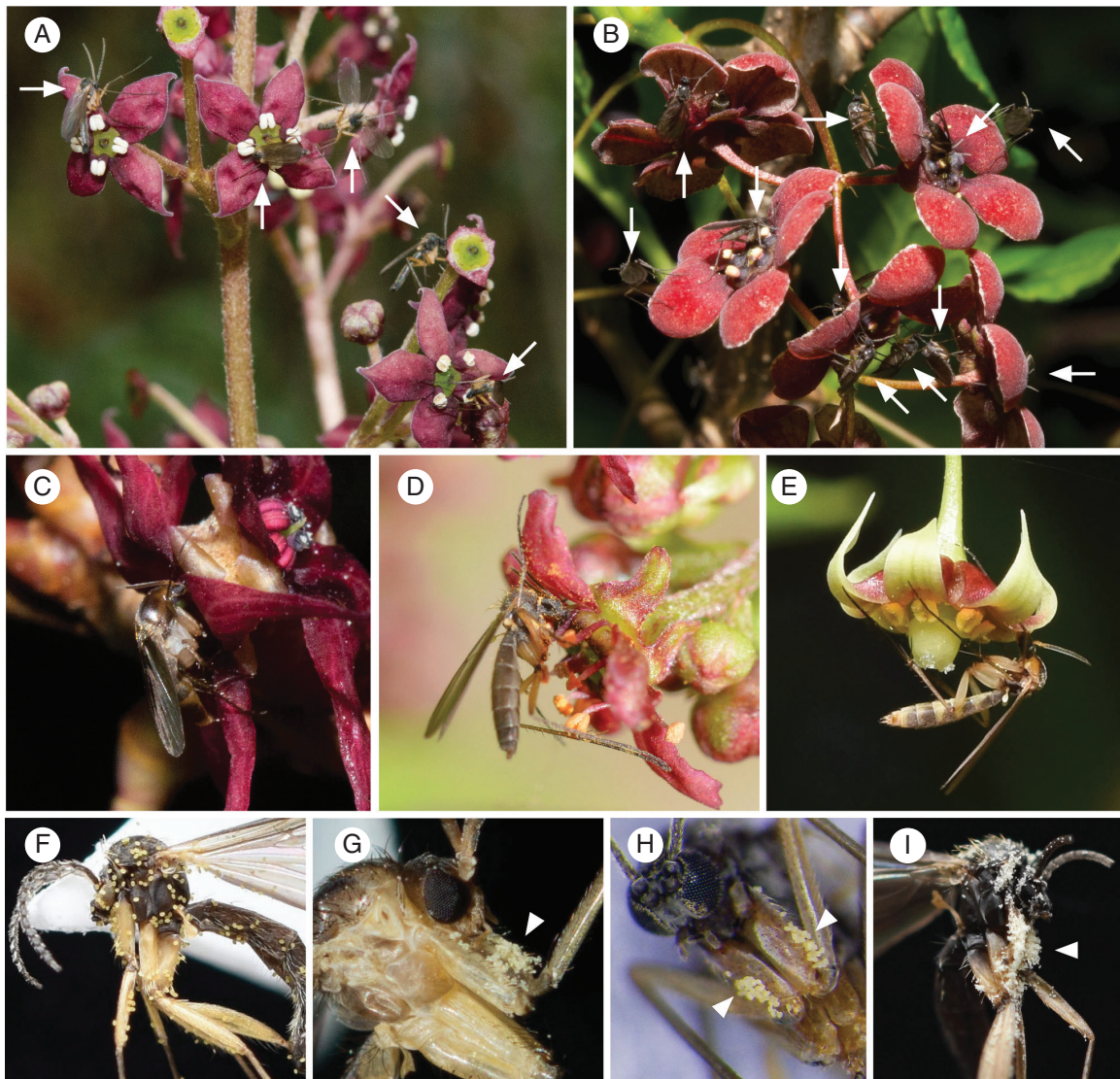


FIG. 3. Fungus gnat visitors of the studied plants: (A) *Boletina* sp. (arrows) visiting a male inflorescence of *A. japonica* at dusk; (B) Sciaridae sp. (arrows) visiting an inflorescence of *E. tricarpus* at dusk; (C) *Mycetophila* sp. visiting *D. cercidifolius*; (D) *Boletina* sp. visiting *M. fusca*; (E) *Brevicornu* sp. 2 visiting *S. streptopoides*; (F) *Boletina* sp. 1 collected on the flower of *A. japonica*. Pollen grains were attached mainly to the head and thorax. (G) An unidentified mycetophilid fungus gnat collected on a *E. melananthus* flower, with massive pollen load on a femur of the forelegs (arrowhead). (H) Sciaridae sp. collected on a *E. tricarpus* flower. Pollen grains were attached to the hairy femur (arrowheads). (I) *Boletina* sp. 7 caught on a *D. cercidifolius* flower, with numerous pollen grains on the femur and mouthparts (arrowhead).

Mycetophilidae and Sciaridae fungus gnats were by far the most frequent visitors (319 individuals: 68.6 % of all visitors). Among the mycetophilids, *Boletina* was the dominant genus; *Boletina* sp. 1 was overwhelmingly more frequent than other mycetophilid species (Table 2). Flowers were visited by sciarid fungus gnats throughout the day and by mycetophilid fungus gnats at dusk (1830–1930 h, Fig. 3A). Although the most frequent visitors were various sciarid species, the numbers of pollen grains carried were much lower than those of mycetophilids. Pollen grains were attached mainly on the ventral side of the thorax and head, and occasionally on the abdomen (Fig. 3F). Pollen-laden fungus gnats were repeatedly observed on female plants, suggesting that fungus gnats are capable of transporting pollen between individuals.

Pollinators of *Euonymus lanceolatus*

During the 67.5-h observation period for the two *E. lanceolatus* populations, 130 insects of 12 species belonging to seven families of four orders were recorded (Tables 1 and S3). The mycetophilid gnats *Neoempheria* spp. and drosophilid flies were the predominant visitors at both study sites. Visitation by *Neoempheria* spp. occurred only during humid afternoons (1400–1600 h), although visitation was highly unpredictable throughout the observation period. *Neoempheria* spp. were observed to forage for nectar. Drosophilid flies were observed during the daytime, but many of these rested on the petals, abaxial surface of the flower or flower stalk. The numbers of pollen grains carried were much higher in *Neoempheria* spp. than in drosophilid flies (Table 1), probably due to

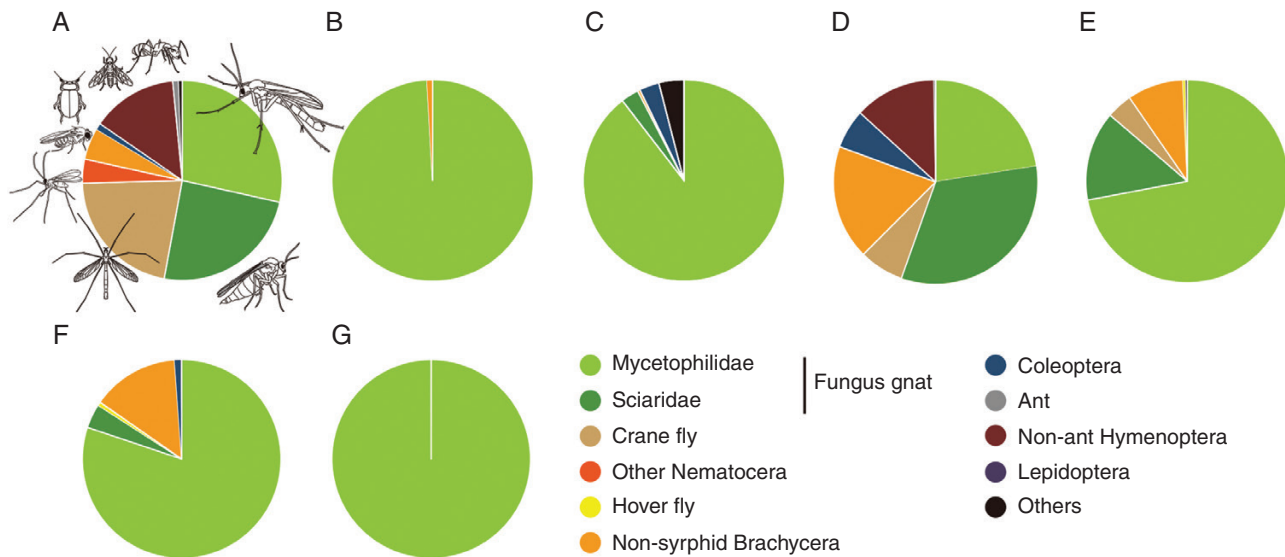


FIG. 4. Pie charts showing the pollination importance index (PII) of functional pollinator groups: (A) *A. japonica*; (B) *E. lanceolatus*; (C) *E. melananthus*; (D) *E. tricarpus*; (E) *D. cercidifolius*; (F) *M. fusca*; (G) *S. streptopoides*.

behavioural differences on the flowers. Pollen grains attached to *Neoempheria* spp. were found mainly on the femurs of the forelegs.

Pollinators of *Euonymus melananthus*

During the 29.25-h observation period, we recorded 37 individuals of 19 species belonging to 11 families of five orders (Tables 1 and S3). During the daytime, flowers were occasionally visited by mayflies and common flies including drosophilids; however, around sunset (1830–1930 h) the flowers were visited by fungus gnats. Mycetophilids, *Myomya* spp., *Neoempheria* spp. and species of an unidentified genus were observed. Fungus gnats carried pollen grains only on the hairy femur (Fig. 3G). The numbers of pollen grains carried by fungus gnats were the highest among the functional groups (Table 1).

Pollinators of *Euonymus tricarpus*

During the 17.25-h observation period, we recorded 779 individuals of 48 species belonging to 27 families of four orders (Tables 1 and S3). Although *E. tricarpus* received visits by a much larger number of insects than the other two *Euonymus* species, the flower visitors were predominantly dipteran insects (751 individuals, 96.4%), the majority of which were fungus gnats (697 individuals; Fig. 3B, Table 1). Visitation by sciarid fungus gnats was observed throughout the day, but visitations by mycetophilids were observed only around sunset (1800–1900 h). Hymenopteran visitors, such as ichneumonoid wasps and saw flies, carried the largest numbers of pollen grains, followed by brachyceran flies, crane flies and mycetophilid fungus gnats (Table 1). Although sciarid flies were frequent visitors, they carried fewer pollen grains than did mycetophilid flies (Table 1). Fungus gnats carried pollen grains on the femurs of the forelegs (Fig. 3H).

Pollinators of *Disanthus cercidifolius*

During the 18.5-h observation period, we recorded 80 insects of 22 species belonging to 11 families of four orders (Tables 1 and S3). The flowers were visited diurnally by hoverflies and flies of other brachyceran families, including Drosophilidae, Lauxaniidae and Tachinidae, which was consistent with observations made by Xiao *et al.* (2009) in China. However, the flies found in the current study generally carried a small pollen load despite their large body sizes (Table 1). Around sunset (1800–1900 h), flowers were frequently visited by fungus gnats (Fig. 3C), which carried considerable amounts of pollen (up to 1154 grains; Fig. 4, Table 1). Pollen grains were attached to the femurs of the forelegs and to the ventral side of the head (Fig. 3I). The dominant fungus gnat taxon was *Boletina*. All visitors foraged on nectar secreted by the petals, except for hoverflies, which also foraged on pollen.

Pollinators of *Micranthes fusca*

During the 14-h observation period, we recorded 171 insects of 31 species belonging to 19 families of three orders (Tables 1 and S3). Insect visits occurred both during the day and at dusk; however, the flowers were heavily visited by mycetophilids at dusk (1730–1830 h). Mycetophilidae and Sciaridae fungus gnats were the most frequent visitors, and carried more pollen than other visitors except one rare hoverfly. Several mycetophilid genera were recorded including *Brevicornu* and *Exechia* (Table 2). Pollen grains were attached on the ventral sides of the legs and head of fungus gnats (Fig. 3D, Table 1).

Pollinators of *Streptopus streptopoides*

During the 14.5-h observation period, very few insect taxa were observed visiting the flowers. The flowers were almost exclusively visited by mycetophilid fungus gnats, *Brevicornu*

TABLE 2. Taxonomic composition of the fungus gnat visitors.

Plant	Fungus gnat family	Species	Individuals collected	Female ratio		
<i>A. japonica</i>	Mycetophilidae	<i>Boletina</i> sp. 1	92	0.51		
		<i>Boletina</i> sp. 2	1	1		
		<i>Boletina</i> sp. 3	1	1		
		<i>Brevicornu</i> sp. 1	2	1		
		<i>Coelosia</i> sp. 1	1	1		
		<i>Mycetophila</i> sp. 1	2	1		
		<i>Mycetophila</i> sp. 2	2	1		
		<i>Mycetophila</i> sp. 3	1	1		
		<i>Mycomya</i> sp. 1	1	1		
		<i>Orfelina</i> sp. 1	5	1		
		<i>Saigusaia</i> sp. 1	1	1		
		unidentified species	1	1		
		<i>E. lanceolatus</i>	Sciaridae	spp.	209	0.9
Mycetophilidae	<i>Epicrypta</i> sp. 1		2	1		
	<i>Epicrypta</i> sp. 2		1	1		
	<i>Epicrypta</i> sp. 3		1	1		
	<i>Mycetophila</i> sp. 4		1	1		
	<i>Neoempheria</i> sp. 1		13	0.46		
	cf. <i>Neoempheria</i> sp. 1 (not captured)		58	–		
<i>E. melananthus</i>	Sciaridae	spp.	4	0.75		
	Mycetophilidae	<i>Mycomya</i> sp. 2	1	1		
		<i>Mycomya</i> sp. 3	2	1		
		<i>Mycomya</i> sp. 4	1	1		
		<i>Mycomya</i> sp. 5	1	1		
		<i>Neoempheria</i> sp. 1	2	0		
		<i>Neoempheria</i> sp. 2	1	1		
		unidentified species	4	0.75		
		unidentified species (not captured)	4	–		
		<i>E. tricarpus</i>	Sciaridae	spp.	5	1
Mycetophilidae	<i>Aphrastomyia</i> sp.		1	1		
	<i>Boletina</i> sp. 4		14	0.57		
	<i>Boletina</i> sp. 5		9	0.78		
	<i>Brevicornu</i> sp. 2		2	0		
	<i>Brevicornu</i> sp. 3		1	0		
	<i>Epicrypta</i> sp. 4		1	0		
	<i>Epicrypta</i> sp. 5		2	1		
	<i>Mycetophila</i> sp. 5		1	1		
	Sciaridae		spp.	663	0.96	
	<i>D. cercidifolius</i>		Mycetophilidae	<i>Boletina</i> sp. 6	2	1
<i>Boletina</i> sp. 7		8		0.5		
<i>Exechia</i> sp. 1		2		1		
<i>Mycetophila</i> sp. 6		4		1		
<i>Mycetophila</i> sp. 7		5		1		
<i>Rymosia</i> sp.		2		1		
unidentified species (not captured)		3		1		
Sciaridae		spp.		5	0.8	
<i>M. fusca</i>		Mycetophilidae		<i>Boletina</i> sp. 8	1	1
				<i>Brevicornu</i> sp. 2	27	0.59
	<i>Epicrypta</i> sp. 6		2	0.5		
	<i>Exechia</i> sp. 2		11	1		
	<i>Mycetophila</i> sp. 8		4	1		
	<i>Mycetophila</i> sp. 9		3	1		
	<i>Mycetophila</i> sp. 10		3	1		
	<i>Orfelina</i> sp. 2		7	0		
	<i>Rymosia</i> sp.		1	1		
	cf. <i>Orfelina</i> sp. 2 (not captured)		21	–		
	unidentified species (not captured)		25	–		
	<i>S. streptopoides</i>		Sciaridae	spp.	16	1
			Mycetophilidae	<i>Brevicornu</i> sp. 2	25	0.92
				<i>Phronia</i> sp.1	4	0.5
<i>Zygomya</i> sp.		2		0.5		
unidentified species (not captured)		62		–		
Sciaridae	spp.	4	0.75			

sp. 1 (Fig. 3E), *Phronia* sp. 1 and *Zygomya* sp., and by Sciaridae spp. Other visits were made only occasionally, by an empidid fly, cerambycid and chrysomelid beetles, a myrid bug and an

unidentified nematoceran fly (Table S3). Fungus gnats were the only insects found to carry pollen grains, although the body pollen counts were not high (about ten on average, Table 1).

TABLE 3. Quantity of nectar secreted per flower during 24 h

Plant	No. of samples (no. of plants)	Nectar volume (mean \pm sd, μ L)
<i>A. japonica</i> (female)	35 (7)	1.64 \pm 1.00
<i>A. japonica</i> (male)	43 (6)	1.02 \pm 0.74
<i>E. lanceolatus</i>	1 (1)	0.80 \pm 0.00
<i>E. melananthus</i>	8 (4)	2.13 \pm 0.90
<i>E. tricarplus</i>	30 (4)	2.11 \pm 1.19
<i>D. cercidifolius</i>	8 (5)	0.49 \pm 0.64
<i>M. fusca</i>	20 (5)	–
<i>S. streptopoides</i>	6 (5)	0.82 \pm 0.75

Visitors were observed foraging for nectar (Fig. 3E); in doing so, they collected pollen grains on the femurs of the forelegs.

Nectar secretion

Nectar secretion was confirmed in all species except *M. fusca*. Although the floral visitors to the flowers of *M. fusca* showed nectar-feeding behaviour, the nectar could not be sampled by the method we used, probably due to the very small amount of nectar produced by the flowers of this species. The amount of nectar accumulated in 24 h ranged from 0.49 μ L in *D. cercidifolius* to 2.13 μ L in *E. melananthus* (Table 3).

DISCUSSION

Our results indicate that the most important pollinator group amongst all plant studies was fungus gnats (Table 1, Fig. 4). In some of the plant species studied, fungus gnats did not have the largest pollen load (e.g. *A. japonica*, *E. tricarplus* and *M. fusca*), or the pollen load was small (e.g. *S. streptopoides*). However, because fungus gnats were by far the most frequent visitors, their overall contribution to pollination was higher than that of other insects (Table 1; Ackerman and Mesler, 1979; Mesler *et al.*, 1980). In other species, the numbers of pollen grains carried by fungus gnats were comparable to or higher than those of other insects (*E. melananthus*, *E. lanceolatus* and *D. cercidifolius*; Table 1). In these cases, the PIIs of fungus gnats were greater than 0.85, suggesting their high effectiveness (Table 1, Fig. 4). The PIIs of fungus gnats were low in *A. japonica* and *E. tricarplus* (0.53 and 0.55, respectively), so these two species may have more generalized pollination systems than other studied species. Although other insects may also contribute to pollination, especially in *A. japonica* and *E. tricarplus*, fungus gnats probably provide the largest contribution to the pollination of all the studied plant species.

A notable characteristic shared among the plants studied is their dark red floral pigmentation, but the ecological role of such a floral display is currently unknown. Dark red floral pigmentation is also present in 17 of the 20 previously known fungus gnat-pollinated genera (Table S1), although greenish flowers, such as those of *S. streptopoides*, are also common. In general, fungus gnats are attracted to bright colours such as yellow, which is used to trap sciarid flies in greenhouses (Cloyd and Dickinson, 2005). A study conducted by Tremblay and Ackerman (2007) showed that there is no difference in

reproductive success between unicolour (yellow) and bicolour (yellow and dark red) variants of fungus gnat-pollinated *Lepanthes rupestris*, suggesting that dark red pigmentation does not increase floral attractiveness. A recent study by Katsuhara *et al.* (2017) found that the dark red, branched petals of fungus gnat-pollinated *Mitella pauciflora* do not function as attractants, but rather as footholds. Although it is possible that dark colour is mimetic of fungus gnat oviposition sites (e.g. fungal fruiting body, rotten wood) in some species, the available information indicates that dark red coloration does not necessarily function to attract fungus gnats. One possibility is that dark red reduces the frequency of visits by unwanted visitors such as bees (or hoverflies), which are heavy pollen consumers, because bees are less sensitive to red (Troje, 1993; Briscoe and Chittka, 2001; Lunau *et al.*, 2011). Besides fungus gnat-pollinated plants, dark red flowers are common among plants with specialized dipteran pollinators, such as those that attract saprophagous, coprophagous or mycophagous flies (van der Niet *et al.*, 2011; Chen *et al.*, 2015; Policha *et al.*, 2016) or gall midges (Luo *et al.*, 2017) for pollination. Behavioural experiments and study of dipteran vision will help us understand the ecological role of dark red pigmentation in pollination. For example, some saprophytic flies have innate preference for dark colours under the presence of oligosulphides (Chen *et al.*, 2015).

Unlike floral colour, floral scent is hypothesized to play a critical role in fungus gnat-pollination systems (Vogel and Martens, 2000; Okamoto *et al.*, 2015; Katsuhara *et al.*, 2017). For example, some plants pollinated by fungus gnats (*Arisaema*, *Corybas* and *Heterotropa*) are thought to employ brood site mimicry because they produce a mushroom-like scent and the eggs and larvae of fungus gnats have been observed inside their flowers [Sugawara, 1988; Vogel and Martens, 2000; Kelly *et al.*, 2013; Woodcock *et al.*, 2014; but Kelly and Gaskett (2014) and Kuitert and Findlater-Smith (2017) refuted fungal mimicry in *Corybas*]. The studied plants also produced floral scents that are detectable to the human nose, which resembled those of fermented dairy products (e.g. yogurt or fresh cheese) or the leaves of fish mint (*Houttuynia cordata*) (Table 4). However, no fungus gnat visitors exhibited oviposition behaviour, and no insect eggs were found on the flowers, suggesting that brood site mimicry is not involved. Most nematoceran flies, including fungus gnats, feed on nectar as adults (Larson *et al.*, 2001), so our observation that the studied plants produce floral nectar further indicates that the system is reward-based. Further study of floral scent chemistry and the natural history of fungus gnat pollinators may reveal how the plant species examined in the current study attract fungus gnats and why the composition of fungus gnat visitors was female-biased (Table 4).

In addition to floral colour and odour, overall floral architecture may also be important in facilitating pollination by fungus gnats. Fungus gnats generally have very short mouthparts that restrict their foraging to open flowers with exposed nectar (Larson *et al.*, 2001; Fig. 3F–I). The fungus gnats observed in this study consumed nectar by pressing the head and thorax onto the flat floral surface, promoting contact between the hairy femur and head and the nearly sessile stamens and pistils (Fig. 3C–E). This foraging behaviour results in the attachment of a massive pollen load to the ventral surface of the fungus gnat body (Fig. 3F–I; Table 1). In contrast, insects with long mouthparts can consume nectar without coming into contact with the

TABLE 4. Summary of fungus gnat pollination in the five studied genera

Genus	<i>Aucuba</i>	<i>Euonymus</i>	<i>Disanthus</i>	<i>Micranthes</i>	<i>Streptopus</i>
Species	<i>A. japonica</i>	<i>E. melananthus</i> , <i>E. lanceolatus</i> , <i>E. tricarplus</i>	<i>D. cercidifolius</i>	<i>M. fusca</i>	<i>S. streptopoides</i>
Family	Garryaceae	Celastraceae	Hamamelidaceae	Saxifragaceae	Liliaceae
Order	Garryales	Celastrales	Saxifragales	Saxifragales	Liliales
Inflorescence type	Compound raceme	Cyme	A pair of adpressed flowers	Raceme	Uniflorous
Floral colour	Dark-red	Dark-red	Dark-red	Dark-red to pinkish	Greenish white with dark-red markings
Filament	<1 mm	<0.5 mm	<0.5 mm	<1 mm	<0.5 mm
Nectary	On disc, exposed	On disc, exposed	On petal	On disc, exposed	On tepal, exposed
Habit	Shrub	Shrub, dwarf shrub	Shrub	Perennial	Perennial
Habitat	Forest understory	Forest understory, subalpine or boreal meadow	Forest edge, understory	Subalpine meadow, streamside	Subalpine or boreal meadow, streamside
Flowering season	Early spring	Summer	Late autumn to winter	Summer	Summer
Sex expression	Dioecious	Hermaphrodite (protandrous)	Hermaphrodite	Hermaphrodite	Hermaphrodite
Floral scent	Tainted flour-like	Yogurt- or fish mint-like	Fish mint-like	Weakly fermented	Faint but slightly foul
Main pollinator	Various sciarid species, <i>Boletina</i> (Mycetophilidae)	Various sciarid species, <i>Boletina</i> (<i>E. tricarplus</i>); <i>Neoempheria</i> (<i>E. lanceolatus</i>); various mycetophilid genera (<i>E. melananthus</i>)	<i>Boletina</i> , <i>Mycetophila</i> (Mycetophilidae)	<i>Brevicornu</i> , <i>Orfelina</i> (Mycetophilidae)	<i>Brevicornu</i> , <i>Phronia</i> (Mycetophilidae)
Pollen attachment site	Entire body	Ventral thorax (fore femur)	Ventral thorax (fore femur), head	Ventral thorax, head	Ventral thorax (fore femur)
Pollinator activity	Throughout the day by sciarids and at dusk by mycetophilids	At dusk (<i>E. melananthus</i>), during the daytime (<i>E. lanceolatus</i>), throughout the day by sciarids and at dusk by mycetophilids (<i>E. tricarplus</i>)	Dusk	Throughout the day with a peak at dusk	Daytime

short stamens and pistils. As a result, these insects carried pollen grains sparsely on various parts of the body (Table 1). The remarkably specific and similar placement of pollen on ventral surfaces of the fungus gnat body in five families suggests that this may be a specific floral adaptation that facilitates pollination by fungus gnats. In support of this view, plants that have similar dark red floral display but lack the above floral architecture, such as *Acer* spp. (Sapindaceae) or *Sanguisorba officinalis* (Rosaceae), are pollinated by bees, syrphid flies or other brachyceran flies (K. Mochizuki, pers. observ.).

Pollination syndrome refers to a pattern of shared floral characteristics among distantly related plants as the result of convergent selection pressure exerted by the shared pollinator (Fenster *et al.*, 2004; Johnson and Wester, 2017). Whether the observed similarity in floral traits among the plants examined in this study (dark red pigmentation, floral architecture) fits the concept of a pollination syndrome cannot be evaluated unless there is information on pollination systems and floral traits of related plant species (e.g. Whittall and Hodges, 2007; Okuyama *et al.*, 2008). However, because floral coloration and gross architecture were good predictors of fungus gnat pollination in the plant species we studied, there may be a previously unnoticed association between fungus gnat pollination and a particular set of floral traits. The genus *Euonymus* may be particularly suited for testing the association between fungus gnat pollination and floral traits because it contains species with both green and dark red flowers of variable floral morphology.

Preliminary observation in *Euonymus* species with greenish flowers indicates that they are pollinated by a much broader spectrum of insects.

Lastly, an important characteristic shared among the studied plant species is their habitat. All of the studied plants are restricted to moist and shady environments, such as temperate forest understory, mountain streamside or the shade of subalpine vegetation (Table 4). Most of the fungus gnat-pollinated plants reported to date flower in similar habitats, and some authors have suggested a relationship between pollination by fungus gnats and plant habitat (Moldenke, 1976; Mesler *et al.*, 1980; Goldblatt *et al.*, 2004; Okuyama *et al.*, 2008). Thus, fungus gnat pollination may be particularly advantageous for plants growing in these continuously moist environments, where fungus gnats are abundant throughout the year but other common pollinators, such as bees, are scarce or unpredictable (Moldenke, 1976; Mesler *et al.*, 1980).

Prior to this study, pollination by fungus gnats was known to occur in 20 genera of eight plant families over a wide geographical range, including North and Central America, the Himalayas, East Asia, New Zealand, Australia and South Africa (Table S1). This study adds five genera of five families in the flora of Japan alone. The role of fungus gnats as pollinators of angiosperms has clearly been underestimated, and examples are likely to be found in regions other than Japan. Indeed, there have been many scattered observations of fungus gnat pollination, such as in *Thismia* (Burmanniaceae; Mar and

Saunders, 2015) and *Osyris* (Santalaceae; Aronne *et al.*, 1993). Pollination by fungus gnats may have been overlooked because many fungus gnats are nocturnal and both flowers and pollinators are inconspicuous. Previously, floral characteristics that are predictive of fungus gnat pollination were unknown, which also limited the discovery of new examples. The set of floral characteristics we found that is shared among fungus gnat pollinated-plants may facilitate the discovery of other examples of fungus gnat pollination.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: Summary of known fungus gnat-pollinated genera. Table S2: Summary of field observations. Table S3: List of floral visitors.

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