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Research

Chemical and morphological filters in a specialized floral mimicry system

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Summary

- Many plant species attract insect pollinators through chemical mimicry of their oviposition sites, often detaining them in a trap chamber that ensures pollen transfer. These plant mimics are considered to be unspecialized at the pollinator species level, yet field observations of a mycoheterotrophic rainforest orchid (*Gastrodia similis*), which emits an odour reminiscent of rotting fruit, indicate that it is pollinated by a single drosophilid fly species (*Scaptodrosophila bangi*).
- We investigated the roles of floral volatiles and the dimensions of the trap chamber in enforcing this specialization, using gas chromatography-mass spectrometry analyses, bioassays and scanning electron microscopy.
- We showed that *G. similis* flowers predominantly emit three fatty-acid esters (ethyl acetate, ethyl isobutyrate and methyl isobutyrate) that were shown in experiments to attract only *Scaptodrosophila* flies. We additionally showed that the trap chamber, which flies enter into via a touch-sensitive 'trapdoor', closely matches the body size of the pollinator species *S. bangi* and plays a key role in pollen transfer.
- Our study demonstrates that specialization in oviposition site mimicry is due primarily to volatile chemistry and is reflected in the dimensions of the trapping apparatus. It also indicates that mycoheterotrophic plants can be specialized both on mycorrhizal fungi and insect pollinators.

Introduction

Ecological specialization is the hallmark of adaptive radiation and is thus a central theme in evolutionary biology. Organisms highly specialized to a particular habitat or resource use are prone to divergent selection acting on their traits, which can potentially lead to rapid isolation between species, as exemplified by Darwin's finches (Schluter, 2000). Among the vast diversity of plants using insects for pollination, floral specialization is most commonly manifest at the level of functional pollinator groups such as euglossine bees or hawkmoths (Johnson & Steiner, 2000; Pellmyr, 2002). Species that are specialized at the pollinator species level have proven to be relatively infrequent, and include plants that accommodate pollinating seed consumers in their reproductive structures (Sakai, 2002a; Song *et al.*, 2014) and orchids that deceive naïve wasp males through mimicry of conspecific females (Schiestl *et al.*, 1999).

Thousands of plant species across a wide range of families are suspected to attract fly and beetle pollinators through chemical mimicry of their oviposition sites (Dobson, 2006; Urru *et al.*, 2011; Jürgens *et al.*, 2013; Schiestl & Johnson, 2013). Studies of the chemical signalling evolved by independent plant lineages – for example in cycads (Proches & Johnson, 2009), aroids (Stensmyr *et al.*, 2002; Urru *et al.*, 2011) and stapeliads (Jürgens *et al.*, 2006) – have led to the identification of several general categories of oviposition sites mimicked by plants, such as carrion (Stensmyr *et al.*, 2002; van der Niet *et al.*, 2011; Jürgens *et al.*, 2013), dung (Johnson & Jürgens, 2010; Urru *et al.*, 2010; Humeau *et al.*, 2011), mushrooms (Kaiser, 2006; Ren *et al.*, 2011), rotting fruits (Goodrich & Raguso, 2009; Proches & Johnson, 2009; Maia *et al.*, 2012) and yeasts (Stökl *et al.*, 2010). These biological substrates are largely ephemeral, and insects that use them have a low probability of finding suitable sites for laying their eggs. Unlike living tissue, decaying material is not defended by chemicals that must be overcome through biochemical arms races. Therefore, insects that seek decaying material as oviposition sites should have a broader host range than counterparts that seek living tissue as food for themselves or their offspring (Pellmyr, 2002). As for plants with flowers that mimic such sites, current evidence shows that they tend to have pollination systems that are relatively generalized at the insect species level (Endara *et al.*, 2010; Stökl *et al.*, 2010; van der Niet *et al.*, 2011).

Many of these plants that mimic insect oviposition sites have flowers with a trapping chamber that may filter out insects according to their size, and also manipulate them into positions that effect pollen transfer (Bolin *et al.*, 2009; Urru *et al.*, 2011). Some plant species even have a touch-sensitive floral organ, which forces insects against the reproductive parts of the flower (Liu *et al.*, 2010; Phillips *et al.*, 2014). The morphometrics of the trapping mechanism could thus enforce some degree of specialization if this is not achieved by the volatile signals alone.

In this study, we explore the roles of floral volatiles and floral morphology in determining the level of specialization for pollinators in a rainforest orchid, *Gastrodia similis*, which has a floral odour reminiscent of rotting fruit. Species of *Gastrodia* are achlorophyllous and colonize forest niches characterized by low light availability, high moisture and rich soil litter. They are fully mycoheterotrophic, that is, they rely solely on root mycorrhizal fungi for carbon uptake throughout their life cycle (Bidartondo, 2005; Martos *et al.*, 2009; Selosse *et al.*, 2010; Selosse & Martos, 2014). Unlike the nutritional strategies, very few empirical studies have been devoted to the reproductive strategies of these plants in their mycoheterotrophic niches (but see Jones, 1985; Lehnebach *et al.*, 2005; Kato *et al.*, 2006), perhaps because mycoheterotrophic plants have often been assumed to be autogamous or to have generalized pollination systems (Bidartondo, 2005; Waterman & Bidartondo, 2008).

We asked the question whether the volatile organic compounds emitted by the flowers, the morphology of the trap chamber, or both, account for the level of pollinator specialization in this new mimicry system. We specifically aimed to: identify the floral scent composition of *G. similis* flowers; test the behavioural responses of drosophilid fly species that are present in the forest habitat to floral volatiles presented both individually and in blends; and test the morphological fit between drosophilid species and the trap chamber of *G. similis*.

Materials and Methods

Study species and sites

Gastrodia similis Bosser grows as scattered, small colonies in rainforest below 500 m a.s.l. on the eastern slopes of Reunion Island. Plants are underground for most of the year. Flowering stems, which appear for 2–3 wk during the cooler season between July and September, reach 10–25 cm above ground and bear 1–10 flowers (Fig. 1a). The sepals are fused throughout more than half of their length, forming an urceolate floral chamber *c.* 12 mm in

Fig. 1 Pollination of the orchid *Gastrodia similis* by the fruit fly *Scaptodrosophila bangi* (Drosophilidae). (a) *G. similis* colony in primary rainforest habitat. (b) Two *S. bangi* flies visiting a *G. similis* flower, one is outside of the floral chamber, the other one inside. (c) *S. bangi* fly leaving the trap chamber in a *G. similis* flower via the anther; note the exact morphological fit between the fly and the trap chamber between the tooth-like appendages on either side of the column. (d) *S. bangi* fly carrying two sectile pollinia of *G. similis* on the upper thorax. (e) *G. similis* flower after pollen deposition; note that only pollen grains are deposited onto the sticky stigma. (f) *S. bangi* fly with *G. similis* pollinia on the thorax visiting rotting fruits in the rainforest habitat. Bars, 2 mm.

depth coloured brownish-white to dull brown and translucent at both ends (Fig. 1b). The labellum *c.* 6.5 × 3.5 mm fits loosely within the chamber, is covered with papillae and bears two fleshy calli at its base; it turns from bright orange basally to yellow and dull brown through green apically (Fig. 1e). The column is as long and as wide as the labellum, winged apically, and bears an ovate stigma at the base and a sub circular anther at the apex (Fig. 1e); a tooth-like appendage is seen on either side of the anther (Fig. 1c). The anther consists of two sectile pollinia attached to a shared viscidium (Fig. 1d). The flower of *G. similis* does not produce nectar. This orchid is self-compatible but requires pollinators to initiate fruit development (Supporting Information Table S1). Pollination and floral scent chemistry in this species have not been reported previously.

Our study was conducted during the flowering seasons of 2011 and 2012 in two populations of *G. similis* on Reunion Island: Mare Longue (ML: 21°21′01″S; 55°44′34″E; elevation 310 m), the best-preserved lowland rainforest, and Sainte-Suzanne (SS: 20°56′59″S; 55°35′02″E; elevation 380 m), a formerly similar habitat today dominated by alien tree species.

Flower visitors

At ML, we recorded the number of visits on three flowers, each on a different inflorescence, between 5:00 h and 19:00 h, using Sony Handycam® (Sony, Tokyo, Japan) camcorders equipped with hard drives and long-life batteries. We later reviewed the videos and calculated the number of flower visits per 1-h interval over 14 h. At both sites, we randomly sampled drosophilid flies on *G. similis* inflorescences, throughout the two flowering seasons, using a mouth aspirator. Flies were stored in ethanol for further identification. When flies carried *G. similis* pollinia, we kept them dry at 4°C in order to identify the attachment site, because the pollinia fall apart in ethanol.

Flies were identified using specialist literature (Burla, 1954; Bock & Wheeler, 1972; Tsacas & Bocquet, 1976; Lemeunier *et al.*, 1997). In the case of the genus *Scaptodrosophila*, diagnoses were based on all characters that are important for this genus (Burla, 1954); that is, the form of the male terminalia and facial carina, the palpus, the coloration and the setation of the frons, thorax, and the pilosity of the eye (Fig. S1), following recommendations by a taxonomic expert (S. McEvey, pers. com.). DNA-based identification methods were not used in the present study, because sequence data available for the genus *Scaptodrosophila* are scarce.

Local drosophilid assemblages

In order to assess the level of pollinator specialization in *G. similis*, we investigated the local communities of fruit-breeding drosophilid species in the forest habitats at ML and SS. This was to establish whether the apparent specialization of the orchid to one particular drosophilid species reflected an absence of other fruit-breeding insects, or alternatively a highly specialized pollination system. We used bait with fermented banana known to attract a wide range of Drosophilidae (Stökl *et al.*, 2010). Six baited traps were set up in both sites. They each consisted of 150 g over-ripe mashed banana at the bottom of a 1.5 l bottle in which a 5 × 5 cm window was cut out from the side. The bottle was then hung on a tree branch at *c.* 30 cm above the ground within a radius of 10 m from a *G. similis* colony and left for 2 wk, thus covering several fermentation stages. We sampled flies within the bottles using a mouth aspirator every 2–3 d and stored them in ethanol.

Chemical mimicry

We sampled the volatile compounds emitted by eight (four per site) intact inflorescences of *G. similis* using the dynamic headspace method. Each inflorescence was enclosed within a polyacetate bag for 30 min, after which air was pumped from the bag for 30 min at 200 ml min^{-1} through a quartz tube containing a 1 : 1 mixture of 3 mg Tenax-TA (mesh 60–80; Supelco; Sigma-Aldrich) and Carbotrap (mesh 20–40; Supelco) using a portable membrane pump (Spectrex PAS-500; Spectrex, Redwood City, CA, USA). Control samples were made using the same procedure but without enclosing *G. similis* inflorescences.

Using the same sampling method, we also sampled the volatile compounds emitted by indigenous fruits undergoing fermentation on the forest floor at ML and SS. We selected three plant species of the local plant communities, *Labourdonnaisia calophylloides*, *Mimusops balata* (both Sapotaceae) and *Ficus mauritiana* (Moraceae), known to attract and host drosophilid flies on Reunion Island (D. Strasberg, pers. comm.).

All scent samples were later analysed by gas chromatography-mass spectrometry (GC-MS). We used a Varian CP-3800 gas chromatograph (Varian, Palo Alto, CA, USA) with a Varian 1079 injector equipped with a 'ChromatoProbe' thermal desorption device and DB-5 column coupled to a Varian 1200 quadrupole mass spectrometer. Identification of the compounds was conducted with the NIST 02 MS database and by comparison of their retention indices with published ones on the DB-5 column. For three volatile compounds dominating the floral scent bouquet of *G. similis* (i.e. ethyl acetate, ethyl isobutyrate and methyl isobutyrate), we confirmed the identity by co-injection of authentic standards.

Fly responses to volatiles

In order to test the effects of the main volatile compounds emitted by *G. similis* flowers on specialization, we carried out attraction experiments with olfactory sticky traps in the best-preserved forest habitat at ML. The trap consisted of a translucent plastic disc (8 cm in diameter) onto which odourless glue (Tangle-Trap® Sticky Coating; Tanglefoot, Grand Rapids, MI, USA) was applied. The disk was placed on top of a 10-ml vial, and a wick was inserted into the vial through the disk centre so that it protruded 1 cm above the disk. We introduced 2 ml of solution of either ethyl acetate, ethyl isobutyrate, methyl isobutyrate, or a 50 : 25 : 25 (respectively) mixture (matching the actual scent composition of the orchid; see Table 1) of these compounds, each

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Table 1 Mean percentage of volatile compounds emitted by flowers of the orchid *Gastrodia similis* and rotting fruits of *Ficus mauritiana*, *Labourdonnaisia calophylloides* and *Mimusops balata*

Compound	Kovats retention index on DB-5	*G. similis* $n = 8$	*F. mauritiana* $n = 4$	*L. calophylloides* $n = 4$	*M. balata* $n = 4$
Fatty acid derivatives					
2-Butanone	615	–	–	–	10.8
Ethyl acetate*	620	45.8	7.3	–	–
Methyl isobutyrate*	655	22.2	–	–	–
2-Pentanone	660	–	–	–	12.8
3-Hydroxy-2-butanone	685	1.0	0.1	–	–
Ethyl isobutyrate*	715	22.9	1.4	–	–
Methyl butyratre	725	–	0.1	–	–
2-Methylpropyl acetate	730	–	–	1.3	–
2-Hexanone	740	–	–	–	16.4
Ethyl butyrate	755	–	23.1	–	–
Hexanol	825	–	–	–	8.6
2-Acetoxy-3-butanone	845	0.6	–	–	–
2-Heptanone	850	–	1.0	–	14.9
2-Heptanol	860	–	1.2	–	–
2-Methyl-4-heptanone	890	–	–	–	2.4
Methyl hexanoate	985	–	0.7	–	–
Ethyl hexanoate	1005	–	35.3	–	–
Hexyl acetate	1010	–	0.9	–	–
2-Heptyl acetate	1030	–	0.1	–	–
Methyl octanoate	1035	–	0.2	–	–
Ethyl 2-hexenoate	1045	–	2.6	–	–
6-Methylheptanol	1055	–	–	–	1.4
Octanol	1075	–	0.5	–	–
2-Nonanone	1095	–	2.8	–	2.4
Ethyl heptanoate	1100	–	0.6	–	–
2-Nonanol	1110	–	2.5	–	–
Ethyl 3-hydroxyhexanoate	1140	–	0.1	–	–
(E,Z)-2,6-Nonadienal	1170	–	–	–	1.3
Ethyl benzoate	1195	–	0.1	–	–
Ethyl 4-octenoate	1205	–	0.1	–	–
Ethyl octanoate	1220	–	11.9	–	–
Octyl acetate	1230	–	0.1	–	–
Ethyl (*E*)-2-octenoate	1275	–	0.6	–	–
2-Undecanone	1325	–	0.8	–	–
2-Undecanol	1335	–	1.2	–	–
Ethyl decanoate	1430	–	0.9	–	–
Benzenoids and phenyl propanoids					
Styrene	860	–	–	18.2	4.1
Methoxymethylbenzene	1000	–	–	15.8	–
2-Phenylethanol	1130	–	–	–	0.6
Methyl 2-hydroxybenzoate	1225	–	–	–	0.3
β-Phenethyl acetate	1290	–	–	0.1	–
Methyl 2-aminobenzoate	1390	–	–	–	0.6
C5-branched compounds					
2-Methylbutanol	710	–	–	2.0	11.8
Methyl 2-methylbutyrate	765	4.1	–	–	–
Ethyl 2-methylbutyrate	800	1.3	2.2	–	–
3-Methylbutyl acetate	840	–	1.1	26.3	–
3-Methylbutyl butanoate	1050	–	0.2	–	–
Hexyl 2-methylbutyrate	1260	–	–	–	0.5
Terpenoids					
α-Pinene	905	–	–	3.7	–
Sabinene	955	–	–	2.4	–
Myrcene	970	–	–	4.8	–
p-Cymene	1015	–	–	–	0.2
(*E*)-Ocimene	1025	–	–	–	0.8
p-Mentatriene	1030	–	–	7.9	–
α-Ocimene	1040	–	–	5.6	5.3

Table 1 (Continued)

Compound	Kovats retention index on DB-5	*G. similis* $n = 8$	*F. mauritiana* $n = 4$	*L. calophylloides* $n = 4$	*M. balata* $n = 4$
γ-Terpinene	1060	–	–	8.4	–
Linalool	1105	–	–	–	0.5
β-Terpineol	1160	–	–	–	0.2
Camphor	1175	–	–	0.2	–
Menthone	1180	–	–	1.3	1.8
Borneol	1200	–	–	1.0	–
4-Terpineol	1210	–	–	0.1	–
α-Terpineol	1225	–	–	1.0	–
Methyl citronellate	1285	–	–	–	0.1
α-Ylangene	1420	–	0.2	–	–
α-Copaene	1425	–	–	–	0.2
α-Santalene	1465	0.3	–	–	–
β-Caryophyllene	1475	–	–	–	2.1
(*E*)-α-Bergamotene	1480	1.8	–	–	–
epi-β-Santalene	1495	0.2	–	–	–
α-Caryophyllene	1515	–	–	–	0.1

*Identification of these volatile compounds was confirmed by comparison of their retention times and mass spectra with co-injected authentic standards.

diluted 1 : 100 in paraffin oil. Paraffin oil is odourless to humans and does not yield gas chromatography peaks in headspace samples. Nevertheless a trap consisting of pure paraffin oil was used as a control. Using the dynamic headspace method, we found release rates of volatile compounds from the traps to be equal or less than five-fold those of a single *G. similis* inflorescence (note that in the natural situation up to ten inflorescences are usually present next to each other; see Fig. 1a). We placed the five traps onto the forest floor in five different positions *c.* 4 m distance apart and switched them randomly between these five positions every hour, removing any drosophilids trapped on the sticky disks and placing them into ethanol before each switch. We repeated this experiment twice on two consecutive days (10 h total).

We analysed the effects of different volatile compounds on the average number of flies trapped per hour by fitting a generalized estimated equation (GEE) model with appropriate distribution for count data (negative binomial), and allowing for the random effect due to the position of the trap, using the gee procedure in SPSS (SPSS Inc., Chicago, IL, USA). Treatment means were compared using *post-hoc* Sidak tests.

Morphological fit

In order to test whether the dimensions of the trap chamber in the flower of *G. similis* constrains the level of specialization, we compared it with the head widths of fruit-breeding drosophilid species found in the local communities. We first measured the width of the channel between the two tooth-like appendages on either side of the anther (see Fig. 4b), in 36 *G. similis* flowers harvested from different colonies in both sites. We then measured the head width in both sexes of eight drosophilid species, including the pollinator species, which were present in the local communities (15–22 individuals per species depending on the relative abundances in banana baits). Samples were examined using scanning electron microscopy (SEM) and measured with a precision up to 10^{-7} m, after dehydrating biological tissues with a critical point dryer (Quorum K850; Quorum Technologies, Lewes, UK) and coating their surface with gold (Eiko IB3; Eiko Corp., Tokyo, Japan). Comparison with alcohol-stored material indicated that critical point drying did not result in any shrinkage of material (data not shown).

Results

Flower visitors and behaviour

We recorded a high number of flower visits by drosophilid flies, with a mean ± SE of 80.7 ± 12.1 visits per flower during 14 h of video observations. Visits occurred at any time of the day,

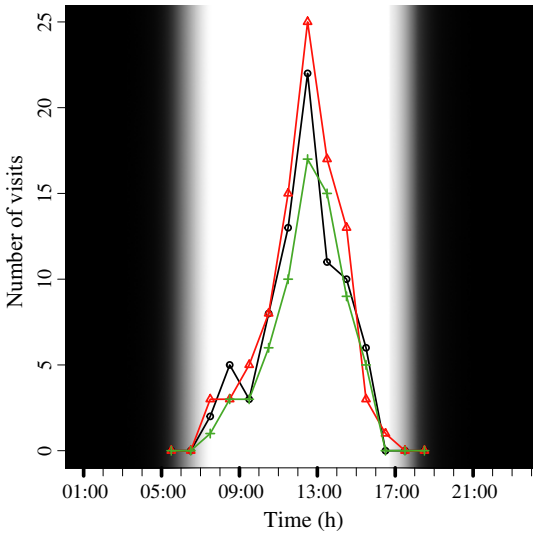


Fig. 2 Numbers of visits by *Scaptodrosophila* flies, recorded on three flowers of the orchid *Gastrodia similis* during 14 h of video observations.

between 06:30 and 16:30 h, but with a consistent peak of visit frequency at *c.* 12:30 h (Fig. 2). We sampled 25 flower visitors at ML ($n = 9$) and SS ($n = 16$) that were later identified as either males ($n = 11$) or females ($n = 14$) of the Drosophilidae species *Scaptodrosophila bangi* (Table S2). Although *S. bangi* flies of both genders were caught on *G. similis* flowers, only females ($n = 10$) were found to carry the sectile pollinia of *G. similis* (Fig. S2).

Similarly to Endara *et al.* (2010), we observed guarding on *G. similis* flowers by *S. bangi* flies. A fly individual could spend a long time on the outer corolla, sometimes > 1 h, charging at any conspecific intruders landing on it. On rare occasions, courtship and mating appeared to take place on *G. similis* flowers (F. Martos, pers. obs.), as was also observed on indigenous fruits undergoing fermentation on the forest floor (Fig. S3). However, we never observed any egg-laying behaviour on the *G. similis* flowers. Not all of the fly visitors went into the floral chamber. Once inside, flies were sometimes seen lapping at the base of the labellum with their proboscis.

Pollination process

Once triggered by a *S. bangi* visitor, the touch-sensitive labellum of the orchid *G. similis* moves upwards, hence lifting the fly up towards the column where the reproductive organs are (Video S1). After *c.* 1.5 min the fly is trapped in a longitudinal channel between the labellum and the column, from which it can only escape by moving forward between the two tooth-like appendages via the anther (Fig. 1c). The two sectile pollinia connected by a shared viscidium are transferred to the upper thorax (Figs 1d, S2), as the fly exits the trap chamber. The labellum returns to its lowest position in *c.* 8 min, which allows the same fly or a different one to deposit pollen onto the stigma through the same process. This touch-sensitive labellum 'trapdoor' can be triggered several times and will eventually stop moving after the stigma has received pollen and has returned to its lowest position, thus releasing the fly (Fig. 1e).

Local drosophilid assemblages

The banana bait attracted eight different species of Drosophilidae, including the pollinator *S. bangi* that visited late fermentation stages exclusively (Table S2). Some species, such as *Drosophila nasuta* and *Zaprionus* spp., were up to thirty times more abundant than *S. bangi* within the bait traps, suggesting high population sizes for these species at the study sites, as is often the case for cosmopolitan species (D. Lachaise & M-L. Cariou, pers. obs.).

Chemical mimicry

Gastrodia similis flowers emit a subtle, fruity odour reminiscent of fermented apple or pineapple to the human nose. The GC-MS analyses (Table 1) revealed that at both sites, the scent of *G. similis* flowers is dominated by three fatty-acid esters, namely ethyl acetate (45.8% total emission), ethyl isobutyrate (22.9%) and methyl isobutyrate (22.2%). The remaining volatile compounds consist of trace compounds (≤ 1%), with the exceptions of the two esters methyl- and ethyl- 2-methylbutyrate (4.1% and 1.3%, respectively), as well as the sesquiterpene (*E*)-α-bergamotene (1.8%). Most of these trace compounds are known to occur widely in floral odours (Knudsen *et al.*, 2006). Scent emission varies little throughout the day (data not shown).

To humans, the odours of fruits of *L. calophylloides* and *M. balata* are reminiscent of melon, but become unpleasant as they reach maturity. The odour of fruits of *F. mauritiana* is more characteristic of a fruit undergoing fermentation. Several drosophilid species showed attraction to the mature fruits of *F. mauritiana* and *M. balata* in the forest habitats, one of which was the pollinator species *S. bangi* (Figs 1f, S3; Table S2). None of the drosophilid species was observed on mature fruits of *L. calophylloides*. Interestingly, the GC-MS analyses (Table 1) revealed that the odour of *F. mauritiana* fruits is dominated by fatty-acid ethyl esters, for example, ethyl hexanoate (35.3%), ethyl butyrate (23.1%) and ethyl octanoate (11.9%), but also contains two key functional compounds in the scent of *G. similis* flowers, namely ethyl acetate (7.3%) and ethyl isobutyrate (1.4%). The scent composition of *M. balata* and *L. calophylloides* fruits is similar, and, relative to the orchid and rotting figs, they both emit fewer fatty-acid esters and more ketones and monoterpenes, as well as aromatic compounds (Table 1).

Fly responses to volatiles

In the bioassays involving sticky traps laced with volatile compounds, 596 flies were trapped over a 10-h period (Table S3). After identification, most were found to be *S. bangi* ($n = 512$) and of these the majority of individuals were female ($n = 323$; 63%). All traps except the paraffin control attracted *S. bangi* flies, and there were significant differences among treatments in the mean numbers of *S. bangi* flies trapped per hour: mixture > ethyl acetate > ethyl isobutyrate > methyl isobutyrate ($P < 0.05$; Fig. 3a). Moreover, although both genders responded to the different traps, the number of females attracted overall was double that of the number of males. Two of the volatile compounds (i.e. ethyl acetate and methyl isobutyrate, and the mixture), attracted the congeneric species *S. triangulifer* ($n = 74$) besides *S. bangi* (Table S3). *Scaptodrosophila triangulifer* flies (Fig. S1), and females in particular, were attracted more to methyl isobutyrate than to ethyl acetate (Fig. 3b). The pollinator *S. bangi* was the only species attracted to the compound ethyl isobutyrate. Other drosophilid species present in the environment hardly responded to the olfactory traps (Table S3).

Morphological fit

The channel between the two tooth-like appendages that shape the trap chamber (see Fig. 4b) had a minimum, median and maximum width value of 615.1, 751.25 and 870.9 μm, respectively (Fig. 4a). A percentage of individuals of the eight fruit-breeding drosophilid species present in the environment, with the exceptions of female *D. immigrans* and *Zaprionus* spp. that were found to be consistently bigger, can fit in the widest channels recorded.

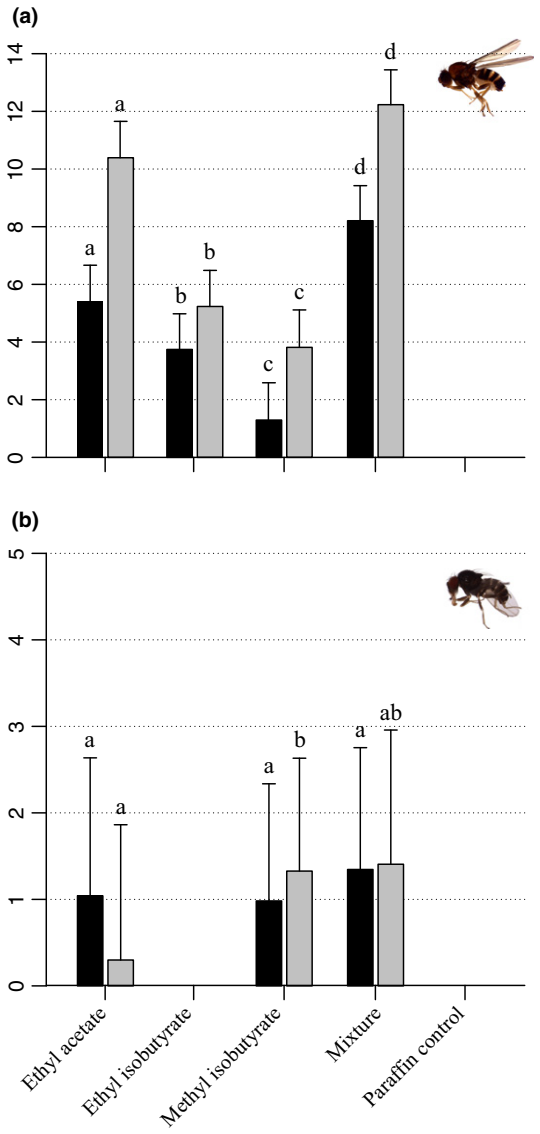


Fig. 3 Estimated marginal means (± SE) of flies of the pollinator species *Scaptodrosophila bangi* (a) and the congeneric species *S. triangulifer* (b), attracted per hour to five olfactory traps laced with ethyl acetate, ethyl isobutyrate, methyl isobutyrate, a 50 : 25 : 25 (respectively) mixture of these compounds, each diluted 1 : 100 in paraffin oil, and a control of pure paraffin oil. Both males (black bars) and females (grey bars) are represented. Lower cases denote significant differences between treatment means.

However, the two *Scaptodrosophila* species, *S. bangi* and *S. triangulifer*, showed the best morphological fit with the *G. similis* trap chamber, as most individuals were narrower than the median size of its channel (Fig. 4a). In addition to the *Scaptodrosophila* flies, another species in the subgenus *Sophophora*, *D. ercepeae*, showed a good morphological fit with the trap chamber of *G. similis*.

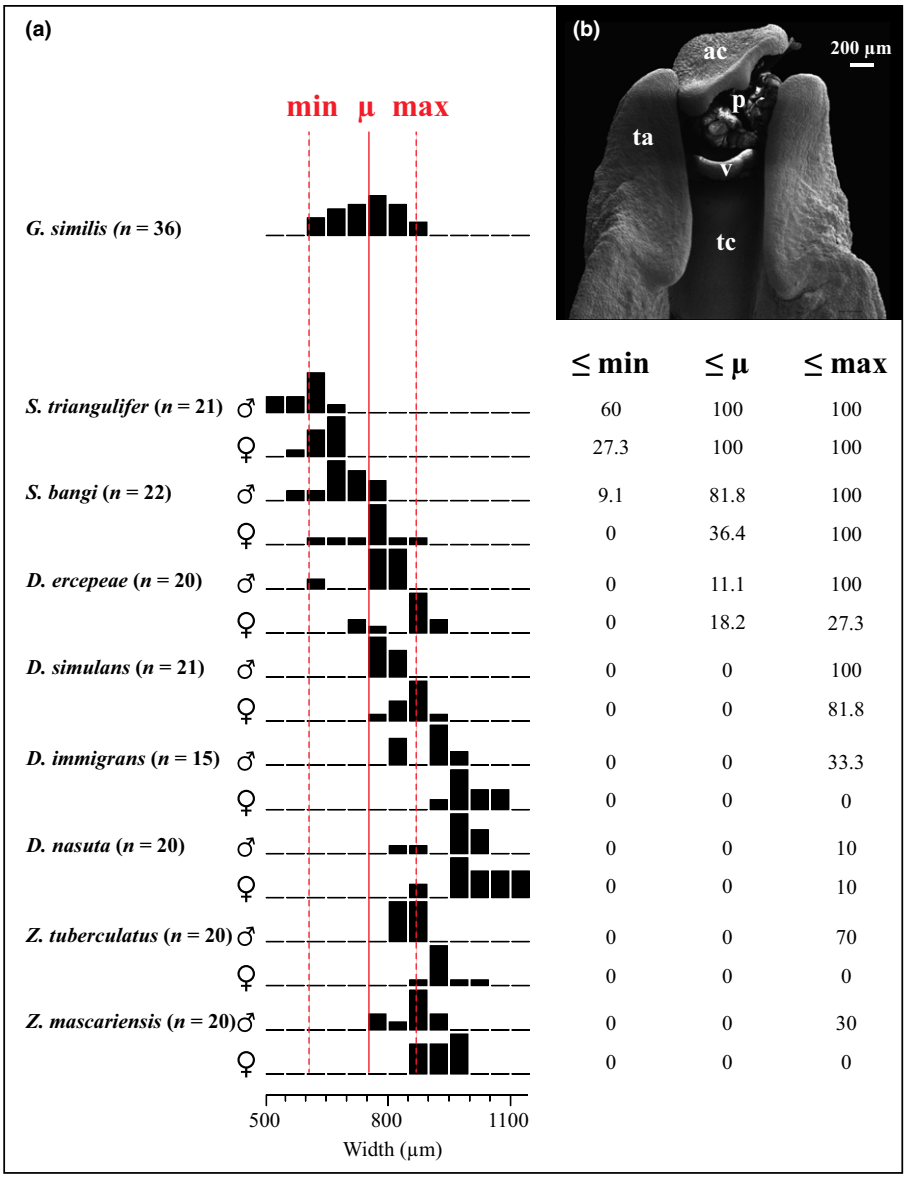
Discussion

The orchid *Gastrodia similis* represents one of the rare examples of monocotyledonous plants mimicking decaying fruit (but see Dobson, 2006). Indeed this type of mimicry is common in more basal angiosperm families, such as Annonaceae (Goodrich & Raguso, 2009; Goodrich *et al.*, 2009; Maia *et al.*, 2012) and Aristolochiaceae (Sakai, 2002b). Although no particular scent profile is associated with rotting fruit mimicry, production of fermentation volatiles, such as ethyl acetate, by *G. similis* is consistent with existing literature (Dobson, 2006). In *G. similis*, drosophilid flies that respond to the key esters are mostly females, and males never seem to carry pollinia in the forest habitat, two indications that the chemical production may be perceived as a signal of an available oviposition site. Some authors recently proposed that the model in oviposition site mimicry should be considered to be yeast rather than rotting fruit, because the functionally active compounds emitted by these flowers, and used by insects to locate rotting fruit, are actually derived from the fermentation process carried out by yeasts (Goodrich & Raguso, 2009; Goodrich *et al.*, 2009; Stökl *et al.*, 2010; Arguello *et al.*, 2013).

Rotting fruit/yeast mimicry has never been suspected in *Gastrodia*. Previous studies rather suggested that bee pollination might be common in this genus. For instance, Jones (1985) reported a spicy floral fragrance in *G. sesamoides*, a species found throughout the temperate regions of Australia and New Zealand, which attracts small native bees. Bee pollinators were also observed in *G. elata* from China (Kato *et al.*, 2006), whereas aphid visitors were seen within flowers of *G. cunninghamii* in New Zealand (Lehnebach *et al.*, 2005). However, some floral traits of *G. similis* associated with drosophilid pollination likely occur in other *Gastrodia* species, especially in African members of this genus (Cribb *et al.*, 2010). Further empirical studies on the reproductive biology of *Gastrodia* are thus needed to confirm this.

Here we mainly addressed the role of floral scent chemistry and the dimensions of the trap chamber in enforcing specialization in oviposition site mimicry. In spite of the occurrence of several other fruit-breeding drosophilid species at the study sites, only *Scaptodrosophila bangi* is attracted to the orchid flowers. This suggests that specialization occurs through a filtering effect rather than a depauperate island pollinator fauna (Olesen & Jordano, 2002). This observation was corroborated by bioassays in which co-occurring species, although abundant in the forest habitat, did not respond to the floral chemical cues – except perhaps the congeneric species, *S. triangulifer*, which was attracted to two out of three floral esters, in particular methyl isobutyrate. Consequently the chemical signalling of *G. similis* flowers targets either a single species or a narrow set of closely related drosophilid species. The finding of this hyper-specialized system challenges the view that rotting fruit/yeast mimicry always exploits widespread basal functions in the fly sensory system (Stökl *et al.*, 2010), and has interesting parallels with the carrion mimicry system in the orchid *Satyrium pumilum*, where only a small subset of the pool of carrion fly species present in the plant habitat are attracted to its flowers (van der Niet *et al.*, 2011).

Our study also revealed that the flower of *G. similis* has an intriguing chamber mechanism to secure entrapment of the *S. bangi* flies that visit it (see Video S1). A touch-sensitive labellum 'trapdoor' that is able to move up and down until the



	≤ min	≤ μ	≤ max
S. triangulifer (*n* = 21) ♂	60	100	100
♀	27.3	100	100
S. bangi (*n* = 22) ♂	9.1	81.8	100
♀	0	36.4	100
D. ercepeae (*n* = 20) ♂	0	11.1	100
♀	0	18.2	27.3
D. simulans (*n* = 21) ♂	0	0	100
♀	0	0	81.8
D. immigrans (*n* = 15) ♂	0	0	33.3
♀	0	0	0
D. nasuta (*n* = 20) ♂	0	0	10
♀	0	0	10
Z. tuberculatus (*n* = 20) ♂	0	0	70
♀	0	0	0
Z. mascariensis (*n* = 20) ♂	0	0	30
♀	0	0	0

Fig. 4 Morphological fit between the width of the trap chamber in the flowers of *Gastrodia similis* and the head width of eight fruit-breeding drosophilid species in the genera *Scaptodrosophila*, *Drosophila* and *Zaprionus*. (a) The distribution of width of the trap chamber is compared with the distribution of head width for each drosophilid species and sex. Vertical lines represent the minimum (min), median (μ) and maximum (max), values recorded for the trap chamber. Percentages of individuals that can fit in the narrowest (≤ min), median (≤ μ) and widest (≤ max) trap chambers have been calculated for each drosophilid species and sex. (b) Scanning electron micrograph of the trap chamber in a *G. similis* flower after critical point drying. ac, anther cap; p, pollinarium; ta, tooth-like appendage on either side of the anther; tc, trap chamber; v, shared viscidium.

stigma receives pollen has not been reported previously in *Gastrodia*. In other orchids, a motile labellum has been reported in *Bulbophyllum penicillium,* which also traps drosophilid flies (Liu *et al.*, 2010), and *Pterostylis sanguinea* and *Paracaleana minor*, which have touch-sensitive labella and are pollinated by mycetophilid gnats (Phillips *et al.*, 2014) and thynnid wasps (Hopper & Brown, 2006; Bower, 2014), respectively. It remains to be determined whether the physiological processes inducing locomotion of touch-sensitive floral organs are the same, between different orchid lineages, but also between orchids and other plant lineages for example, the carnivorous genera *Dionaea* and *Drosera* (Braam, 2005).

The morphology of the *G. similis* trap chamber will clearly filter out some flies from the local community (Fig. 4), but it cannot be the primary explanation for the specialized pollination system, as another species in the genus *Scaptodrosophila* and some individuals in the genus *Drosophila* and the subgenus *Sophophora* could pass through the chamber. There is currently no evidence from our video and direct observations that flies belonging to species other than *S. bangi* ever visit *G. similis* flowers or carry the orchid pollinia in the forests habitats. This is also evidenced from the bioassay experiments in which orchid olfactory cues were not found attractive to flies other than *Scaptodrosophila*. Furthermore, the good morphological fit between the orchid and *S. bangi* is suggestive that the trapping chamber is adapted to make use of this fly only as a pollinator. So why does *S. triangulifer* not apparently visit *G. similis* flowers? Interestingly, neither males nor females of this species respond to the trap laced with ethyl isobutyrate, but both genders are attracted to the blend (Fig. 3). This indicates that this compound should not be inhibitory to *S. triangulifer*, and that this congeneric species may be an occasional but undetected visitor of *G. similis* flowers.

The chemical mimicry accomplished by *G. similis* flowers appears to rely on a pre-existing bias (Schiestl & Johnson, 2013). Indeed *S. bangi* had already developed sensory and behavioural responses to orchid olfactory cues in the context of locating host

fruits. This is strongly suggested by the fact that indigenous figs undergoing fermentation on the forest floor are highly attractive to *S. bangi* flies, and moreover produce two key functional compounds deployed by the orchid flower (ethyl acetate and ethyl isobutyrate). This drosophilid species was described from West Africa (Burla, 1954) and is known from Madagascar (M-L. Cariou & M. Schiffer, pers. obs.) and Reunion Island (Lachaise, com. pers.; Tsacas & David, 1975; Tsacas & Chassagnard, 1990), but its breeding sites are unknown over its range. Our fruit trapping assays nevertheless indicate that it could feed and breed on certain types of fruits, such as fermenting figs. These preferences may or may not be specific to the island populations of *S. bangi*. For instance, *Drosophila sechellia*, which is endemic to the Seychelles, evolved host specialization on *Morinda* fruits, most likely at the time of speciation (Dekker *et al.*, 2006; Legrand *et al.*, 2009; Stensmyr, 2009). Whether *G. similis* chemically mimics only one particular type of host fruit awaits further investigations, in particular focusing on the reproductive biology of the pollinator species *S. bangi*.

Finally, the discovery of a fully mycoheterotrophic orchid species with a very specific fungal association (Martos *et al.*, 2009) and a highly specialized pollination system (this study), suggests that specialization in both kinds of interactions may be evolutionary stable (Bidartondo, 2005; Waterman & Bidartondo, 2008). More research on the reproductive biology of mycoheterotrophic plant species is required, particularly for plant families in which there is still a large imbalance in our knowledge of reproductive vs nutritional strategies.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Two of the most common species of *Scaptodrosophila* (Diptera: Drosophilidae) found in the forest habitat of the orchid *Gastrodia similis*.

Fig. S2 A female *Scaptodrophila bangi* with sectile pollinia of the orchid *Gastrodia similis* attached to its upper thorax.

Fig. S3 Indigenous fruits visited by the pollinator species *Scaptodrosophila bangi* in the primary forest habitat growing at Mare Longue (Reunion Island).

Table S1 Breeding system experiments in two populations of the orchid *Gastrodia similis* on Reunion Island

Table S2 Drosophilid species recorded on flowers of the orchid *Gastrodia similis,* on fermenting fruits of *Ficus mauritiana* (Moraceae) and *Mimusops balata* (Sapotaceae), and on banana baits

Table S3 Drosophilid species trapped for each volatile treatment over a 10-h period

Video S1 Touch-sensitive labellum 'trapdoor' of the orchid *Gastrodia similis*, triggered by the drosophilid species *Scaptodrosophila bangi*.

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