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Further Insights into the Floral Biology of *Asarum tamaense* (sect. Heterotropa, Aristolochiaceae)

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Abstract The sect. *Heterotropa* of the genus *Asarum* (Aristolochiaceae) comprises 49 endemic species in Japan. Species of *Heterotropa* are predominantly outcrossing and exhibit remarkable diversity in floral traits. This suggests that pollinator interactions may play critical roles in their diversification. A previous study found that *A. tamaense* did not produce seeds via autonomous self-pollination and was primarily pollinated by female individuals of a fungus gnat (*Cordyla* sp.; family Mycetophilidae) without providing any reward. Because the pollinator oviposits inside the calyx tube when visiting the flower, and the fungus gnat genus *Cordyla* is known to use mushrooms as brood sites, it has been hypothesized that the plant species adopts a mushroom mimicry system for pollination. Here, we revisited the floral biology of *A. tamaense* and investigated the hypothesized mushroom-mimicry system using time-lapse photography, pollinator identification via DNA barcoding, pollination experiments, and floral scent analyses. We confirmed that *A. tamaense* seldom sets fruit by autonomous self-pollination and that *Cordyla* sp. are likely the principal pollinators, as determined by DNA barcoding of adult insects that visited the flowers and eggs laid within the calyx tube. *Cordyla* sp. and other potentially pollinating dipteran species typically visited flowers from daytime to dusk. The major floral volatile compounds of *A. tamaense* were dimethyl disulfide that is a well-known major component of carrion scent, 2,3-butanediol diacetate, 2,3-butanediol, and several esters that are typical components of fermenting fruit scents. Although these volatile compounds are not typical in mushroom-mimicking flowers, it is possible that they are involved in attracting pollinators.

Key words: *Asarum tamaense,* brood-site mimicry, dimethyl disulfide, floral volatile, fungus gnats, mitochondrial COI, mushroom mimicry, pollination.

Introduction

Asarum (Aristolochiaceae) is a genus of perennial herbs comprising 128 species distributed throughout the temperate region of the Northern Hemisphere. Recent studies have recognized six sections in the genus based on morphological, cytological, and phylogenetic evidence (Sinn *et al.*, 2015a; Okuyama *et al.*, 2020). Among these six sections, sect. *Heterotropa*, characterized by base chromosome number $x = 12$ (Sugawara 1981, 1987; Zhou, 1998), contains the largest number of species, i.e., 62 (Okuyama *et al.*, 2020). All species of *Heterotropa*, excluding two that are native to mainland China, are distributed in Japan and Taiwan; 49 species are endemic to the Japanese Archipelago (Okuyama *et al.*, 2020). *Heterotropa* species are predominantly outcrossing (Sinn *et al.*, 2015b; Matsuda *et al.*,

2017), and their floral traits, including size, shape, color, and scent, are highly diversified (Azuma *et al.*, 2010; Kakishima and Okuyama, 2018a), suggesting that pollinator interactions play a critical role in their diversification. Moreover, their pollination systems are considered to be a typical brood-site mimicry system wherein flowers provide no reward to their pollinators (Sugawara, 1988). The reported pollinators of several species of *Asarum* are mycophagous dipterans such as fungus gnats (Sugawara, 1988; Mesler and Lu, 1993). Accordingly, it has been hypothesized that *Asarum* has a mushroom-mimicry pollination system because pollinators have been known to oviposit in the flowers, which somewhat resemble mushrooms (Sinn *et al.*, 2015b).

Asarum tamaense Makino is endemic to Japan and is distributed in the western half of the Kanto district, specifically the Tokyo, Kanagawa, and Saitama Prefectures (Sugawara, 2006). *A. tamaense* is listed in both the National (vulnerable) and Prefectural Red Lists (vulnerable to critically endangered) (Kanagawa Prefecture, 2006; Saitama Prefecture, 2011; Tokyo Prefecture, 2013; Ministry of the Environment, Japan, 2020). To date, *A. tamaense* is the only *Heterotropa* species whose pollination biology has been documented in detail (Sugawara, 1988). Sugawara (1988) reported that the species did not produce seeds via autonomous self-pollination and was primarily pollinated by female *Cordyla* sp. (Mycetophilidae); these gnats visited the flowers, laid eggs inside the calyx tubes, and were observed carrying pollen grains on their dorsal thorax following flower visitation. Thus, these gnats should contribute to the pollination of *A. tamaense*.

As part of our ongoing study linking the remarkable diversity of floral traits within *Heterotropa* to pollinator diversity, we revisited the floral biology of *A. tamaense* to more deeply investigate the hypothesized brood-site mimicry system. To this end, we monitored the timing and frequency of insect visitations to *A. tamaense* flowers using time-lapse photography. We examined the diversity of flower-visiting insects using DNA barcoding of both visitors and their deposited eggs. Finally, we analyzed the composition of floral volatile compounds using gas chromatography/mass spectrometry (GC/MS) to characterize the potential chemical components of the pollination system.

Materials and Methods

Time-lapse photography and specimen collection

This study was conducted on the Hachioji campus of the Tokyo University of Pharmacy and Life Sciences (N35°38′, E139°22′), Tokyo Prefecture, Japan. To monitor the insects visiting the flowers of *A. tamaense* (flower visitors), we employed time-lapse photography using WG-4 or WG-50 cameras (Ricoh, Tokyo, Japan). Eleven and ten flowering individuals were monitored during 4–5 April and 11–13 April 2018, respectively (Table 1). The cameras were set up at a minimum distance of 5 cm in front of the target flowers and the time-interval between photos was set to 2 min. We discarded photos in which more than half of the flower was obscured by overlapping leaves. An animal individual touching the upper surface of the calyx or remaining inside the calyx was counted as a single visit, and subsequent photos of the same animal on the same flower were not counted. To characterize temporal patterns of insect visitation, we compared the number of flower visitors observed at dawn (from one hour before sunrise to one hour after sunrise), daytime (from one hour after sunrise to one hour before sunset), dusk (from one hour before sunset to one hour after sunset), and overnight (from one hour after sunset to one hour before sunrise).

Flower visitors were collected on 12 and 13 April 2018. Once an animal entered the calyx, we collected it using an insect aspirator. Collected animals were stored dry to inspect pollen on their bodies or in 99.5% ethanol for identification using morphology and DNA barcoding, as described below. To collect insect eggs laid inside the calyx tubes, we sampled 26 flowers on

Table 1. The detailed information of the time-lapse photography

Plant ID	Number of flowers	Date	Time	Number of photos	Note
1	1	4–5 Apr. 2017	11:19–12:33	679	Exclude obscured pictures
2	2	4–5 Apr. 2017	11:31–13:29	782	
3	1	4–5 Apr. 2017	11:49–13:37	777	
4	2	4–5 Apr. 2017	11:42–13:48	329	Exclude obscured pictures
5	1	4–5 Apr. 2017	11:28–13:30	132	Exclude obscured pictures
6	3	4–5 Apr. 2017	11:20–13:26	787	
7	2	4–5 Apr. 2017	11:26–13:36	571	Exclude obscured pictures
8	1	4–5 Apr. 2017	11:44–13:44	784	
9	2	4–5 Apr. 2017	11:46–13:48	785	
10	2	4–5 Apr. 2017	11:50–14:00	789	
11	4	4–5 Apr. 2017	11:59–13:56	778	
12	6	11–12 Apr. 2017	11:31–12:55	714	Exclude obscured pictures
		12–13 Apr. 2017	13:36–12:23	685	
13	3	11–12 Apr. 2017	11:49–13:49	781	
		12–13 Apr. 2017	13:54–12:21	395	Exclude obscured pictures
14	2	11–12 Apr. 2017	11:49–13:41	777	
		12–13 Apr. 2017	13:49–12:37	685	
15	1	11–12 Apr. 2017	11:42–13:32	775	
		12–13 Apr. 2017	13:40–12:34	688	
16	2	11–12 Apr. 2017	11:59–13:43	773	
		12–13 Apr. 2017	13:47–12:39	687	
17	2	11–12 Apr. 2017	12:08–13:52	772	
		12–13 Apr. 2017	13:57–12:43	684	
18	4	11–12 Apr. 2017	12:11–13:53	678	Exclude obscured pictures
		12–13 Apr. 2017	13:59–12:43	683	
19	5	11–12 Apr. 2017	12:20–13:58	769	
		12–13 Apr. 2017	14:04–12:43	457	Exclude obscured pictures
20	5	11–12 Apr. 2017	12:25–14:03	676	Exclude obscured pictures
		12–13 Apr. 2017	14:08–12:44	479	Exclude obscured pictures
21	4	11–12 Apr. 2017	12:26–13:56	765	
		12–13 Apr. 2017	14:06–12:41	677	
Total				20793	

13 April 2018. We counted the number of eggs using a microscope and identified the eggs using DNA barcoding.

Identification of floral visitors by DNA barcoding

DNA barcoding was based on sequences of mitochondrial cytochrome oxidase subunit I using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). DNA extraction and sequencing were performed as reported in Kakishima and Okuyama (2018b) and Kakishima *et al.* (2020). New nucleotide sequences obtained in this study were deposited in the DNA Data Bank of Japan under accession numbers LC550539–LC550565. Species identification of collected floral visitors was based on nucleotide sequences and obtained using the Barcode of Life Data System (Ratnasingham and Hebert, 2007). We adopted a 4% uncorrected genetic distance as the threshold for differentiating species using DNA barcoding (Okuyama *et al.*, 2018). A phylogenetic analysis was conducted using RAxML version 8 (Stamatakis, 2014) using a maximum likelihood (ML) method with a GTR + G likelihood model for nucleotide substitutions. The ML tree and bootstrap branch supports were obtained by running a rapid bootstrapping algorithm with 1,000 replicates followed by a search for the ML tree.

Bagging experiments

To examine the effect of insect pollination in a natural population of *A. tamaense*, we conducted flower-bagging experiments from 5 April 2018 to 22 May 2018. We arbitrarily selected 22 flower buds that were expected to bloom in the days fol-

lowing selection to assess autonomous self-pollination in *A. tamaense*. We covered these flower buds with unwoven polyester fabric bags to prevent insect pollination (i.e., bagging treatments). We then arbitrarily selected and labeled 21 flowers and monitored these as open-pollination controls (i.e., open treatments). The fruit and seed set of all selected flowers were determined on 22 May 2018, 39–47 days after the bags were placed.

Volatile compound analyses

Floral scents of *A. tamaense* were examined using five samples from three individuals. The floral scents of the three individuals were first examined during daytime. One individual (plant ID: TBG160651) was sampled again in a different years and another individual (SK18097) was sampled again during the night. The scents from a cut leaf of one individual (TBG165603) were also assessed to identify the volatile compounds that were unique to flowers. For sampling, 1–2 flowers (0–7 days after opening) or one leaf was collected and placed in a 50- or 100-mL glass vial sealed with aluminum foil. Volatile compounds were collected for 30 min using headspace-solid phase microextraction (SPME) with 100-μm fibers of divinylbenzene/carboxen/polydimethylsiloxane (Supelco, Bellfonte, PA, USA). Some of the data, including the relative abundance of the individual compounds within a sample, will have been influenced by various factors that affect the sensitivity and selectivity of the small surface area of the SPME fiber and, therefore may not always represent the exact compositions of volatile compounds in the sample. Nevertheless, the data on volatile compound composition data obtained by SPME under the standardized sampling conditions were highly repeatable and consistent with those obtained by absorbent-based trapping methods in terms of both quality and quantity (e.g., Friberg *et al.*, 2013). To distinguish the volatile compounds of flowers from those of the ambient air, volatiles from an empty vial were used as a control.

All samples were subjected to GC/MS with settings equivalent to those reported in Okamoto *et al.* (2015) and Kakishima and Okuyama (2018a). We used a GCMS-QP2010SE system (Shimadzu, Kyoto, Japan) equipped with an Rtx-5SilMS capillary column (30 m × 0.25 mm; film thickness, 250 μm; Restek, Bellefonte, PA, USA). Helium was used as the carrier gas at a velocity of 48.1 cm s^{-1}, and the injector temperature was 250°C. The injector was operated in splitless mode for 1 min. Electron ionization mass spectra were obtained at a source temperature of 200°C. The oven temperature was programmed to the following sequence: 40°C for 5 min, an increase of 5°C/min to 210°C, an increase of 10°C/min to 280°C, and holding at 280°C for 5 min. The relative peak area in the total ion chromatogram (TIC) was used as a rough estimate of the relative content of each compound in each sample.

For all volatile compounds, retention indices were calculated with n-alkane (C6–C20) standards (Wako, Tokyo, Japan). Tentative identification was made by comparing the mass spectra with those in the libraries (NIST14 and NIST14s, National Institute of Standards and Technology, USA) using a cutoff of 94% similarity. The mass spectra, as well as the retention indices for the compounds, were compared with authentic standards. When authentic standards could not be obtained, the retention indices were compared with those reported in the National Institute of Standards and Technology Chemistry WebBook (Linstrom and Mallard, 2012).

Results

Floral visitors of Asarum tamaense

We took a total of 20,793 photographs of *A. tamaense* flowers (Table 1). We detected 0–36 flower visitors to each *A. tamaense* individual and a total of 228 flower visitors across the monitoring period (Table 2). We collected 1,680, 9,818, 1,800, and 7,495 photographs at dawn, daytime, dusk, and overnight, respectively (Table 3). The time-lapse photography allowed us to detect multiple photos of three individuals of *Cordyla* sp. bearing a large number of pollen

Table 2. A list of the flower visitors photographed on each *Asarum* plant individual

Category of visitor		Plant ID																					
		4–5 Apr. 2017											11–13 Apr. 2017										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total
Coleoptera		0	0	0	0	0	0	0	0	0	0	0	1	1	4	0	0	0	9	3	0	0	18
Diptera	Cecidomyiidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
	Drosophilidae	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	5	7
	Mycetophilidae	0	0	0	0	0	0	0	1	1	1	0	0	0	3	0	0	0	0	0	0	0	6
	Sciaridae	0	0	0	0	0	0	0	0	0	0	0	1	2	3	2	4	6	7	3	2	1	31
	Tipulidae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	2
	Unidentified Nematocera*	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	1	3	0	1	0	8
	Unidentified Brachycera*	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	3
	Unidentified Diptera*	0	0	0	0	0	0	0	0	0	0	1	3	1	2	2	0	0	1	0	1	3	14
Hemiptera	Aphidoidea (aphids)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Hymenoptera	Formicidae (ants)	4	8	3	0	0	5	1	0	0	3	7	0	29	12	0	2	1	6	3	9	12	105
	Unidentified Hymenoptera*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	3
Unidentified insects		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Amphipoda		0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	3
Collembola		0	0	0	0	0	0	1	0	0	1	0	0	1	2	0	0	2	2	2	0	1	12
Isopoda		0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	2	0	5
Myriapoda	(millipedes and centipedes)	0	0	0	0	0	0	0	0	0	1	3	1	0	1	0	0	1	0	0	0	1	8
Total		4	8	3	0	0	5	2	2	3	6	14	9	36	27	6	6	11	29	13	20	24	228

*Including those with difficulty in identification because of obscure images.

Table 3. The number of flower visitors per 100 hours (3,000 photographs)

Category of visitor		Morning	Daytime	Dusk	Night
Coleoptera	(beetles)	3.6	1.8	3.3	3.2
Diptera	Cecidomyiidae	0	0.3	0	0
	Drosophilidae	0	2.1	0	0
	Mycetophilidae	0	1.2	3.3	0
	Sciaridae	0	6.1	5.0	3.2
	Tipulidae	0	0.6	0	0
	Unidentified Nematocera	1.8	1.2	3.3	0.4
	Unidentified Brachycera	0	0.9	0	0
	Unidentified Diptera	1.8	2.4	1.7	1.6
Hemiptera	Aphidoidea (aphids)	0	0.3	0	0
Hymenoptera	Formicidae (ants)	12.5	13.4	23.3	16.0
	Unidentified Hymenoptera	0	0.6	1.7	0
Unidentified insects		0	0.3	0	0
Amphipoda		1.8	0	1.7	0.4
Collembola		3.6	0.6	3.3	2.4
Isopoda		1.8	0	0	1.6
Myriapoda	(millipedes and centipedes)	1.8	0	1.7	2.4
Total		28.6	32.1	48.3	31.2
Nuber of photos		1680	9818	1800	7495

grains on their bodies (Fig. 1A). We did not recognize pollen grains on the bodies of other dipterans such as Cecidomyiidae, Drosophilidae, or Sciaridae in any photos (Fig. 1B–D). All six collected individuals of *Cordyla* sp. that were stored dry had a large number of pollen grains (>20) on their bodies (Fig. 1G, H), but we also found about ten pollen grains on the dried body of one *Scaptomyza* sp. (Drosophilidae) individual collected from an *A. tamaense* flower (Fig. 1I). Although ants were the most frequently observed animal in the photographs (Table 2), there was no evidence that they entered the calyx tubes or transported pollen grains on their body. There was no clear temporal pattern of flower visitation by ants. By contrast, Amphipoda, Coleoptera (beetles), Collembola, and Myriapoda (millipedes and centipedes) primarily visited flowers from dusk to dawn. Dipterans (Drosophilidae, Mycetophilidae, Sciaridae, and others) primarily visited flowers from daytime to dusk.

We collected 18 dipteran flower visitors from *A. tamaense* flowers. Six individuals of *Cordyla* sp., which has been reported as a principal pollinator of *A. tamaense* (Sugawara, 1988), seven individuals of Cecidomyiidae, one Drosophilidae, three Sciaridae, and one Tipulidae were collected (Table 4). For *Cordyla* sp., five females and one male were collected (Fig. 1G, H). Sixteen of the 18 collected individuals were successfully sequenced by DNA barcoding (Fig. 2). Based on our DNA barcoding criterion, specimens of *Cordyla* sp. and Cecidomyiidae were indicated as including only one species each. The three Sciaridae individuals were recognized as three different species. The single Drosophilidae individual was identified as *Scaptomyza* sp. (Fig. 1I).

We found 18 eggs inside the calyx tubes of six of the 26 collected flowers. Twelve eggs were successfully sequenced by DNA barcoding; all were identified as dipterans, of which nine were *Cordyla* sp. and three were *Drosophila* sp. (Drosophilidae) (Table 4; Fig. 1E, F). The *Cordyla* sp. was indicated to be the same species that was collected from flower visitor samples. All three *Drosophila* sp. eggs were collected from a single calyx tube (Fig. 1F).

Bagging experiments

Only one of the 22 bagged flowers produced fruit (5% fruit set), whereas five fruits were produced from the 21 control (open treatment) flowers (24% fruit set). Although the difference between the two treatments was not significant (Fisher's exact test, one-sided, $P = 0.08$), the fruit

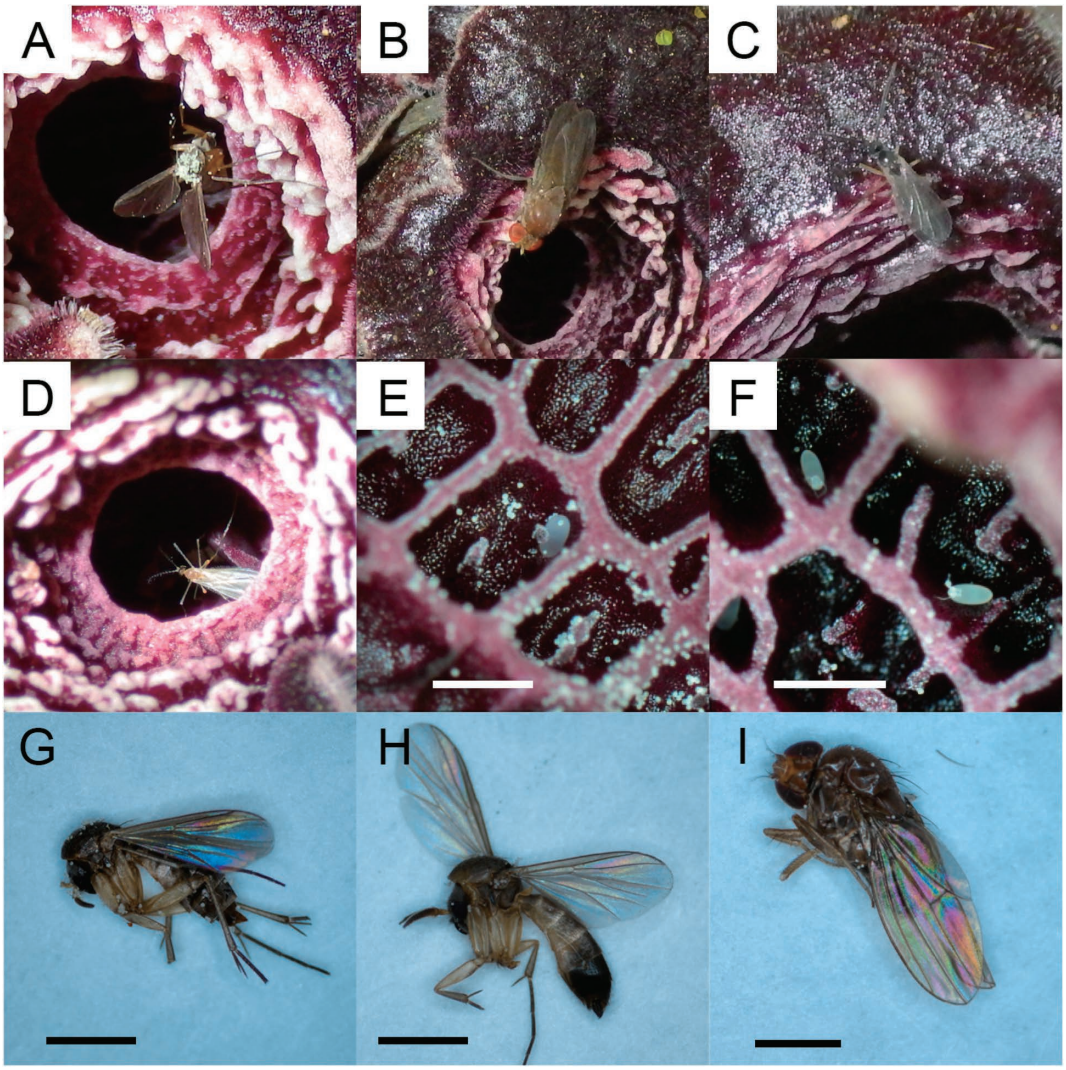

Fig. 1. Images of floral visitors and eggs laid on the inner surfaces of the calyx tubes. A: *Cordyla* sp. (Mycetophilidae) heavily coated with pollen grains departing from a calyx tube. B: Drosophilidae sp. C: Sciaridae sp. D: Cecidomyiidae sp. E: An egg of *Cordyla* sp. F: Eggs of *Drosophila* sp. (Drosophilidae). G: A female *Cordyla* sp. H: A male *Cordyla* sp. I: *Scaptomyza* sp. (Drosophilidae). Scales=1 mm.

set in the open-pollination treatment was slightly higher than the bagging treatment. Thus, it remains possible that pollinator-mediated outcrossing facilitates fruit set of *A. tamaense*. The number of seeds per fruit was 16 and 23 in the bagged and open treatments, respectively.

Floral volatile compounds in Asarum tamaense

Overall, 86 floral volatile compounds were found in *A. tamaense*. We detected 35–48 volatile compounds in each individual headspace sample (Table 6). These compounds consisted of 29 aliphatics, two benzenoids and phenylpropanoids, 18 monoterpenoids, 33 sesquiterpenoids, two diterpenes, and two sulfur-containing compounds. The volatile compounds common among all flower samples were isopropyl acetate, 2,3-butanediol isomer, 2,3-butanediol diacetate isomer, α-pinene, camphene, β-pinene, α-phellandrene, cymene isomer, one unidentified

Table 4. The number of collected adult insects and eggs

	Cecidomyiidae	Drosophilidae	Mycetophilidae	Sciaridae	Tipulidae	Total
Adult	7	1	6	3	1	18
Egg	9	3	0	0	0	12

Table 5. Fruit set and seeds per fruit in the pollination experiments

Treatment	Individuals	Flowers	Fruit set	Fruiting rate	Seeds per fruit
Open	16	21	5	0.24	16.0
Bagging	22	22	1	0.05	23.0

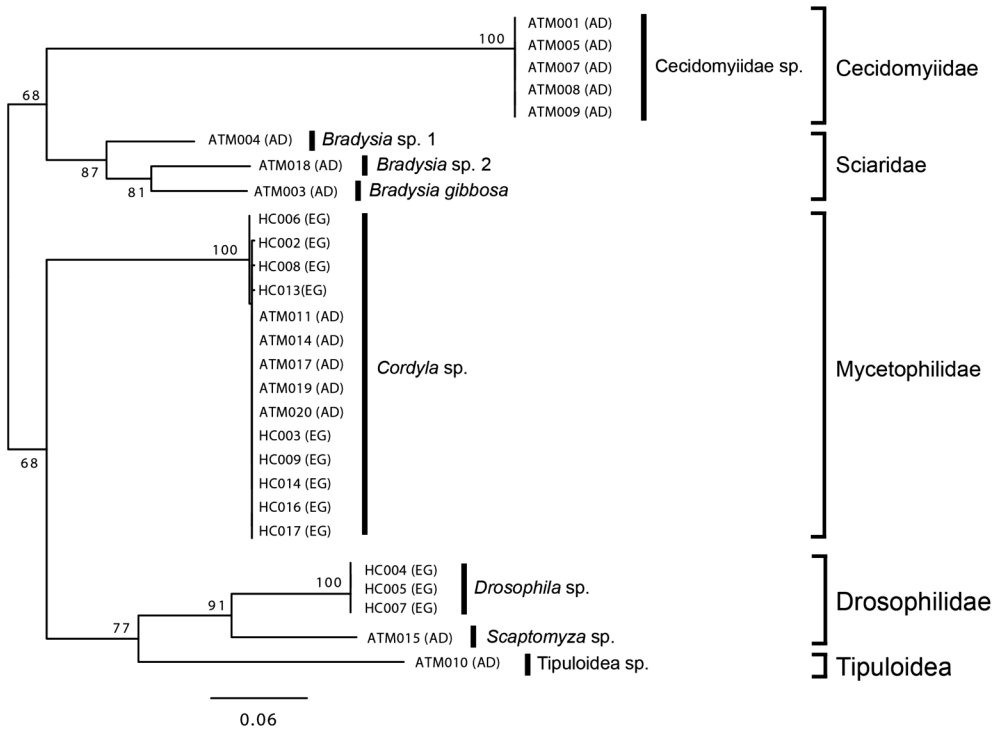

Fig. 2. A maximum likelihood phylogenetic tree of 28 dipteran individuals based on 617-base pairs (bp) of mitochondrial cytochrome oxidase subunit I (COI). Bootstrap supports are shown for nodes above the species level. Each operational taxonomic unit (OTU) label represents the sample name, followed by the sample type: adult (AD) or egg (EG).

monoterpene, humulene, four unidentified sesquiterpenes, and dimethyl disulfide. Among these, *α*-pinene, camphene, *β*-pinene, cymene isomer, one unidentified monoterpene, humulene, and one unidentified sesquiterpene were also detected in the leaf sample. The volatile composition, represented by the TIC peak area ratios, was variable among flower samples and was dominated by one sulfur-containing compound, dimethyl disulfide (15.77–53.03%), and three aliphatics, namely, 2,4,5-trimethyl-1,3-dioxolane isomer (0.00–15.14%), 2,3-butanediol isomer (1.93–9.05%), and 2,3-butanediol diacetate isomer (0.37–30.43%).

Discussion

Our bagging experiments, although not statis-

Table 6. Relative amount (%) of volatile compounds detected from the flower of *Asarum tamaense*

Name	Ret. Index	Identification†	Leaf	TBG160651-1	TBG160651-2	TBG165603	SK18097 (daytaime)	SK18097 (night)	Average
Aliphatics									
Isopropyl acetate	648	A		0.19	0.55	0.43	0.08	0.12	0.27
Acetoin	703	A		—	—	—	0.47	—	0.09
n-Propyl acetate	705	A		—	—	0.44	—	—	0.09
Aliphatics-1	708			—	7.97	—	—	—	1.59
2,4,5-trimethyl-1,3-Dioxolane isomer	715	B		4.73	5.09	—	11.36	15.14	7.26
2,4,5-trimethyl-1,3-Dioxolane isomer	737	B		—	0.06	—	0.08	0.11	0.05
sec-Butyl acetate	741	A		—	0.09	0.05	—	—	0.03
Isobutyl acetate	759	A		—	0.15	0.10	—	—	0.05
2,3-Butanediol isomer 1	775	A		1.93	6.25	7.53	9.05	5.53	6.06
2,3-Butanediol isomer 2	785	A		—	0.52	0.54	0.45	0.23	0.35
Ethyl butyrate	794	A		—	2.19	0.28	0.14	—	0.52
Aliphatics-2	810			—	0.19	0.22	0.09	—	0.10
Isoamyl acetate	877	B		—	0.36	0.05	—	—	0.08
2-Methylbutyl acetate	879	A		—	1.55	0.51	—	—	0.41
2-Acetoxy-3- butanone	889	B		—	1.56	—	—	—	0.31
Propyl Butyrate	899	A		—	—	0.36	—	—	0.07
Ethyl 3-methylcrotonate	924	A		—	—	—	0.30	0.42	0.14
Aliphatics-3	929			—	0.36	0.14	—	—	0.10
Aliphatics-4	977			—	0.21	—	—	—	0.04
Aliphatics-5	1005			—	0.31	—	—	—	0.06
Hexyl Acetate	1013	A		—	0.25	0.06	—	—	0.06
2,3-butanediol diacetate isomer	1056	B		—	0.08	0.26	—	—	0.07
Aliphatics-6	1065			—	—	0.06	—	—	0.01
2,3-butanediol diacetate isomer	1069	B		0.37	30.43	28.23	1.72	1.30	12.41
Aliphatics-7	1139			—	0.12	0.39	0.32	0.21	0.21
Aliphatics-8	1144			—	—	0.24	—	—	0.05
2-Ethylhexyl acetate	1148	A		—	0.25	0.30	—	0.08	0.13
Aliphatics-8	1154			—	—	0.96	—	—	0.19
2,2,4-Trimethyl- 1,3-pentanediol diisobutyrate	1586	A	*	—	0.20	—	0.11	—	0.06
Benzenoids and phenylpropanoids									
Benzyl acetate	1160	A		—	0.07	—	—	—	0.01
Elemicin	1544	B	*	0.15	0.11	—	0.32	0.09	0.13
Monoterpenoids									
α-Pinene	931	A	*	0.34	0.65	0.18	1.51	0.58	0.65
Camphene	946	A	*	0.11	0.29	0.07	0.69	0.19	0.27
Sabinen	970	A		—	—	—	—	0.15	0.03
β-Pinene	974	A	*	0.24	0.58	0.13	1.17	1.12	0.65

Table 6. continued

Name	Ret. Index	Identification†	Leaf	TBG160651-1	TBG160651-2	TBG165603	SK18097 (daytaime)	SK18097 (night)	Average
β-Myrcene	989	A		—	2.18	—	0.36	0.38	0.58
2-(2-ethoxyethoxy)-ethanol	998	A	*	—	0.41	0.07	0.00	0.52	0.20
α-Phellandrene	1003	A		1.15	3.49	0.74	7.97	3.10	3.29
Cymene isomer	1022	A	*	0.32	0.34	0.11	0.82	0.11	0.34
Monoterpene-1	1027		*	0.84	2.23	0.50	5.04	1.68	2.06
(E)-*β*-Ocimene	1035	A		—	—	—	0.12	—	0.02
(Z)-*β*-Ocimene	1046	A		0.07	1.08	—	2.86	1.38	1.08
Terpinolene	1083	A		—	0.06	—	0.15	0.06	0.06
Linalool	1098	A	*	—	0.16	—	0.09	0.05	0.06
Bornyl acetate	1282	A		—	0.26	0.09	0.33	0.15	0.17
Safrole	1286	A		—	—	—	—	0.24	0.05
Monoterpene-2	1340			—	—	—	0.25	—	0.05
Monoterpene-3	1345			—	0.28	—	0.28	—	0.11
Monoterpene-4	1347			—	—	—	0.43	—	0.09
Sesquiterpenoids									
Sesquiterpene-1	1344			0.17	—	—	—	—	0.03
Sesquiterpene-2	1368			0.17	—	—	—	—	0.03
Sesquiterpene-3	1369		*	—	—	—	0.23	—	0.05
Sesquiterpene-4	1371			—	—	0.04	—	—	0.01
α-Copaene	1374	B	*	0.22	0.24	—	0.38	0.16	0.20
Sesquiterpene-5	1378			1.34	0.06	0.91	0.18	0.29	0.56
β-Elemene	1389	B	*	—	—	0.06	—	—	0.01
Sesquiterpene-6	1397			0.28	—	—	—	—	0.06
Sesquiterpene-7	1403			0.12	—	0.12	—	—	0.05
Sesquiterpene-8	1416			1.06	—	0.70	—	0.13	0.38
Sesquiterpene-9	1418			—	—	0.61	—	—	0.12
Caryophyllene	1418	A	*	3.98	6.31	—	4.54	2.77	3.52
β-Cedrene	1423	A		0.20	—	0.23	—	—	0.09
Sesquiterpene-10	1429			0.45	0.30	—	0.28	0.22	0.25
Sesquiterpene-11	1431			0.14	—	—	—	—	0.03
Sesquiterpene-12	1448			—	0.07	0.08	—	—	0.03
(E)-*β*-Farnesene	1451	A		—	—	—	—	0.60	0.12
Humulene	1454	A	*	4.17	4.90	0.74	6.72	4.03	4.11
Sesquiterpene-13	1460		*	—	—	—	0.22	0.06	0.06
Sesquiterpene-14	1461			0.25	—	—	—	—	0.05
Sesquiterpene-15	1468		*	0.39	—	0.14	—	—	0.11
Sesquiterpene-16	1471			0.35	—	0.11	—	—	0.09
Sesquiterpene-17	1475			—	—	0.09	—	—	0.02

Table 6. continued

Name	Ret. Index	Identification†	Leaf	TBG160651-1	TBG160651-2	TBG165603	SK18097 (daytaime)	SK18097 (night)	Average
Sesquiterpene-18	1479		*	0.41	0.46	0.09	1.91	1.51	0.88
Sesquiterpene-19	1487		*	—	—	—	0.11	0.07	0.04
Sesquiterpene-20	1494			—	—	—	0.34	0.21	0.11
Sesquiterpene-21	1495		*	—	—	0.08	—	—	0.02
Sesquiterpene-22	1499			5.14	0.28	1.64	0.49	0.93	1.70
α-Farnesene	1502	B	*	—	—	—	0.13	0.13	0.05
δ-Cadinene	1516	B	*	—	—	—	0.14	0.10	0.05
Sesquiterpene-24	1526			9.34	0.56	3.45	0.82	2.37	3.31
Sesquiterpene-25	1585			0.41	—	—	—	—	0.08
Sesquiterpene-26	1587			0.14	—	—	—	—	0.03
Diterpenes									
Diterpene-1	1647			0.09	—	—	—	—	0.02
Diterpene-2	1951			1.44	0.10	0.22	—	—	0.35
Sulphur-containing compounds									
2-(methylthio)-propane	660	A		—	—	1.20	0.92	0.44	0.51
Dimethyl disulfide	730	A		59.27	15.77	46.43	36.02	53.03	42.10

†Identification was based on A: mass spectrum and retention index of authentic compound, B: similarity of mass spectrum to those in the libraries and previously reported RI index in the NIST Chemistry WebBook. * Indicates those compounds were detected in a leaf sample.

tically significant, suggested that *A. tamaense* seldom sets fruit by autonomous self-pollination (Sugawara, 1988). Our pollinator observations provided further evidence that *Cordyla* sp. is the principal pollinator of *A. tamaense*, as has been previously reported (Sugawara, 1988). DNA barcoding indicated that a single *Cordyla* species was collected as both eggs and adults (Fig. 2) from *A. tamaense* flowers, suggesting that this dipteran plays an important role in the pollination of this species. We collected one male *Cordyla* sp. of the six specimens collected. Although only female individuals were reported as flower visitors previously (Sugawara, 1988), this result implies that both male and female individuals of *Cordyla* sp. can be pollinators of *A. tamaense*, while the visitation frequency is female-biased. Female-biased visitation and the presence of eggs inside the calyx tubes also suggests that the flowers of *A. tamaense* mimic the brood-site of this pollinator, i.e., mushrooms (Sugawara, 1988; Sinn *et al.*, 2015b), because *Cordyla* species are known to utilize mushrooms, especially ground-inhabiting fungi such as *Russula* and *Lactarius*, as their larval food source (Stone *et al.*, 1965; Hackman and Meinander, 1979; Jakovlev, 2012). Although Sugawara (1988) did not report any other dipteran visitors of *A. tamaense*, we observed visitation by members of Cecidomyiidae, Drosophilidae, Sciaridae, and Tipulidae, both with time-lapse photography and direct observation. Furthermore, we observed pollen grains on the body of a collected *Scaptomyza* sp., which indicates that dipterans other than *Cordyla* sp. may play some role in the pollination of *A. tamaense*. Although further observation would be necessary to reach a clear conclusion, we suggest that other flower visitors, including ants, coleopterans, collembolans, and isopods, are unlikely to function as effective pollinators of *A. tamaense* because we never observed these groups transporting pollen.

Most dipterans, including *Cordyla* sp., visited flowers during daytime and dusk. Therefore, it is likely that pollination of *A. tamaense* occurs mainly from daytime until dusk. This is likely a result of the behavioral circadian rhythms of the pollinators, because the floral scents of *A. tamaense* were not found to differ between day and night. Our previous field observations of *A. costatum* on Shikoku Island and *A. minamitanianum* on Kyushu Island found that flowers were mainly visited by flightless animals (Kakishima and Okuyama, 2018a). The difference in flower visitors between *A. tamaense* and these two *Asarum* species may be the result of strikingly different floral scent compositions between these species, as discussed below, although differences in climate or geographical distribution may also be important factors.

We consider it plausible that *A. tamaense* uses mushroom-mimicry to attract pollinators (Sugawara, 1988; Sinn *et al.*, 2015b). However, our analyses also indicated that *A. tamaense* does not emit C8 aliphatics such as 1-octen-3-ol, 3-octanol, and 3-octanone, which are the typical volatile components of mushrooms or so-called mushroom-mimicking flowers (Jürgens *et al.*, 2013; Policha *et al.*, 2016; Kakishima *et al.*, 2019). Instead, the most abundant floral volatile compound of *A. tamaense* was dimethyl disulfide, accounting for, on average, 42.1% of the relative floral volatile compounds, which is the typical scent component of carrion or carrion-mimicking flowers (Johnson and Jürgens, 2010; Jürgens *et al.*, 2013). This compound has never been reported from the flowers of sect. *Heterotropa* (Azuma *et al.* 2010; Kakishima and Okuyama, 2018a). Dimethyl oligosulfides, including dimethyl disulfide, are characteristic volatile components of truffle (*Tuber* spp.) and shiitake (*Lentinus edodes*) mushrooms, as well as some species bearing mushroom-mimicking flowers, such as *Duguetia cadaverica* (Annonaceae) (Pelusio *et al.*, 1995; Yang *et al.*, 1998; Teichert *et al.*, 2012). Therefore, although the flowers of *A. tamaense* do not appear to have volatile profiles typical of mushroom-mimicking flowers, they may mimic ground-growing mushrooms by emitting dimethyl disulfide. Because the emission of dimethyl disulfide by shiitake mushrooms is known to increase upon drying (Yang *et al.*,

1998), it is also possible that *A. tamaense* might mimic degraded mushrooms under wet and dry cycles that are typical in natural settings. Because the known hosts of the fungus gnat genus *Cordyla* are mostly confined to Russuaceae and Boletaceae (Sasakawa and Ishizaki, 2003, Jacovlev, 2012), it would be worth investigating whether these mushroom species emit dimethyl disulfide.

We also detected 2,3-butanediol diacetate (mean composition: 12.41%), 2,3-butanediol (6.06%), and other esters such as isobutyl acetate and isoamyl acetate from the flowers of *A. tamaense*. These compounds are characteristic of the volatile blends of fermenting fruits (Procheş and Johnson, 2009; Goodrich and Jürgens, 2018). We further detected 2,4,5-trimethyl-1,3-dioxolane (7.26%), which is characteristic of fermented fruit juice (Günther *et al.*, 2019). Some of these compounds are also known as floral scent components of certain *Gastrodia* (Orchidaceae), *Arum* (Araceae), and *Asimina* (Annonaceae) species, whose pollination systems are considered to be based on fermenting-fruit mimicry (Goodrich and Raguso, 2009; Stökl *et al.*, 2010; Martos *et al.*, 2015). Beetles or drosophilids are the typical pollinators of these species (Goodrich and Jürgens, 2018). Accordingly, these volatile compounds may also play some role in luring pollinators to *A. tamaense*, and thus this species may be adopting some traits of fermenting-fruit mimicry as well as mushroom-mimicry.

The floral volatile compounds of *A. tamaense* are strikingly different from those of the four taxa of *Asarum* Series Sakawanum in sect. *Heterotropa* (Kakishima and Okuyama, 2018a). The main components of the floral scent of *A. tamaense*, i.e., dimethyl disulfide, 2,3-butanediol diacetate, 2,3-butanediol, 2,4,5-trimethyl-1,3-dioxolane, isobutyl acetate, and isoamyl acetate, are not found in other species of *Asarum* Series Sakawanum. In addition, the principle component of the floral scents of species of *Asarum* Series Sakawanum, methyl angelate, was not detected in *A. tamaense*. However, many terpenoids found in this study are common to *Asarum* Series Sakawanum. We note that some of these terpenoids were also detected in the cut leaves, indicating that these compounds are not unique to the flowers. Nevertheless, because terpenoids are generally considered to be important in determining the specificity of pollinator attraction (Okamoto *et al.*, 2015; Pichersky and Raguso, 2018), careful inspection is needed to determine their function in the pollination systems of sect. *Heterotropa*.

Floral scent profiles are highly variable within sect. *Heterotropa* (Azuma *et al.*, 2010; Kakishima and Okuyama, 2018a); our study has provided yet another distinct example. It is thus becoming evident that the floral scent profiles of sect. *Heterotropa* species are remarkably diverse, as are other floral traits within this genus. Further study is required to comprehend the full scope of the relationships between pollinator diversity and floral traits, including scent profiles, within this genus, and to clarify if pollinators have played a central role in the radiation of sect. *Heterotropa* in the Japanese archipelago.

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