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Flowers of Deceptive *Aristolochia microstoma* Are Pollinated by Phorid Flies and Emit Volatiles Known From Invertebrate Carrion

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Deceptive flowers decoy pollinators by advertising a reward, which finally is not provided. Numerous deceptive plants are pollinated by Diptera, but the attractive cues and deceptive strategies are only identified in a few cases. A typical fly-deceptive plant genus is *Aristolochia*, which evolved sophisticated trap flowers to temporarily capture pollinators. Though rarely demonstrated by experimental approaches, *Aristolochia* species are believed to chemically mimic brood sites, food sources for adult flies, or utilize sexual deception. Indeed, for most species, studies on scent composition and attractive signals are lacking. In this study, we focused on *Aristolochia microstoma*, a peculiar Greek endemic with flowers that are presented at ground level in the leaf litter or between rocks and are characterized by a unique morphology. We analyzed flower visitor and pollinator spectra and identified the floral scent composition using dynamic headspace and gas chromatography coupled to mass spectrometry (GC/MS). Female and male phorid flies (Phoridae) are the exclusive pollinators, although the flowers are also frequently visited by Sciaridae, as well as typical ground-dwelling arthropods, such as Collembola and arachnids. The carrion-like floral scent mainly consists of the oligosulphide dimethyldisulfide and the nitrogen-bearing compound 2,5-dimethylpyrazine. These compounds together are known to be released from decomposing insects, and thus, we conclude that pollinators are likely deceived by chemical imitation of invertebrate carrion, a deceptive strategy not described from another plant species so far.

Keywords: Aristolochiaceae, deceptive pollination, dimethyldisulfide, 2,5-dimethylpyrazine, floral scent, Phoridae, sapromyiophily, *Megaselia*

INTRODUCTION

Deceptive pollination evolved in 4–6% of angiosperms (Renner, 2006), and relies on the inability of pollinators to distinguish between a true resource (e.g., mating partners, brood-sites, and food) and the flower/inflorescence that imitates the reward (Brodmann et al., 2008, 2009; Urru et al., 2011). Pollinators are cheated by deceptive flowers through sophisticated olfactory, visual, and tactile

traits (Vogel, 1978; Dafni, 1984; Stensmyr et al., 2002; Schiestl et al., 2003; Schiestl, 2005; Stökl et al., 2010; Woodcock et al., 2014). In such systems, Diptera are common pollinators (Renner, 2006; Woodcock et al., 2014). Fly-deceptive pollination strategies include mimicry of brood-sites (Stensmyr et al., 2002; Urru et al., 2011; Jürgens et al., 2013), food (e.g., Heiduk et al., 2016), and mating partners (Martel et al., 2016). However, the specific signals involved in fly attraction and the deceptive strategies are identified in a few cases only (Stökl et al., 2010; Heiduk et al., 2015, 2016; Oelschlägel et al., 2015).

A prominent example of fly-pollinated deceptive plants is the genus *Aristolochia* (Aristolochiaceae). The different species are visited by a wide range of dipteran families, but often information on the actual pollinators is lacking (reviewed in Berjano et al., 2009). However, there is evidence that each *Aristolochia* species is specialized in just one or few pollinator families (e.g., Phoridae, Drosophilidae, and Chloropidae), and in some species fly attraction is sex-specific (Hime and Costa, 1985; Wolda and Sabrosky, 1986; Hall and Brown, 1993; Rulik et al., 2008; Berjano et al., 2009). *Aristolochia* species are long known for their spectacular, highly derived trap flowers (Knoll, 1929). To assure cross-pollination, the plants have evolved elaborate micro- and macromorphological features, enabling them to trap, retain, and release insects [described in detail by Oelschlägel et al. (2009)]. Pollinators enter the protogynous flower in the female phase through the tube, where downward-bending trichomes lead them to the utricle, and prevent them from escaping during the female flower phase (Oelschlägel et al., 2009). The trapped pollinators are able to deposit pollen, previously picked up from another flower, on the receptive stigmatic lobes before the flower enters the male phase. In the early male phase the pollen is released, but trapping trichomes still block the exit, before they finally shrink and allow pollinators to leave the trap, loaded with pollen (Oelschlägel et al., 2009).

Due to their often obvious and strong scents during the female phase, many authors suggested that *Aristolochia* flowers generally attract their pollinators by floral scent (Vogel, 1978; Hall and Brown, 1993; Bänziger and Disney, 2006; Trujillo and Séric, 2006; Rulik et al., 2008; Martin et al., 2017), which indeed was substantiated by behavioral assays in a few species (Cammerloher, 1923; Daumann, 1971; Oelschlägel et al., 2015). Based on the type of scent released, it is believed that the flowers generally mimic brood-sites of their respective pollinators, such as carrion, feces, decaying plants, or fungi, by chemical deception (Cammerloher, 1923; Vogel, 1978; Proctor et al., 1996; Martin et al., 2017). In some weakly odored species with strongly male- or female-biased pollinator attraction, mimicry of sex pheromones was suggested (Wolda and Sabrosky, 1986; Hall and Brown, 1993). First attempts to identify floral scent compounds in *Aristolochia* date back almost 100 years (Cammerloher, 1923, 1933), but the scent composition was only studied recently in four species by quantitative chemical analytics (Stashenko et al., 2009; Johnson and Jürgens, 2010; Oelschlägel et al., 2015; Martin et al., 2017). Among other compounds (e.g., citral), all these studies identified substances characteristic of brood-site deceptive plants (e.g., dimethyldisulfide), with one exception. Oelschlägel et al. (2015) mainly identified aliphatic hydrocarbons and esters in the Mediterranean *A. rotunda*. More detailed physiological and behavioral analyses with the pollinators of this species rejected brood-site deception and discovered a novel pollination strategy in plants, called kleptomyiophily (Oelschlägel et al., 2015). *A. rotunda* deceives its chloropid pollinators by mimicking alarm pheromones of preyed-upon mirid bugs, which are a food source of these kleptoparasitic adult flies (Oelschlägel et al., 2015).

Most of the approximately 500 *Aristolochia* species are native to tropical and subtropical regions, but about 50 species occur in the Mediterranean and adjacent Near East (Nardi, 1984, 1991; Neinhuis et al., 2005; Wanke et al., 2006). Among those, *Aristolochia microstoma* Boiss. & Spruner, a species endemic to Greece, stands out due to its unique perianth morphology and flower presentation (Wanke, 2006). The limb of the small, purplish-brownish flowers (**Figures 1A–C**) is reduced to a small beak or missing, and the entrance into the floral tube is reduced to a small pore, responsible for the name of the species (Nardi, 1991). While most *Aristolochia* species display their often showy flowers above the ground, *A. microstoma* flowers are presented close to or partly buried in the ground, among leaf litter (**Figures 1A,B**) or between rocks (**Figures 1C,D**), often hidden from above (Nardi, 1991; Wanke, 2006). Another unusual feature is the more or less horizontal orientation of the floral tube, which is vertical in other species. Pollinators were hypothesized to be small arthropods living near the ground or in leaf litter (Nardi, 1991; Wanke, 2006). So far, the flower visitors and pollinators, the reproductive biology, and the floral scent of *A. microstoma* remained unknown.

In this study, we recorded flower visitors and pollinators, and analyzed floral scents in three natural populations of *A. microstoma*. Specifically, we asked: (1) Are the flowers, as in congeners, also pollinated by flies, or by other ground-dwelling arthropods, and is the pollinator spectrum similar among populations? (2) Do the flowers produce scent, and if so, what is the composition, and is it similar among populations? Based on the obtained data, we discuss possible deceptive strategies of this unusual flower.

MATERIALS AND METHODS

Study Sites

Aristolochia microstoma is endemic to Central Greece and Northern Peloponnesus, where it colonizes dry, stony, calcareous places in open woodlands, garrigue, and macchia (Nardi, 1991). Samples were collected during field trips in March 2019 and 2020, around the peak of flowering, in "Egaleo" (Athens, Mt. Egaleo, 37.999377N, 23.641652E, 225 m a.s.l.), "Arachneo" (surroundings of Arachneo, near Moní Panagías Talantíou, 37.6714204N, 22.9114465E, 420 m a.s.l.), and "Methana" (Methana peninsula, south of Kypseli, 37.6002630N, 23.4050652E, 65 m a.s.l.). Voucher specimens of the plants are deposited at Herbarium Dresdense (DR).

FIGURE 1 | *Aristolochia microstoma* and its flowers in their natural habitat in Greece (Athens, Mt. Egaleo): The small flowers (length = 2 cm to 3 cm) are presented on ground-level, either well hidden in the leaf litter **(A,B)**, or between rocks **(C)**, where they are not visible from above (**D**; rock removed to see the flowers).

Flower Visitor and Pollinator Collection and Identification

A total of 1,457 flowers (1,044 female phase, 413 male phase) were randomly collected at the three study sites. As a rhizome may or may not produce several shoots (Nardi, 1991), it was difficult to identify a plant individual. Thus, we recorded the visitors at the level of populations. The flowers were opened and checked for trapped arthropods in the field or stored in 80% isopropanol for later processing in the lab. For each flower, we recorded the flowering phase (female or male), and the number of trapped arthropods with and without pollen. Applying the most conservative approach, only arthropods collected from female phase flowers that carried *Aristolochia* pollen were treated as pollinators (Rulik et al., 2008; Oelschlägel et al., 2015). The inaperturate exine is characteristic of *Aristolochia*-pollen (unpublished data), and since no other *Aristolochia* species were co-flowering at the study sites, we assumed that all *Aristolochia* pollen belonged to *A. microstoma*. Collected arthropods were conserved in 80% isopropanol and identified to the order level. Diptera were further identified to family level. The sex of visitors was determined in the two main visitor families, i.e., Phoridae and Sciaridae (see section "Results"). Morphological determinations were performed mainly with the help of Disney (1994) and Oosterbroek (2006). Voucher specimens of the collected arthropods are deposited at the Department of Biosciences, University of Salzburg.

Molecular Analyses of Pollinators

In addition to the identification based on morphological characters, pollinators were characterized by molecular data as well. DNA was extracted from all specimens carrying pollen in female stage flowers (total 25 individuals). In order to preserve the specimens as intact as possible for subsequent morphological identification only one single hind leg of each isopropanol conserved fly was used for DNA isolation.

Genomic DNA was extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. The extracted DNA samples were stored at −20°C until use. The quality and quantity of each extracted DNA sample was assessed using Invitrogen™ Qubit 3 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, United States).

The barcoding marker COI, a 658 bp fragment of cytochrome oxidase I, was amplified using the two primer pairs COI-Dip-F5 (CWACWAAYCAYAARGATATTGG)/COI-Dip R3 (TNGTRATAAAATTWACDGCNCC) and COI-Dip-F7 (CWATTATAATTGGDGGDTTYGG)/COI-Dip-R4 (CCAAARAATCARAATARRTGTTG), respectively (newly designed for this study). The PCR reactions were performed in a total volume of 20 μL containing 5.5 μL ddH_2O, 4 μL 1 × GoTaq Flexi buffer, 0.1 μL GoTaq G2 Flexi DNA polymerase (Promega, Fitchburg, MA, United States), 3.2 μL dNTPs (1,25 mM each), 1.2 μL 25mM $MgCl_2$, 1 μL of 10mM F- and R-primer, and 4 μL of 1:10 diluted genomic DNA. Amplification was performed in Biometra

T3000 thermocycler (Analytic Jena, Jena, Germany) according to the following protocol: initial denaturation for 30 s at 98°C, 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 60 s, and a final extension step at 72°C for 5 min. Quality of PCR products was assessed by gel electrophoresis employing a 1% agarose gel. 5 μL of each PCR, 1.5 μL Gelstar (Bio-RAD Laboratories Inc., Hercules, CA, United States), 2 μL 6× loading dye was run at 80V and amplicons were visualized under UV light (Biometra BioDoc, Analytic Jena, Jena, Germany).

PCR products were purified employing the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Düren, Germany). The manufacturers protocol was followed. Samples were diluted in 30 μL elution buffer and directly sequenced using the Macrogen Europe sequencing service (Amsterdam, Netherlands). Sequence quality and trimming was done by eye accessing the pherograms. Forward and reverse sequences for each PCR product were aligned manually using PhyDE® – Phylogenetic Data Editor version 0.9971[1]. Thus, each COI region for each fly individual was amplified and sequenced in two broadly overlapping parts resulting in up to 4x coverage for each nucleotide. Quality controlled sequences were submitted to NCBI nucleotid blast (megablast) as well as BOLD. First 100 hits were checked for query coverage, and percentage identity. Only BLAST search results with at least 90% query coverage and >95% identity were considered.

[1]http://www.phyde.de/

Floral Scent Sampling

The volatiles emitted by single female phase flowers were collected by dynamic headspace methods (Dötterl et al., 2005) in the field during daytime (11:00–17:30) at the three study sites (Egaleo: $n = 7$; Arachneo: $n = 10$; Methana: $n = 6$). Due to their short fragile stems and hidden position, it was often necessary to cut the flowers for scent sampling. The effect of cutting was found to be minor, as scent collected *in situ* from still attached flowers ($n = 4$) yielded the same compounds in comparable ratios (see section "Results," **Table 1** and **Figure 2**). Therefore, cut and uncut flower samples were pooled for further analyses. Single flowers were inserted into oven bags (10 × 5 cm; Toppits®, Minden, Germany), and scent collection was initiated immediately after bagging. The air containing the volatiles was sucked through an adsorbent tube for 30 min at a flow rate of 200 ml min^{-1} by a membrane pump (G12/01 EB; Rietschle Thomas Inc., Puchheim, Germany). Adsorbent tubes consisted of a microvial (ChromatoProbe quartz microvials; Varian Inc., Palo Alto, CA, United States: length 15 mm, inner diameter 2 mm) filled with 3 mg of a 1:1 mixture of Tenax-TA (mesh 60–80) and Carbotrap

TABLE 1 | Median relative (%) and total absolute (ng/h) amounts of scent (compounds) emitted by single female phase *Aristolochia microstoma* flowers, collected at three natural sites in Greece: Egaleo, Arachneo, and Methana.

		Egaleo ($n = 7$)		Arachneo ($n = 10$)		Methana ($n = 6$)	
KRI	**Relative amounts of scent compounds (%)**	**Median (min – max)**					
Sulfur-bearing compounds							
746	Dimethyldisulfide	78.6	(15.9–97.2)	54.7	(10.4–76.9)	40.4	(12.5–62.8)
979	Dimethyltrisulfide	1.2	(0.0–6.6)	5.4	(1.5–14.7)	3.8	(tr–6.3)
Nitrogen-bearing compounds							
824	2-methylpyrazine	0.0	(0.0–0.6)	0.0	(0.0–0.2)	0.1	(0.0–0.6)
912	2,5-dimethylpyrazine	7.5	(0.4–76.8)	25.8	(11.0–64.8)	46.9	(36.1–76.8)
1140	2-isobutyl-3-methylpyrazine	0.0	(0.0–7.2)	0.0	(0.0–24.3)	0.0	(0.0–0.1)
C5-branched chain compounds							
731	3-methyl-1-butanol	1.4	(0.0–7.9)	0.3	(0.0–1.6)	0.1	(0.0–2.0)
876	3-methylbutyl acetate	0.0	(0.0–1.5)	0.0	(0.0–0.0)	0.0	(0.0–0.1)
Irregular terpenes							
1233	β-cyclocitral	0.0	(0.0–3.9)	0.0	(0.0–0.5)	0.2	(0.0–0.7)
Aromatic compounds							
1598	Methyl-3,4-dimethoxybenzoate	0.3	(0.0–7.9)	0.5	(0.0–1.9)	0.1	(0.0–2.5)
Unknown compounds							
702	Unknown (m/z: 45, 77, 59, 81, 43, and 44)	0.0	(0.0–2.7)	0.0	(0.0–23.6)	0.0	(0.0–6.2)
809	Unknown (m/z: 92, 45, 77, 57, 47, and 44)	tr	(0.0–0.2)	0.1	(0.1–0.3)	0.2	(tr–0.3)
1048	Unknown (m/z: 121, 108, 136, 135, 69, and 83)	0.0	(0.0–0.0)	tr	(0.0–2.2)	0.1	(0.0–0.7)
1068	Unknown (m/z: 47, 126, 63, 79, 64, and 46)	tr	(0.0–0.2)	2.3	(0.3–7.4)	0.3	(tr–3.2)
1133	Unknown (m/z: 122, 121, 135, 108, 150, and 39)	0.0	(0.0–tr)	tr	(0.0–3.8)	tr	(0.0–1.4)
1139	Unknown (m/z: 61, 43, 138, 95, 123, and 85)	0.0	(0.0–0.2)	0.1	(0.0–0.5)	0.1	(tr–1.0)
1283	Unknown (m/z: 79, 108, 93, 99, 127, and 155)	0.0	(0.0–1.4)	0,0	(0.0–0.0)	0.0	(0.0–0.0)
	Total amount of scent per flower (ng/h)	84.2	(2.9–108.9)	48.3	(10.5–139.4)	33.6	(24.1–145.3)

Compounds are sorted by compound class, and within class by the Kovats retention index (KRI). tr, compounds occurring only in trace amounts (<0.05%); m/z, mass-to-charge ratio of unknown compounds.

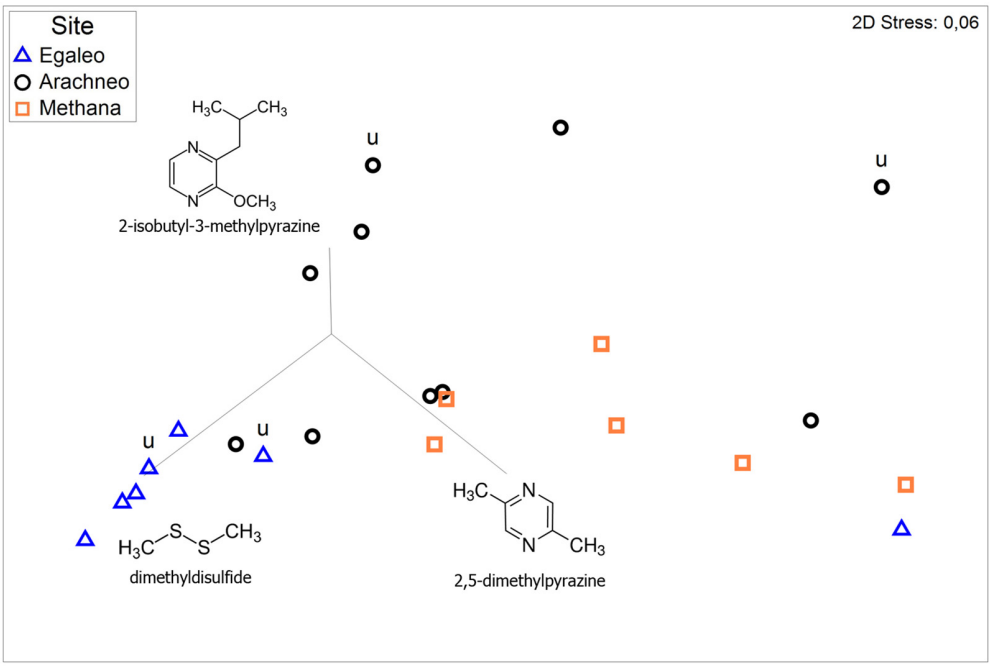

FIGURE 2 | Non-metric multidimensional scaling (NMDS) to visualize semi-quantitative dissimilarities among individual floral scent samples from three *Aristolochia microstoma* populations in Greece: Egaleo (*n* = 7); Arachneo (*n* = 10); and Methana (*n* = 6). The ordination is based on pairwise Bray-Curtis similarities. The vectors depict the volatiles most correlating with the axes. "u" marks samples collected from uncut flowers, all other samples were collected from cut flowers.

B (mesh 20–40) (both Supelco, Bellefonte, PA, United States) fixed by glass wool plugs (Heiduk et al., 2015). Subsequently to sampling, the flowers were dissected to determine their sexual phase. To unambiguously identify compounds as floral volatiles, control samples of leaves, as well as ambient air, were sampled in a similar way. Samples were stored at 4°C during fieldwork and at –20°C in the laboratory before GC/MS analyses.

Gas Chromatography Coupled to Mass Spectrometry (GC/MS)

The adsorbent tubes containing the trapped volatiles were analyzed by GC/MS on an automatic thermal desorption (TD) system (TD-20, Shimadzu, Japan) coupled to a Shimadzu GC/MS-QP2010 Ultra equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; length = 60 m, inner diameter = 0.25 mm, film thickness = 0.25 µm, Phenomenex), as described by Heiduk et al. (2015). At a consistent helium carrier gas flow of 1.5 ml/min, the samples were processed at a split ratio of 1:1. The GC oven temperature started at 40°C, then increased by 6°C/min to 250°C and was held for 1 min. The MS interface worked at 250°C. Mass spectra were taken at 70 eV (EI mode) from m/z 30 to 350. GC/MS data were analyzed using the GCMSolution package, Version 4.41 (Shimadzu Corporation 1999-2015). Compounds were tentatively identified by comparison of Kovats retention indices (KRI, based on a series of n-alkanes) and mass spectra to data available in the databases ADAMS, ESSENTIALOILS-23P, FFNSC 2, and W9N11. All compound identities were confirmed by authentic reference standards available at the Plant Ecology lab of the University of Salzburg. Compounds also detected in leaf and ambient air controls were excluded from the analyses. Total scent emission was estimated by injecting known amounts of alkane standards.

Statistical Analyses

We used chi-square tests to compare sex-ratios in Phoridae and Sciaridae among populations. Similarities and dissimilarities in scent bouquets among the samples were visualized by non-metric multidimensional scaling (NMDS), based on pairwise Bray-Curtis dissimilarities calculated on the relative amounts of compounds. We performed analyses of similarities (ANOSIM; 10,000 permutations) to test for differences in floral scent among study sites, and PERMDISP (Anderson et al., 2008) to test for differences in dispersion among populations (10,000 permutations). All multivariate statistical analyses were performed with the software PRIMER 6.1.0.5 (Clarke and Gorley, 2006).

RESULTS

Flower Visitors and Pollinators

The 248 flower visitors recorded in this study (**Table 2**) originated from 11 to 17% of investigated female-phase and 13–20% of male-phase flowers, respectively, depending on the study site. The majority of flowers had no visitors. Females and males of the dipteran families Phoridae (99 individuals in total) and

Sciaridae (52) were the most abundant flower visitors at all study sites (**Table 2** and **Figure 3**). In flower-visiting Phoridae, sex ratios differed between sites (chi-square$_2$ = 10.3; P = 0.006). They were female-biased at Arachneo and Methana and balanced at Egaleo. In contrast, Sciaridae were male-biased at all sites (chi-square$_2$ = 2.65; P = 0.265). Further visitors included other Diptera (Sphaeroceridae, Drosophilidae), and a range of other arthropods, such as Collembola, Acari, Myriapoda, Isopoda, and Coleoptera. The only visitors that carried pollen in female- and/or male-phase flowers were Diptera. In all flies, pollen was attached dorsally to the thorax, mainly around the wing-base, only occasionally on the front and central parts (see **Figure 3B**). Only Phoridae (25 individuals) carried pollen in female-phase flowers (**Figures 3A,B**), and thus classified as the exclusive pollinators at all three study sites (**Table 2**). At the sites Egaleo and Arachneo, Phoridae of both sexes were recorded as pollinators, at Methana only females. Additional pollen-carrying Phoridae of both sexes (10 females and 4 males in total) were found in male-phase flowers at each site. Morphological identification showed that all but one pollinator belonged to the genus *Megaselia*, mostly the *Megaselia angusta/longicostalis* complex, and *Megaselia scalaris* (Loew, 1866). The BLAST hits on NCBI and BOLD confirmed the presence of species of the *Megaselia angusta/longicostalis* complex [three individuals, each up to >99.7% identity with GenBank accessions of *Megaselia longicostalis* (Wood, 1912)], of *M. scalaris* (one individual; 99.7% identity to GenBank accession HM399356), and also suggests the presence of *Conicera similis* (Haliday, 1833) (one individual) as pollinator (95 to 97% identity with GenBank accessions). COI sequences of the pollinators are provided as a **Supplementary Data Sheet 1**.

Although frequent visitors, Sciaridae never carried pollen in female phase flowers (**Figures 3C,D**), however, pollen grains were found on three individuals collected from male phase flowers at Egaleo.

TABLE 2 | Flower visitors of *Aristolochia microstoma* collected at the three study sites in Greece (Egaleo, Arachneo, and Methana), shown overall (sum) and per site.

	sum	Egaleo	Arachneo	Methana
# female phase flowers	1,044 [13%]	366 [13%]	251 [17%]	427 [11%]
# male phase flowers	413 [15%]	189 [13%]	71 [20%]	153 [15%]
Taxa				
Arachnida				
Acari	16	8	4	4
Araneae	1	0	1	0
Pseudoscorpiones	4	0	1	3
Crustacea				
Isopoda	6	6	0	0
Insecta				
Coleoptera	5	2	3	0
Collembola	26	7	12	7
Diptera				
Drosophilidae	1	0	0	1
Phoridae ♀	66 (19/10)	19 (2/6)	22 (5/2)	25 (12/2)
Phoridae ♂	30 (6/4)	19 (5/2)	5 (1/1)	6 (–/1)
Phoridae unknown sex	3	1	1	1
Sciaridae ♀	11 (–/1)	8 (–/1)	0	3
Sciaridae ♂	41 (–/2)	21 (–/2)	7	13
Sphaeroceridae	15	1	5	9
Hemiptera	3	0	1	2
Hymenoptera	4	3	1	0
Thysanoptera	1	1	0	0
Unidentified larvae	7	3	4	0
Myriapoda	8	1	7	0

Arthropod individuals carrying pollen are given in brackets (in female/male phase flowers), as a subset of visiting individuals. Sexes were determined only in Phoridae and Sciaridae. The total numbers of flowers sampled per site are given, with the percentage of flowers containing arthropods in square brackets. ♀ mean females, ♂ means males.

Floral Scent

Aristolochia microstoma flowers emitted an unpleasant, carrion-like scent, which was typically well noticeable by the human nose from a few centimeters distance to the flowers. The total scent emission per flower varied considerably among flowers (2.9–145.3 ng/h), with a median between 34 and 84 ng/h, depending on the study site. A total of 16 compounds was found (**Table 1**), including nitrogen-bearing (3 compounds), sulfur-bearing (2) and C5-branched chain compounds (2), one aromatic compound, one irregular terpene, and seven unknown substances. The main compounds were dimethyldisulfide, with a median relative amount between 40 and 79%, and 2,5-dimethylpyrazine (8–47%), followed by dimethyltrisulfide (1–5%). Those three compounds were present in all samples, except dimethyltrisulfide, which was not detected in one sample. All further compounds, such as 3-methyl-1-butanol and methyl-3,4-dimethoxybenzoate, were minor. Interestingly, the nitrogen-bearing compound 2-isobutyl-3-methylpyrazine, as well as an unknown compound, were particularly strong in some flowers (both up to 24%), although absent in the majority of samples. The relative amount of compounds differed among sites (**Figure 2**) (ANOSIM: R = 0.826; P = 0.004) and cannot be explained by differences in dispersion among populations (PERMDISP: $F_{2,20}$ = 0.443; P = 0.830). While there were no significant differences between the sites Arachneo and Methana, the site Egaleo differed from both other sites (ANOSIM: R > 0.265; P < 0.019), due to a higher relative amount of dimethyldisulfide (see **Figure 2**).

DISCUSSION

Flower Visitors and Pollinators

Aristolochia microstoma was mainly visited by the dipteran families Phoridae and Sciaridae, and less frequently by Sphaeroceridae and Drosophilidae. Further flower visitors included a range of other arthropods, most frequently members of Collembola, Acari, Myriapoda, Isopoda, and Coleoptera. Among flower visitors, Phoridae were the exclusive pollinators at all study sites. The carrion-like floral scent comprised 16 compounds, and was dominated by the oligosulphides dimethyldisulfide and dimethyltrisulfide, and the nitrogen-bearing compound 2,5-dimethylpyrazine. Absolute and

FIGURE 3 | Two specimens of the two most frequent Diptera families visiting flowers of *Aristolochia microstoma*: **(A)** a pollinating female *Megaselia* sp. (Phoridae) carrying pollen on its thorax **(B)**; and **(C)** a male of an unidentified species of Sciaridae not carrying pollen **(D)**.

relative amounts of the main compounds were variable, and flowers from the site Egaleo differed in scent patterns from Arachneo and Methana.

Our findings show that *A. microstoma* is not pollinated by non-dipteran ground- or litter-dwelling arthropods, as Wanke (2006) hypothesized, but by flies, as all other *Aristolochia* species studied so far (Berjano et al., 2009). To which extent the pollinating phorid flies are ground-associated could not be determined. Of the 25 pollinating phorid flies, 24 belong to the megadiverse genus *Megaselia*, and the remaining individual to the genus *Conicera (C. similis)*, but determination to species level remained difficult. While several COI sequences of the pollinating *Megaselia* specimens showed high accordances to GenBank accessions of the *M. angusta/longicostalis* complex, as well as *M. scalaris*, others did not match any identified accessions. Unfortunately, most individuals of *Megaselia* in BOLD and Genbank are identified to genus level only (if at all). This is due to the difficult identification and the large number of species in the genus *Megaselia*, with the majority of species still undescribed or known from one sex only (Disney, 1994). Therefore, the species mentioned here have to be viewed as provisional, and demand further investigations (ongoing research).

Phoridae are well-documented pollinators and flower visitors in *Aristolochia*. Numerous species in this genus are preferentially or exclusively pollinated by members of this family, including tropical and Mediterranean species, some of them with male or female sex bias (Hime and Costa, 1985; Hall and Brown, 1993; Bänziger and Disney, 2006; Rulik et al., 2008; Berjano et al., 2009; Hipólito et al., 2012; Martin et al., 2017). In our study, the observed sex-ratio in Phoridae could be the result of differing abundances of sexes in the respective fly populations during the collection period. However, a balanced sex-ratio in flower-visiting Phoridae was only found at the site Egaleo, where the floral scent bouquet differed significantly from the other two sites, in which the flower visitors of this family were female-biased. Therefore, it might be possible that these differences in scent lead to sex-biased attractiveness in phorid visitors. In contrast, the flower-visiting Sciaridae were male-biased at all sites, suggesting that the observed differences in floral volatiles did not affect sex-specific attractiveness in this family. Although Sciaridae, and to a lesser extent Sphaeroceridae, were frequently found in the flowers of *A. microstoma*, they were not classified as pollinators. The occurrence of significant numbers of non-pollinating Diptera families is not unusual in *Aristolochia*, since several species attract and trap different Diptera, with only a subset of taxa actually pollinating them (e.g., Cammerloher, 1933; Brantjes, 1980; Hilje, 1984; Burgess et al., 2004; Berjano et al., 2009). The spectrum of flower visitors of *A. microstoma* is remarkably similar to that of *A. pallida* (Rulik et al., 2008), another Mediteranean species. Apart from the pollinating male Phoridae (*Megaselia longicostalis*, *M. pumila*, *M. superciliata*), flowers of *A. pallida* are visited – but not pollinated – predominantly by

Sciaridae of both sexes, and occasionally by other visitors also found in *A. microstoma* flowers, including Sphaeroceridae, Acari, Coleoptera, and Collembola (Rulik et al., 2008; Disney and Rulik, 2012). Preliminary morphometric measures of the narrowest part of the floral tube, and the distance between gynostemium and utricle wall of *A. microstoma* suggest that they are similar to those of *A. pallida* (mean = 1.37 mm and 1.68 mm, respectively; see Rulik et al., 2008). These two major morphological floral filters in *Aristolochia* assure that only visitors sharing a specific body size range – small enough to enter the flower, but big enough to physically interact with the gynostemium – can act as pollinators (Brantjes, 1980; Rulik et al., 2008).

In addition to body size, differences in thoracic bristles could contribute to pollinator specialization, as suggested by Cammerloher (1933). As in other *Aristolochia* species (Bänziger and Disney, 2006; Rulik et al., 2008; Oelschlägel et al., 2015), *A. microstoma* pollen was generally deposited dorsally on the thorax. The majority of the pollen was concentrated around the wing base, where the pollinating Phoridae are covered by pronounced, stiff bristles (**Figure 3B**) that probably facilitate pollen adherence. On the front and central parts of the thorax, where bristles are usually very short, pollen grains were hardly found. The lack of such bristles (**Figure 3D**) might exclude Sciaridae as pollinators of *A. microstoma*, or at least make them less efficient, as three pollen-carrying specimens collected from male flowers indicate. The less frequent dipteran flower visitors of the families Sphaeroceridae and Drosophilidae, which possess thoracic bristles similar to those of Phoridae, were never found with attached pollen. Whether this was due to their low abundance in our samples, or other reasons, i.e., different body size or non-recurrent visitation of flowers, remains unanswered. Anyhow, the importance of thoracic bristles for pollination of *Aristolochia* should be experimentally tested in the future. Non-dipteran arthropods were most likely accidental flower visitors, as reported in other *Aristolochia* species (Cammerloher, 1923, 1933; Trujillo and Séric, 2006; Rulik et al., 2008). Generally, the number of flowers containing visitors was strikingly low across all sites, which could be the result of low pollinator availability, of a low attractiveness of the floral signals, or of a small proportion of attracted animals that entered the flowers through the small pore. This pore might have evolved as a morphological filter, i.e., to limit the number of ground-dwelling animals not appropriate as pollinators, that accidentally fall or crawl into the flower, potentially blocking the flower's reproductive organs.

Floral Scent and Possible Deceptive Strategies

The floral scent of *A. microstoma* was strongly dominated by oligosulphides, which are widespread among plants pollinated by carrion-flies and bats, and alkylpyrazines, which are rare floral volatiles (Knudsen et al., 2006). Especially the high amounts of oligosulphides (dimethyldisulfide and dimethyltrisulfide), suggest a sapromyiophilous pollination strategy, as those compounds are the two most common and characteristic volatiles in carrion and carnivorous dung-mimicking flowers, across several plant families (Jürgens et al., 2006, 2013). In contrast, the second main compound of *A. microstoma*, the alkylpyrazine 2,5-dimethylpyrazine, was rarely found in saprophilous flowers. In lower relative amounts than in the present study, it is emitted by the sapromyiophilous South African stapeliads *Orbea variegata* (11%) and *Stapelia leendertzia* (1%), which also emit high amounts of dimethyldisulfide and dimethyltrisulfide, among other compounds, most prominently indole (Johnson and Jürgens, 2010; Jürgens et al., 2013). Both stapeliads, however, were observed to be visited by flies of the families Calliphoridae and Sarcophagidae, and not by Phoridae (Meve and Liede, 1994; Johnson and Jürgens, 2010). Other pyrazines (3-isopentyl 2,5-dimethylpyrazine and 2,6-dimethyl-3-(2-methyl-1-butyl)-pyrazine) are the main compounds in another stapeliad, *Echidnopsis montana*, the biological function of which remains unclear (Jürgens et al., 2006). Sapromyiophily was proposed for several *Aristolochia* species (Cammerloher, 1923, 1933; Vogel, 1978; Johnson and Jürgens, 2010), but chemical analyses of floral scent remain scarce, limiting comparisons within the genus. Nevertheless, *A. microstoma* shares dimethyldisulfide and dimethyltrisulfide with the sapromyiophilous *A. cymbifera*, which, in cultivation, attracts carrion flies (Johnson and Jürgens, 2010). However, the scent of this species is overall dominated by benzenoids. Dimethyldisulfide is also found in smaller amounts in the neotropical phorid-pollinated *A. gigantea*, the odor of which is dominated by sweet lemon-scented citronella-like compounds (Martin et al., 2017). Compared to those and other *Aristolochia* species, which comprise between 63 and 168 floral scent compounds (Johnson and Jürgens, 2010; Oelschlägel et al., 2015; Martin et al., 2017), the odor of *A. microstoma* with only 16 compounds is strikingly less complex. Although the main floral scent compounds of *A. microstoma* were present throughout all samples, their absolute amounts were variable among individuals, and their relative amounts at the sites Arachnea and Methana differed from the site Egaleo. Such intraspecific variation in floral scent is a widespread phenomenon in both deceptive and rewarding plant species, and can be caused by multiple factors, such as local adaptation and genetic drift (Delle-Vedove et al., 2017). In dichogamous plants or plants with unisexual flowers, floral scents sometimes vary between the sexual phases/flower sexes. Preliminary data of *A. microstoma*, however, suggest that the scent of male-phase flowers is similar in both total amount and composition to that of female-phase flowers (**Supplementary Table 1**), and thus might also attract insects, likely to increase pollen export. In *A. gigantea*, the only *Aristolochia* species with such data available, the scent emission is strongly reduced in the male compared to the female phase, with strong differences in composition between the sexual phases (Martin et al., 2017).

Dimethyldisulfide and dimethyltrisulfide are common volatiles in degrading meat (carcasses and carnivore/omnivore feces), that, however, do not emit 2,5-dimethylpyrazine (Jürgens et al., 2006, 2013). Instead, 2,5-dimethylpyrazine was found in the scent of dead bark beetles (*Ips typographus*), alongside dimethyldisulfide, 3-methyl-1-butanol, and other compounds (Zhang et al., 2003). Future studies have to show whether those compounds are also released from other invertebrate

carrion, e.g., other arthropods, and why 2,5-dimethylpyrazine is obviously not released by decomposing vertebrate carrion (Stutz et al., 1991; Johnson and Jürgens, 2010; Jürgens et al., 2013).

Various pyrazines are important volatiles in animal pheromones, such as urinal pheromones in mammals like mice, voles, and hamsters (Novotny et al., 1986; Boyer et al., 1989; Soini et al., 2005), and sex pheromones of fruit flies (Robacker et al., 2009) and thynnine wasps, the latter being exploited by the sexually deceptive orchid *Drakaea glyptodon* (Bohman et al., 2014). Best known, however, is the role of alkylpyrazines as key volatiles in alarm- and trail pheromones in several genera of ants, including 2,5-dimethylpyrazine (Attygalle and Morgan, 1984; Jackson et al., 1990; Morgan et al., 1992; Hölldobler et al., 2001). Pyrazines are key volatiles in host-localization in specialized myrmecophilous Phoridae (*Pseudacteon* spp.), so called ant-decapitating flies (Sharma et al., 2011; Sharma and Fadamiro, 2013; Ngumbi and Fadamiro, 2014). However, to the best of our knowledge, dimethyldisulfide and dimethyltrisulfide were never reported in context with ant pheromones, and no typical ant-associated (myrmecophilous) phorid genera were found among the pollinators of *A. microstoma*. Although there are also cases of myrmecophilous behavior found in *Megaselia* (Disney, 1994), often described as "one of the largest, most biologically diverse and taxonomically difficult genera in the entire animal kingdom" (Marshall, 2012), the pollinators recorded in the present study are probably unspecifically saprophagous. Larvae and adults of *Conicera similis* and members of the *M. angusta/longicostalis* complex (i.e., *M. longicostalis*) are known to feed on vertebrate (e.g., rabbit) and invertebrate (snail) carrion, decomposing plants, but also fungi (Disney, 1994, 1999; Buck, 1997, 2001). The cosmopolitan *M. scalaris* even utilizes the broadest spectrum of larval substrates known in all insects, including numerous dead and living animals, fungi and plants (reviewed in Disney, 2008). Larvae of Sciaridae, which were frequent flower visitors but not pollinators in *A. microstoma*, are usually feeding on living or decomposing plants and fungi, as well as on herbivore excrements, and are frequently found among detritus and forest litter (Menzel and Mohrig, 2000).

CONCLUSION AND OUTLOOK

The spatial position of *A. microstoma* flowers suggests that the pollinating Phoridae probably search for breeding sites or food close to the ground, in leaf litter, or between rocks. This hidden presentation of the flowers also points toward scent as the attractive cue to lure the pollinators. Our data on pollinators and floral scent indicate that *A. microstoma* deceives its phorid pollinators by employing a sapromyiophilous strategy, as proposed for other *Aristolochia* species. The co-occurrence of high amounts of oligosulphides and 2,5-dimethylpyrazine is novel among plants and suggests a so far undescribed type of sapromyiophilous mimicry. Due to the high similarity to carrion scents of dead beetles, and the absence of either 2,5-dimethylpyrazine or dimethyldisulfide in vertebrate carcasses and carnivorous feces, or ant pheromones, we hypothesize that brood-site mimicry of invertebrate carrion is the most likely deceptive strategy. Studies testing the attractiveness of the scent compounds of *A. microstoma* flowers and different potential substrates to the pollinators are currently carried out to test this hypothesis.

DATA AVAILABILITY STATEMENT

All data used for the study are presented in the manuscript.

AUTHOR CONTRIBUTIONS

BO, CN, SW, and SD designed and planned the study. TR, SW, and BO conducted the field work and collected the samples. TR, KR, BO, and HM processed the flower visitors. KR and TR identified the arthropods to order/family level. HM, BO, TW, and SW performed the molecular lab work and characterization of phorid flies. RD morphologically identified phorids flies. TR performed and SD supported the chemical and statistical analyses. TR drafted the manuscript. All authors contributed to the final manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.658441/full#supplementary-material

Supplementary Table 1 | It contains the floral scent dataset.

Supplementary Data Sheet 1 | It contains the COI sequence data of the pollinators.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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