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Floral Scent Profiles and Flower Visitors in Species of *Asarum* Series Sakawanum (Aristolochiaceae)

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Abstract To understand the potential link between the variation in floral scents and pollinators in *Asarum*, a diverse plant genus of Japan, we conducted analyses of floral volatile compositions as well as field monitoring of flower visitors in species of the genus series Sakawanum. We detected a remarkably large number of floral volatile compounds, and found they are dominated by aliphatics and terpenoids but poor in benzenoids. However, despite a relatively intensive effort, we failed to identify specific species of flower visitors likely contributing well for the cross pollination of these plants. Contradicting to the genetic evidence that these species are generally outcrossing, the visitation frequency of the winged insects for their flowers was likely to be low and thus it remained enigmatic how they successfully cross-pollinate in the wild population.

Key words: *Asarum*, floral scent, *Heterotropa*, pollination, SPME.

The Japan archipelago harbors a rich endemic flora and has been designated as one of the biodiversity hotspots (Boufford *et al.*, 2005; Mittermeier *et al.*, 2011). There are 1862 species and 847 varieties of endemic land plants in Japan, accounting a quarter of the plant species native to Japan (Kato and Ebihara, 2011). Although these endemic plants are scattered across 515 plant genera and 161 families, with their close relatives are often found in the surrounding area of Japan, some plant lineages are especially species rich in Japan.

Among these, the genus *Asarum* is a plant genus with the third highest number of the endemic species in Japan, followed by *Carex* (Cyperaceae) and *Cirsium* (Asteraceae) (Sugawara, 2006; Okuyama, 2010; Kato and Ebihara, 2011). The species richness of the genus is represented by the marked diversity of floral forms including color, shape, and size, suggesting that varieties of plant-pollinator interactions have shaped the diversification of the genus. Nevertheless, only a few species are examined for the plant-pollinator interactions in the genus (Sugawara, 1988; Mesler and Lu, 1993). The paucity of the information on pollination system of *Asarum* in Japan is probably due to several reasons. First, the frequency of pollinator visitation is not high, second, the floral organs are enclosed with calyx tube so that the pollinator behaviors are invisible after they entered inside the flower, and lastly, many of the species are now endangered and not abundant enough to conduct field observations. All of the documented pollination system of the genus so far, including that of *A. tamaense* in Japan, involve mushroom-feeding dipterans as the principal pollinator (Sugawara, 1988), thereby mushroom mimic has been suggested for their pollination strategy (Sinn *et al.*, 2015). However, we noticed that the floral scents of the species in Japan are markedly variable and most of them are far from the mushroom-like note, which is expected for the mushroom-mimicking flowers. Nevertheless, very little is known about the variation of the floral scent profile of the genus, with only those of 7 species in Japan and Taiwan are documented so far (Azuma *et al.*, 2010).

Here, as the initial attempt to illustrate the diversity of the pollination systems and associated floral scent profiles within the genus, we report the flower visitors and the floral scent profiles of the species of the *Asarum* series Sakawanum (hereafter Sakawanum species), a well characterized small subclade of the genus in Japan.

Materials and Methods

Chemical analyses

We studied all the species and varieties of the genus *Asarum* series Sakawanum, i.e., *Asarum costatum*, *A. sakawanum* var. *sakawanum*, *A. sakawanum* var. *stellatum*, and *A. minamitanianum*, where 3, 2, 2, 3 individuals of each were sampled for floral scent analyses, respectively. For each plant individual, 1–2 flowers (0–5 days after opening) was collected and placed in a 100 ml glass vial sealed with aluminum foil. Volatile compounds were collected for 30 min using head space-solid phase microextraction (HS-SPME) with fibers of 100 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS; Supelco, Bellfonte, PA, USA). To distinguish volatile compounds of flowers from those of the ambient air, the volatiles from an empty vial was used as the control. To examine the volatile compounds emitted from the vegetative part, a leaf of an individual of *A. costatum* (TBG160624) was also cut from the petiole and was analyzed for the volatile using the same method as for the flower. All the plants used in this study are cultivated in Tsukuba Botanical Garden of the National Museum of Nature and Science, Japan.

The samples were subjected to gas chromatography/mass spectrometry (GC/MS) with the equivalent settings to those reported previously (Okamoto *et al.*, 2015). Specifically, we used 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 the 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, followed by an increase of 5°C/min to 210°C and then 10°C/min to 280°C, at which the oven was held for 5 min. The rerative peak area in the total ion chromatogram (TIC) was used as a rough estimate of the relative content of each compound in the samples.

For every volatile compound, retention indices were calculated with n-alkane (C6–C20) standards (Wako, Tokyo, Japan). Then identification was made by comparing the mass spectra with those in the libraries (NIST14 and NIST14s, National Institute of Standards and Technology, USA) at the cutoff of 90% similarity and the retention indices with those reported in the NIST Chemistry WebBook (Linstrom and Mallard, 2012). Where available, the mass spectra as well as the retention times for the individual compounds were also compared with the authentic standards.

Time-lapse photography

To monitor flower visitors, time-lapse photography using Optio WG-4 cameras (Ricoh, Tokyo, Japan) were conducted at a wild population of *A. costatum* and *A. minamitanianum*. For *A. costatum*, a total of 12 individuals were monitored on 20–21 April and 14–16 May, 2016 at Nahari site in Kochi prefecture (N33° 25′ 26.9″, E134° 2′ 13.6″). For *A. minamitanianum*, 3 individuals were monitored on 24–25 March, 2014 at Hyuga site in Miyazaki Prefecture (exact location of the site is not shown for conservation reasons). The camera was set in front of a plant individual, keeping $>$5 cm distance from the plants. The time-intervals between the shots were set to 2 min, as this is almost the minimum time-interval for camera battery to be sustained overnight

(>20 h). Any animal individual touching the upper surface of the calyx was count as a single visit, and the animal on the same flower taken in the subsequent shot was not count.

Results

Floral volatile compounds in Sakawanum species

We detected 35–73 volatile compounds in the individual headspace samples. Overall, 133 floral volatile compounds were found from three *Asarum* species and one variety in the series Sakawanum, of which 12 were aliphatics, 11 were C5-branched chain compounds including hemiterpenes, 30 were monoterpenes, 71 were sesquiterpenes, two were diterpenes, and one was the other class of compound, while the remaining six were unidentified compounds (Table 1). Among these, methyl angelate, *α*-pinene, camphene, *β*-pinene, limonene, trans- and cis-*β*-ocimene, *β*-caryophyllene, trans-*α*-bergamotene, and *β*-selinene were common among all samples. We did not detect any benzenoids from the samples, although they constitute a major class of volatile compounds in most of the insect-pollinated flowers. Although the volatile composition represented by the TIC peak area ratios was variable among the plant individuals, there was a clear tendency among the species. The TIC peak area of *A. costatum* is dominated by the aliphatic methyl angelate (37–87% of the total), while that of *A. minamitanianum* is dominated by sesquiterpenes such as humulene (16–18%) and *γ*-curcumene (7–18%). The TIC peak area of *A. sakawanum* is dominated by both methyl angelate (6–22%) and sesquiterpenes such as *β*-caryophyllene (6–26%), somewhat an intermediate pattern between the former two species. The headspace volatile compositions of a young leaf of *A. costatum* were dominated by sesquiterpenes, and the major compounds were trans-*α*-bergamotene (28%), unidentified compound 5 (13%), trans-*β*-farnesene (9%), sesquicineole (6%), sesquiterpene 5 (5%), and methyl angelate (5%).

Flower visitors in the wild populations of A. costatum *and* A. minamitanianum

Using time-lapse photography, we monitored flower visitors of *A. costatum* and *A. minamitanianum* for 19–44 h (586–1338 photographs) per plant individual (Table 2). As the result, we detected 8–54 flower visits per plant individual for *A. costatum* while detected only 5–8 flower visits per plant individual for *A. minamitanianum*. Most (>88%) of the flower visitors were flightless or ground dwelling animals (Table 3). Judging from the contiguous shots of the photographs, some of the pictured animals clearly entered inside the calyx tube. This suggests their potential as pollinators, although it was unclear whether the pollen attached on their body (Fig. 1).

Discussion

We detected 156 floral volatile compounds, which is a remarkably large number for only three closely-related plant species, i.e., *A. costatum*, *A. minamitanianum*, and *A. sakawanum*. This was somewhat unexpected because the volatile compounds usually detected in a set of closely related species are usually far less. For example, Okamoto *et al.* (2015) found only 27 volatile compounds from the headspace of 13 species of the genus *Mitella* (Saxifragaceae), although the results are not directly comparable as absorbents used for volatile collection are different [SPME with fibers of DVB/CAR/PDMS in the present study vs. Tenax-TA in Okamoto *et al.* (2015)].

In the present study, we could not discriminate the floral volatiles from volatiles of the damaged tissue such as the cut peduncle. However, the contents of volatile compounds detected in the present study are generally congruent with the previous study using seven *Asarum* species (*A. yaeyamense*, *A. lutchuense*, *A. hypogynum*, *A. fudsinoi*, *A. dissitum*, *A. senkaku-insulare*, and *A. tokarense*) in that C5-branched chain ester and/or sesquiterpenes were the dominant compounds (Azuma *et al.*, 2010). In Azuma *et al.* (2010), the

Table 1. Relative amount (%) of volatile compounds detected from the flower of *Asarum* species of the series Sakawanum

Compound	Identification*	RI	*A. costatum*			*A. minamitanianum*			*A. sakawanum* var. *sakawanum*		*A. sakawanum* var. *stellatum*	
			TBG160623	TBG160624	Acos1701	TBG152617	TBG152618	TBG152619	TBG146306	TBG160619	TBG160620	TBG160626
Aliphatics												
2,3-Epoxybutane	B	591	0.43	0.15	0.12	0.77	—	—	—	—	—	—
2-Butanone	A	597	3.04	1.01	0.81	—	—	—	0.17	—	—	—
1,2-Epoxybutane	B	600	—	—	—	2.31	3.85	—	—	—	—	—
2-Butanol	B	601	4.46	2.70	2.75	—	—	—	—	—	—	—
2-Methyl-1-propanol	A	620	0.06	—	0.03	—	—	—	—	—	—	—
Aliphatics 1		630	—	—	—	—	—	—	15.80	—	—	—
Aliphatics 2		665	—	—	—	0.19	—	—	—	—	—	—
Methyl isobutyrate	B	676	0.03	—	—	—	—	—	—	—	—	—
Aliphatics 3		755	—	—	—	—	0.44	—	—	—	—	—
Aliphatics 4		796	—	—	—	0.14	—	—	—	—	—	—
Aliphatics 5		903	—	0.08	—	—	—	—	—	—	—	—
3-Octanol	A	997	—	—	—	—	—	—	0.09	—	—	—
C5-branched chain compounds												
2-Methylfuran	B	608	—	—	—	—	—	—	—	0.08	—	—
2-Methyl-3-buten-2-ol	B	608	2.86	9.77	—	—	—	—	—	—	—	—
2-Methyl-butanal	B	653	0.15	1.15	0.19	—	—	—	0.47	—	1.44	0.27
sec-Butyl formate	C	671	0.06	0.07	0.04	—	—	—	—	—	—	—
2-Methyl-1-butanol	B	728	4.53	4.16	0.78	—	—	—	0.07	—	—	—
2-Methyl-2-butenal	B	733	0.07	—	—	—	—	—	0.12	—	—	—
C5-branched chain compounds 1		737	—	—	—	—	—	—	0.07	—	—	—
Methyl 2-methylbutyrate	B	765	0.28	—	0.38	—	—	—	—	—	—	—
Methyl angelate	A	822	71.31	37.73	87.35	1.14	0.51	1.11	16.50	6.75	23.46	6.51
Methyl tiglate	A	863	—	—	0.07	—	—	—	—	—	—	—
3-Methyl-2(5H)-furanone	B	969	—	—	0.09	—	—	—	—	—	—	—
Monoterpenes												
Tricyclene	A	918	0.05	0.20	0.06	—	—	—	0.09	0.36	—	—
α-Thujene	A	924	0.10	0.07	0.03	—	—	—	—	0.11	—	0.18
α-Pinene	A	930	1.49	7.16	1.13	0.99	0.33	1.08	3.49	10.57	7.02	2.57
Camphene	A	946	0.80	3.42	0.73	0.54	0.13	0.41	1.79	5.91	3.63	0.94
Sabinene	A	970	—	—	—	—	—	—	0.64	0.42	0.46	0.53
β-Pinene	A	974	1.37	6.17	1.14	0.39	0.31	0.35	3.80	9.23	4.51	2.26
α-Phellandrene	A	1003	1.52	7.70	—	—	0.22	—	0.18	0.98	4.66	4.62
3-Carene	A	1005	—	—	—	0.90	0.15	0.67	—	—	6.78	2.09
4-Carene	B	1014	—	0.12	—	—	—	—	0.11	—	—	0.36
p-Cymene	A	1022	0.26	0.72	0.04	—	—	—	—	0.39	1.74	0.60
Limonene	A	1027	1.35	5.06	0.12	0.31	0.09	0.22	0.45	0.98	4.35	2.88
Eucalyptol	A	1029	—	0.55	0.32	—	—	—	3.46	2.68	—	—
trans-*β*-Ocimene	A	1036	0.15	0.11	0.07	1.96	1.67	1.40	0.12	0.07	0.37	0.31

Table 1. (Continued)

Compound	Identification*	RI	*A. costatum*			*A. minamitanianum*			*A. sakawanum* var. *sakawanum*		*A. sakawanum* var. *stellatum*	
			TBG160623	TBG160624	Acos1701	TBG152617	TBG152618	TBG152619	TBG146306	TBG160619	TBG160620	TBG160626
cis-β-Ocimene	A	1046	0.83	0.78	0.44	9.13	8.64	6.83	0.62	0.44	1.69	2.52
γ-Terpinene	A	1056	—	—	—	—	0.11	—	—	0.05	—	—
Terpinolene or Isoterpinolene	B	1083	0.07	0.68	—	—	0.04	—	—	0.22	0.44	0.53
Linalool	A	1098	—	—	—	0.81	0.19	8.86	0.20	0.14	—	—
trans-Alloocimene	B	1128	—	—	—	0.36	0.31	—	—	—	—	—
4-Acetyl-1-methylcyclohexene	B	1128	—	—	—	—	—	—	0.05	—	—	—
Alloocimene isomer	C	1139	—	—	—	—	0.21	—	—	—	—	—
Camphor	A	1143	—	0.06	—	—	—	—	0.45	0.27	—	—
Ectocarpene	C	1149	—	—	—	—	—	—	—	0.23	—	—
endo-Borneol	B	1169	—	—	—	—	—	—	0.16	0.57	—	—
Menthol	B	1176	—	—	—	—	—	—	—	2.10	—	—
Monoterpene 1		1186	—	—	—	0.31	—	—	—	—	—	—
Monoterpene 2		1193	—	—	—	—	—	—	—	0.05	—	—
Thymol methyl ether	B	1228	—	0.17	—	—	—	—	—	0.14	—	—
Bornyl acetate	A	1282	0.49	1.73	0.43	0.17	0.06	—	0.86	0.88	2.71	0.51
Monoterpene 3		1345	—	0.08	—	—	—	—	0.89	0.44	1.75	—
Monoterpene 4		1352	—	—	—	—	—	—	0.07	—	—	—
Sesquiterpenes												
Elemene isomer	C	1330	—	—	—	—	—	—	0.26	0.45	—	—
Elemene isomer	C	1334	—	—	—	0.32	0.06	0.96	2.05	3.52	—	—
α-Cubebene	B	1345	—	—	—	0.47	0.52	0.58	—	—	—	0.45
Sesquiterpene 1		1352	—	—	—	—	0.11	—	—	—	—	—
Sesquiterpene 2		1367	—	—	—	—	0.07	—	—	—	—	—
α-Copaene	B	1374	—	—	0.10	1.53	0.57	0.50	0.09	0.08	1.40	0.55
Sesquiterpene 3		1381	—	—	—	—	—	—	—	0.11	—	—
β-Bourbonene	B	1382	—	—	—	3.37	1.22	0.96	0.13	0.11	1.31	0.57
7-epi-Sesquithujene	B	1386	0.17	0.38	0.11	3.60	3.08	2.80	—	—	1.56	1.97
β-Elemene	B	1388	—	—	—	—	—	—	1.53	2.36	—	1.57
Sesquiterpene 4		1392	—	—	—	0.13	0.39	—	0.10	—	—	0.24
Sesquiterpene 5		1401	—	—	—	—	0.57	—	—	0.23	—	—
Italicene	B	1403	—	—	—	2.43	0.38	0.41	—	0.27	0.57	0.95
α-Gurjunene	B	1405	—	—	—	—	—	—	0.35	0.71	—	0.66
Sesquiterpene 6		1408	—	—	—	—	0.26	—	—	—	—	—
cis-α-Bergamotene	B	1412	0.10	0.22	0.07	0.28	—	—	—	0.07	—	—
α-Cedrene	B	1414	—	—	—	1.02	0.47	0.75	0.11	0.18	—	0.46
β-Caryophyllene	A	1418	1.02	0.85	0.58	2.05	6.11	2.73	6.32	26.95	9.64	24.53
Sesquiterpene 7		1422	—	—	—	—	—	—	—	0.21	—	—
β-Caryophyllene isomer	C	1425	0.04	0.09	—	0.28	1.23	0.73	0.46	0.57	0.71	1.74
Sesquiterpene 8		1428	—	—	—	0.47	0.77	0.58	0.26	0.31	—	0.38
trans-α-Bergamotene	B	1431	1.32	2.69	0.86	6.43	3.26	4.67	1.01	2.30	1.62	3.10

Table 1. (Continued)

Compound	Identification*	RI	*A. costatum*			*A. minamitanianum*			*A. sakawanum* var. *sakawanum*		*A. sakawanum* var. *stellatum*	
			TBG160623	TBG160624	Acos1701	TBG152617	TBG152618	TBG152619	TBG146306	TBG160619	TBG160620	TBG160626
Aromandendrene	B	1436	—	—	—	—	—	—	0.09	0.19	—	—
Sesquisabinene isomer	C	1439	0.15	0.44	0.15	0.76	0.56	0.28	0.28	0.36	—	0.49
Cadina-3,5-diene	C	1443	—	—	—	—	0.95	—	0.20	—	—	—
Sesquiterpene 9		1445	—	—	—	0.34	—	1.03	0.44	—	—	0.74
Seychellene	B	1447	0.22	—	—	0.14	—	—	—	—	—	—
Sesquiterpene 10		1447	—	—	—	—	0.33	—	—	—	—	—
Sesquiterpene 11		1449	—	—	—	—	0.20	—	—	0.16	—	—
trans-*β*-Farnesene	B	1452	0.38	1.35	0.41	—	—	—	—	—	—	—
Humulene	B	1454	0.07	—	—	18.21	18.29	16.16	0.87	1.82	1.75	3.03
cis-Muurola-4(15),5-diene	B	1459	—	—	—	0.65	2.25	—	0.35	—	—	0.49
9-epi-Caryophyllene	B	1459	—	—	—	—	—	—	—	1.04	—	—
Sesquiterpene 12		1466	—	—	—	—	0.22	—	—	—	—	—
Sesquiterpene 13		1466	—	—	—	—	—	—	—	0.09	—	—
Sesquiterpene 14		1467	—	—	—	0.43	0.10	—	0.07	0.12	—	0.18
γ-Muurolene	B	1473	—	—	—	0.51	1.16	1.16	0.46	—	—	—
γ-Curcumene	B	1476	—	—	—	18.26	7.72	16.68	2.49	1.33	3.90	7.10
α-Curcumene or Germacrene D	B	1479	0.10	0.07	—	6.90	14.61	8.38	3.09	3.32	5.09	6.08
Sesquiterpene 16		1482	0.07	0.18	0.05	0.39	0.35	—	0.15	0.22	—	0.19
β-Selinene	B	1487	0.20	0.17	0.08	0.41	1.90	0.73	1.01	1.44	1.11	2.81
Viridiflorene	B	1489	—	—	—	—	—	—	0.61	—	—	0.70
γ-Amorphene	B	1490	—	—	—	—	1.32	—	—	—	—	—
Sesquiterpene 17		1493	0.16	0.13	0.04	4.82	5.68	10.10	1.53	4.32	2.67	8.80
α-Muurolene or *α*-Amorphene	B	1496	0.05	—	—	0.09	0.52	0.39	0.15	—	—	0.25
Sesquiterpene 18		1499	0.06	—	—	—	0.35	—	0.35	—	—	—
α-Farnesene	B	1503	—	—	—	0.40	0.81	0.67	0.08	0.13	—	0.42
Sesquiterpene 19		1506	—	—	—	—	—	—	0.42	—	—	—
β-Curcumene	B	1508	—	—	—	2.96	1.74	3.45	0.66	0.90	1.03	1.48
γ-Cadinene	B	1511	—	—	—	0.33	0.97	0.76	0.33	0.14	0.59	0.28
Sesquicineole	B	1512	0.11	0.69	0.14	—	—	—	—	—	—	—
δ-Cadinene	B	1516	—	—	—	0.62	1.61	1.47	0.56	0.33	1.22	0.65
β-Sesquiphellandrene	B	1522	—	0.25	0.07	0.89	1.00	1.88	0.14	1.27	0.81	1.22
Sesquiterpene 20		1525	—	—	—	—	0.06	—	—	—	—	—
Cadina-1,4-diene	B	1530	—	—	—	—	0.21	—	—	—	—	—
α-Cadinene	B	1535	—	—	—	0.09	0.38	0.24	0.10	0.05	—	—
Sesquiterpene 21	B	1539	—	—	—	—	0.08	—	—	—	—	—
α-Bisabolene	B	1539	—	—	—	—	—	—	0.36	—	—	—
7-epi-trans-Sesquisabinene hydrate	C	1542	—	—	—	—	—	—	0.14	—	—	—
Germacrene B	B	1556	—	—	—	—	—	—	0.09	0.15	—	—
Sesquiterpene 22		1581	—	—	—	—	—	—	—	0.05	—	—
Sesquiterpene 23		1582	—	—	—	—	0.03	—	—	—	—	—

Table 1. (Continued)

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			TBG160623	TBG160624	Acos1701	TBG152617	TBG152618	TBG152619	TBG146306	TBG160619	TBG160620	TBG160626
Sesquiterpene 24		1588	—	—	—	—	0.04	—	—	—	—	—
Sesquiterpene 25		1594	—	—	—	—	0.04	—	—	—	—	—
Sesquiterpene 26		1594	—	—	—	—	—	—	0.14	—	—	—
Sesquiterpene 27		1605	—	—	—	—	—	—	0.55	—	—	—
Sesquiterpene 28		1644	—	—	—	—	—	—	0.51	—	—	—
Sesquiterpene 29		1652	—	—	—	—	—	—	0.11	—	—	—
Sesquiterpene 30		1667	—	—	—	—	—	—	0.07	—	—	—
α-Bisabolol	B	1684	—	—	—	—	—	—	19.28	—	—	—
Sesquiterpene 31		1721	—	—	—	—	—	—	0.18	—	—	—
Diterpenes												
Manool oxide	B	2005	—	0.18	—	—	—	—	0.38	—	—	—
Verticilla-4(20),7,11-triene	C	2013	—	—	—	—	—	—	0.39	—	—	—
Other compounds												
2-Methoxy-3-methyl-pyrazine	C	965	—	—	0.08	—	—	—	—	—	—	—
Unidentified compounds												
unidentified 1		1152	—	—	—	—	0.18	—	—	0.04	—	—
unidentified 2		1395	—	—	—	—	—	—	—	—	—	0.22
unidentified 3		1583	—	0.11	—	—	—	—	—	—	—	—
unidentified 4		1658	—	—	0.16	—	—	—	—	—	—	—
unidentified 5		1673	—	0.12	—	—	—	—	—	—	—	—
unidentified 6		1882	—	0.48	—	—	—	—	—	—	—	—

* Identification are 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. For C, identification are based solely on mass spectrum to those in the libraries and thus it is regarded as temporary identification.

Table 2. The detailed information of time-lapse photography in the present study

Plant species	Plant Individual	Number of flowers	Photographing date	Time of day	Number of photographs	Note
A. costatum	1	2	April 20–21, 2016	12:19–12:55	741	Rainy for the last 3 h
	2	2	April 20–21, 2016	12:13–13:27	758	Rainy for the last 3 h
	3	1	April 20–21, 2016	11:59–12:25	734	Rainy for the last 2 h
	4	2	April 20–21, 2016	11:55–12:01	724	Rainy for the last 2 h
	5	1	April 20–21, 2016	12:25–13:27	752	Rainy for the last 3 h
	6	1	May 14–15, 2016	15:29–13:27	660	
			May 15–16, 2016	13:37–11:27	656	Rainy for the last 8 h
	7	1	May 14–15, 2016	14:59–14:21	702	
			May 15–16, 2016	14:32–11:40	635	Rainy for the last 8 h
	8	2	May 14–15, 2016	14:36–13:40	693	
			May 15–16, 2016	14:03–11:31	645	Rainy for the last 8 h
	9	1	May 14–15, 2016	15:13–14:39	704	
			May 15–16, 2016	14:44–11:36	627	Rainy for the last 8 h
	10	1	May 14–15, 2016	14:22–13:04	682	
			May 15–16, 2016	13:12–11:18	664	Rainy for the last 8 h
	11	1	May 14–15, 2016	14:18–13:16	690	
			May 15–16, 2016	13:23–11:19	659	Rainy for the last 8 h
	12	2	May 14–15, 2016	14:52–12:58	664	
			May 15–16, 2016	14:38–9:40	572	Rainy for the last 8 h
A. minamitanianum	1	2	March 24–25, 2014	14:25–13:21	689	
	2	4	March 24–25, 2014	14:32–10:02	586	
	3	1	March 24–25, 2014			

Table 3. A list of the flower visitors photographed on each *Asarum* plant individual

Category of visitor	Plant individual															Total count
	A. costatum												*A. minamitanianum*			
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	
Diptera	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	2
Ants	1	0	1	3	5	8	3	38	3	0	0	4	0	0	0	66
Curculionidae	2	0	0	0	4	0	0	0	0	0	1	0	1	0	3	11
Other Coleoptera	1	0	0	2	0	0	4	1	5	0	1	0	0	0	0	14
Other Hexapoda	1	7	4	1	0	1	4	7	2	9	22	9	6	6	1	81
Isopoda	0	0	0	1	0	0	2	1	4	1	1	2	0	0	0	12
Myriapoda (millipedes and centipedes)	0	1	0	2	0	1	0	1	2	0	0	3	0	0	0	10
Araneae and other arachnids	0	1	0	0	0	0	3	1	2	1	2	4	1	0	1	16
Land snail	2	0	0	0	1	3	0	2	0	0	1	1	0	0	0	10
Land planarians	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
Unknown	2	1	3	1	3	0	2	3	3	2	3	4	0	0	0	27
Total	9	11	8	10	13	15	18	54	21	13	31	28	8	6	5	250

flowers were not removed from the plant body and therefore the volatiles from the damaged tissue were unlikely to be included. We also found that the leaf of *A. costatum* emitted sesquiterpene-dominated volatiles, the compositions of which are mostly shared with the flower of the same plant individual (Fig. 2). Therefore, monoterpenes and C5-branched chain compound, methyl angelate could be the major flower-specific volatile compounds.

To understand the potential association between the variation of floral scents and the pollination systems in *Asarum* species, we also conducted monitoring of flower visitors in two species of *Asarum* series Sakawanum. Especially in *A. costatum*, a relatively intensive effort was made for the monitoring, with a total of 12 plant individuals observed for $>$40 h each. Neverthe-

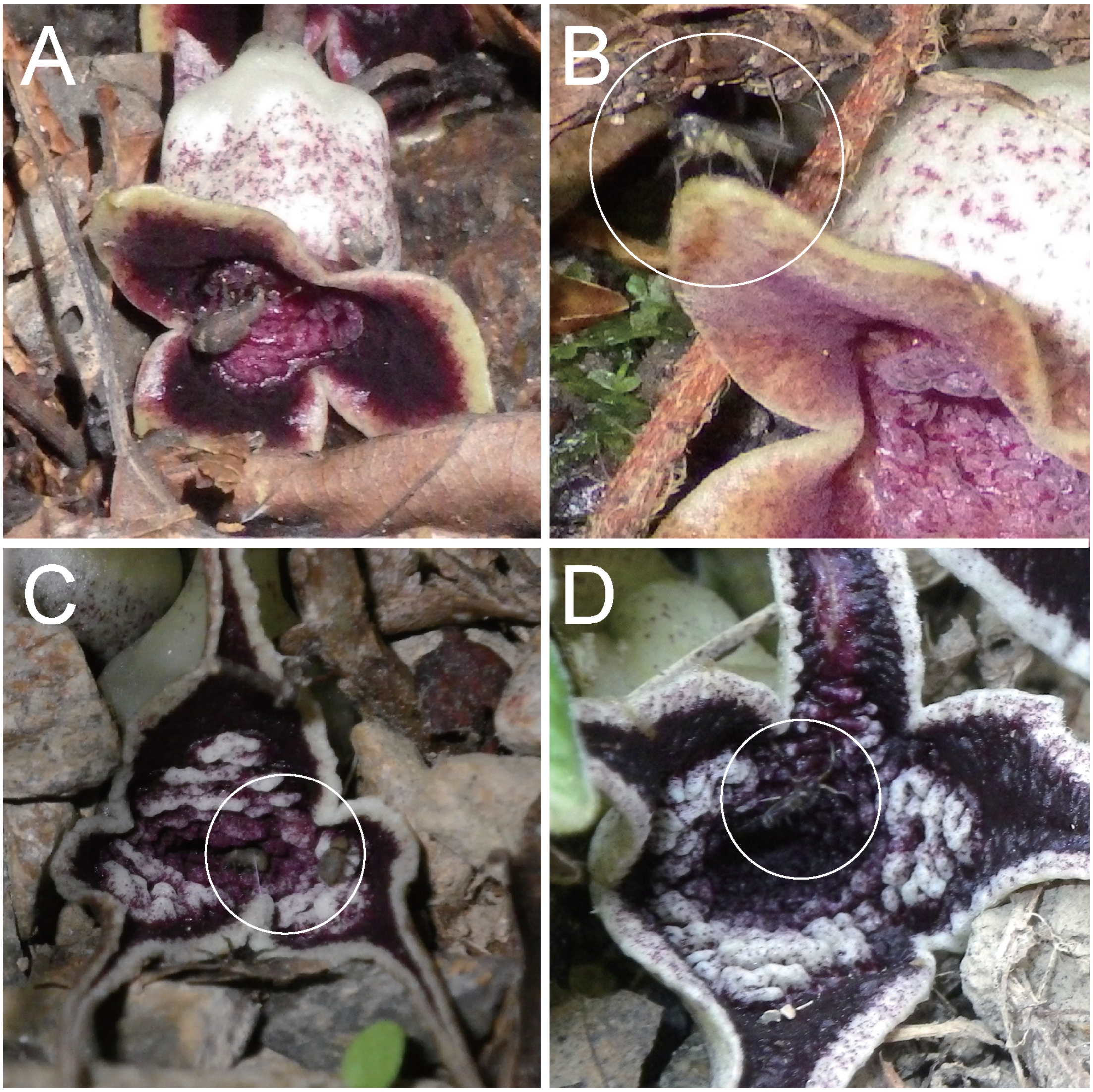

Fig. 1. A part of the flower visitors photographed in the present study. A: a ground-dwelling weevil visiting on *A. costatum*, B: a sciarid fly visiting on *A. costatum*, C: two weevils on *A. minamitanianum*, D: a collembolan visiting on *A. minamitanianum*.

less, we failed to observe repeated visits by winged insects that are presumably effective as pollinator. Thus we could not identify a specific, potential pollinator species in this study. The visitation frequency of the flowers of the two *Asarum* species were low, and most of the flower visitors were flightless animals (Table 3), which are unlikely to contribute to the long-distance transportation of the pollen. Therefore, we failed to address how the plant-pollinator interactions are associated with the floral scent profiles of the Sakawanum species. Mushroom-mimic or other type of brood-site mimicry is considered as the pollination system of the *Asarum* species in general (Vogel, 1973; Sinn *et al.*, 2015), and a few documented examples of the pollinators are indeed mushroom-feeding flies (Sugawara, 1988; Mesler and Lu, 1993), yet we could not find any

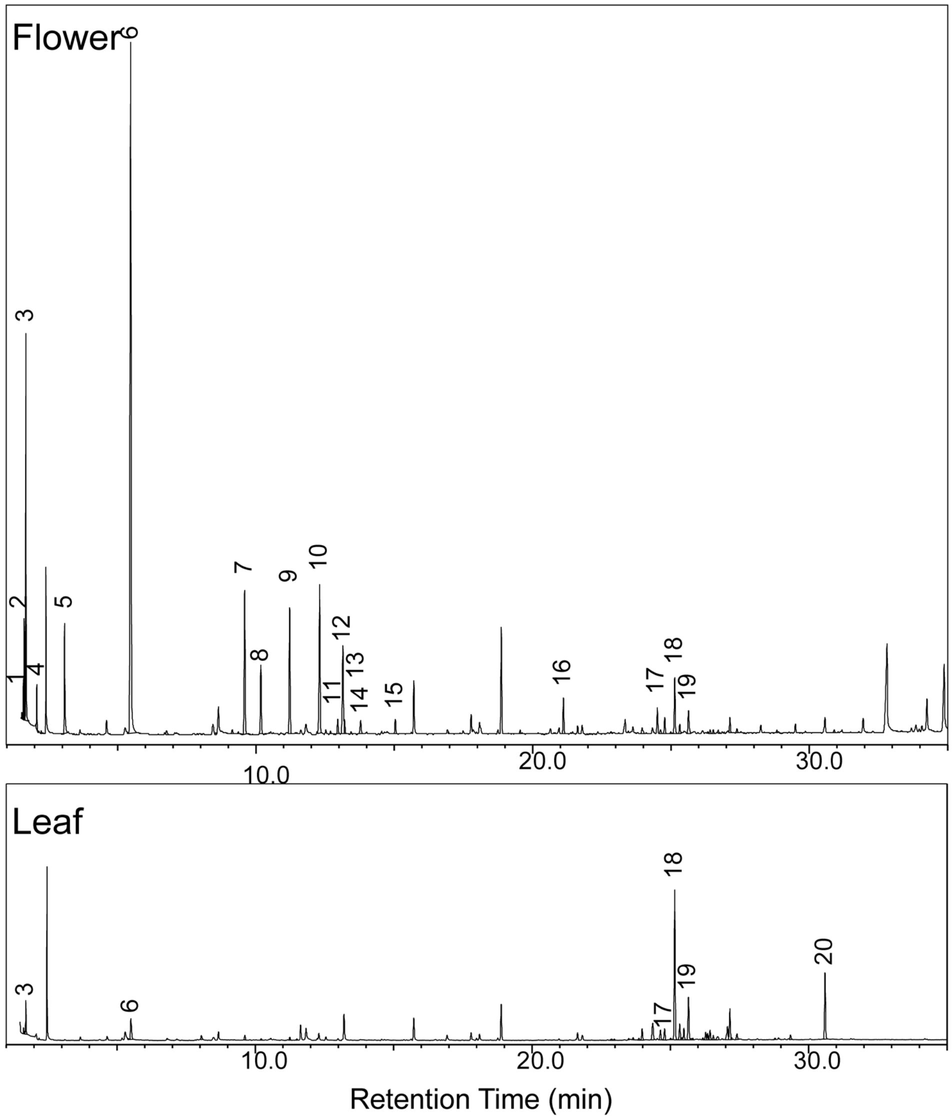

Fig. 2. Chromatograms of the volatile samples collected from a flower and a leaf of *A. costatum* (TBG160624). The scale for signal intensity of the two chromatograms are adjusted. The peaks marked with numbers are the major volatile compounds. 1: 2-butanone. 2: 2-butanol. 3: 2-methyl-3-buten-2-ol. 4: 2-methyl-butanal. 5: 2-methyl-1-butanol. 6: methyl angelate. 7: *α*-pinene. 8: camphene. 9: *β*-pinene. 10: *α*-phellandrene. 11: p-cymene. 12: limonene. 13: eucalyptol. 14: cis-*β*-ocimene. 15: terpinolene or isoterpinolene. 16: bornyl acetate. 17: *β*-caryophyllene. 18: trans-*α*-bergamotene. 19: trans-*β*-farnesene. 20: unidentified compound 4.

evidence that Sakawanum species are mimicking mushrooms [i.e., no mushroom-feeding insects were observed visiting on the flowers (Fig.1, Table 3)]. Floral scent profiles of these species further suggest that they are not mushroom-mimicking flowers. In mushroom mimicking flowers, the floral scents were typically characterized by the C8 alcohols and ketones, which are common in true mushroom fruiting bodies (Johnson and Schiestl, 2016) and we did not find any in the present study.

Recently, Takahashi *et al.* (2017) developed 17

EST-SSR markers for the *Asarum* series Sakawanum in Japan. Using these markers, they calculated a basic genetic diversity metrics for all of the Sakawanum species and found that they seldom deviated from Hardy-Weinberg equilibrium. This indicates that they are largely outcrossing, or at least, only the seeds produced by outcrossing could give rise to the flowering individuals. Therefore, it remained enigmatic how these plants sustain their reproduction with the very-low pollinator visitation frequency.

It might be still early to conclude that their pollinator visitation frequency is extremely low, because the efficient observation of flower visitors could be hindered by the floral characteristic of *Asarum*. *Asarum* flowers often bloom at the ground and the floral organs are hidden inside the calyx tube, which could discount the possibility of the time-lapse photography to detect the flower visitors. Therefore, further research effort might be necessary to elucidate their reproductive system. Such information will also help designing the strategy for conservation of the all four taxa of *Asarum* series Sakawanum, given that they are listed in the Red List of Japan (*A. costatum*: NT, *A. minamitanianum*: CR, *A. sakawanum* var. *sakawanum*: VU, *A. sakawanum* var. s*tellatum:* EN).

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