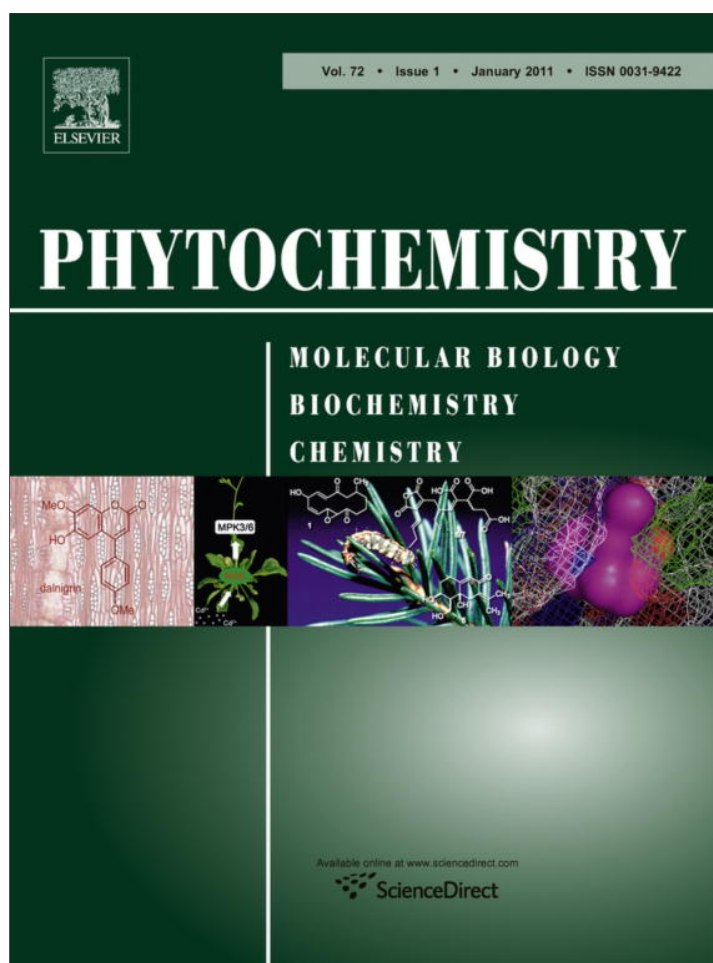


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Geographic variation of floral scent in a highly specialized pollination mutualism

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ABSTRACT

Floral scents are important signals for communication between plants and pollinators. Several studies have focused on interspecific variation of these signals, but little is known about intraspecific variation in flower scent, particularly for species with wide geographic distributions. In the highly specific mutualism between *Ficus* species and their pollinating wasps, chemical mediation is crucial for partner encounter. Several studies show that scents, i.e. blends of volatiles, are species-specific, but no studies address interpopulation variation of scents in fig pollination mutualisms, which often have broad geographic distributions. In this study, using absorption/desorption headspace techniques, we analyzed variation in floral scent composition among three populations of each of two widely distributed Asian *Ficus* species. We identified more than 100 different volatile organic compounds, predominantly terpenes. In both species, significant differences were found between scent bouquets of East Asian and Indian populations. These differences are discussed in relation to geographical barriers that could disrupt gene exchange between these two areas, thereby isolating Indian population populations from those of Eastern Asia.

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1. Introduction

Volatile organic compounds (VOCs) emitted by plants often mediate interactions between plants and animals, as for example in many pollination systems (reviewed by Raguso (2008)) and other mutualistic interactions, such as animal-mediated seed dispersal (Borges et al., 2008; Hodgkison et al., 2007) and ant/plant protection mutualisms (Schatz et al., 2009). Among the different classes of compounds-fatty acid derivatives, benzenoids and terpenes-the last-named group is the largest, with the widest assortment of structural types (Degenhardt et al., 2009). Floral bouquets are usually mixtures of numerous components, varying among species in their composition, blend ratio, and overall concentrations (Raguso, 2008). In specific pollination mutualisms, such differences in floral scents, and in animal responses to them, contribute to reproductive isolation between closely related sympatric species (Levin et al., 2001).

Contrary to interspecific variation, variation among populations of a single species in production of floral scent has been studied only very recently and in a limited number of examples (D otterl et al., 2005b; F ussel et al., 2007; Hossaert-McKey et al., 2010; Ibanez et al., 2010; Knudsen, 2002; Majetic et al., 2009;

Schlumpberger and Raguso, 2008; Svensson et al., 2005). In a few cases, no interpopulation differences in floral scents were found (Dormont et al., 2010; Knudsen, 2002), or only quantitative differences could be observed (Svensson et al., 2005). However, in most studies, consistent interpopulation differences in floral scent have been reported (Azuma et al., 2001; Schlumpberger and Raguso, 2008). Why floral volatiles vary among different populations of the same plant species remains poorly understood. Intraspecific variation has been notably explained by hypotheses such as relaxed selective pressure, genetic drift, introgression of scent traits through hybridization, pleiotropic effects of plant defense on scent biosynthesis, or phenotypic plasticity resulting from edaphic or climatic differences (Raguso, 2008). Another explanation is that not all compounds contribute to the signal used by the pollinator, and only compounds without a signalling function are variable (D otterl et al., 2006; Mant et al., 2005). In the literature, the two most frequently proposed explanations are (i) relaxed selection pressure on floral scents as visual cues assume an important role in pollinator attraction (Azuma et al., 2001; Ellis and Johnson, 2009), and (ii) the occurrence of different pollinators in different geographic areas, as in the case of species visited by a spatially variable set of generalist pollinators (Schlumpberger and Raguso, 2008). The extent to which selection on specificity of the plant-insect interaction leads to selection pressure on scent production is unclear. For example, different populations of *Echinopsis*

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ancistrophora (Cactaceae), pollinated by a broad spectrum of insects, were shown to emit different floral scents that attracted different pollinators (Schlumpberger and Raguso, 2008). But surprisingly, in the case of the interaction between *Yucca* and their pollinating moths (*Tegeticula yuccasella* and *Tegeticula cassandra*), in which specificity is high (but not complete), no difference of scent appeared among populations of *Yucca filamentosa* pollinated by different yucca moths (Svensson et al., 2005).

However, none of the cases mentioned above are entirely species-specific. Interpopulation variation of floral scents has never been investigated in obligatory, strictly species-specific pollination mutualisms (Hossaert-McKey et al., 2010). We can expect that in highly specific interactions, in which scents promote partner encounter, stabilizing selection should tightly constrain interpopulation variation of scent. Indeed, selection pressure could lead to strong conservatism of scents over a wide geographic scale, allowing the specific pollinator to recognize its host throughout its range, which can be quite large if the pollinator is a good disperser and often encounters host individuals a long distance from where it was born (Ahmed et al., 2009; Zavodna et al., 2005). The objective of the present study was to test whether intraspecific variation of scent exists in a highly specific plant-insect pollination interaction, the fig/fig wasp mutualism.

The genus *Ficus* (Moraceae) comprises about 800 species, most of them living in the intertropical region. The pollination of *Ficus* inflorescences (the fig or syconium) is carried out solely by agaonid wasps (Hymenoptera:Chalcidoidea:Agaonidae), which reproduce inside the figs. The relationship between *Ficus* and agaonid wasps is obligate for both partners and generally species-specific [one fig species is pollinated by one species of wasp [(Janzen, 1979; Wiebes, 1979); but see (Cook and Rasplus, 2003)]. In such a close-knit nursery pollination mutualism, the encounter between partners is a crucial step for the continuity of the life cycle of each partner. In tropical forests, where several *Ficus* species can live in sympatry, partner encounter is particularly problematic and requires specific chemical signals for each species pair. Several studies have shown that different species of *Ficus* emit clearly distinct chemical messages to attract only their specific pollinating wasp (Grison-Pigé et al., 2002b; Grison et al., 1999; Hossaert-McKey et al., 2010; Song et al., 2001). Behavioral evidence was also found for specific fig pollinator attraction to host volatiles (Chen et al., 2009; Grison-Pigé et al., 2002a; Hossaert-McKey et al., 1994; Proffitt and Johnson, 2009; Song et al., 2001).

In general, variation in plant traits involved in pollinator attraction would be expected only when the most effective pollinators in each population exhibit divergent sensory preferences (Ellis and Johnson, 2009), even if some scent variation can be in part conditioned by phenotypic plasticity, in response to environmental variation on a large geographic scale, as shown in the family Brassicaceae (Majetic et al., 2009). In fig/fig wasp interactions, where (i) the plant is generally pollinated by only one pollinator species throughout its distribution, and (ii) scent is known to be responsible for the attraction of the obligate mutualistic partner, the interpopulation variation of the olfactory message might be limited (Hossaert-McKey et al., 2010). Indeed, a change in floral scents could disrupt partner recognition, leading to a drastic reduction in fitness of both partners. In this study, we examined the variation of floral scents emitted at receptivity in two *Ficus* species, one monoecious and one dioecious, and investigated variation among three Asian populations of each species. Our aim here is to test the hypothesis that, even on large geographical scales, the scent of both studied species is species-specific and invariant among populations. To our knowledge, this study is one of the rare ones (Svensson et al., 2005) to examine interpopulation variation in chemical signals in a pollination mutualism that is both obligatory and specific.

2. Results and discussion

A total of 114 different VOCs, including six unidentified compounds, were found in the bouquets emitted by receptive figs (i.e. those at the developmental stage ready to be pollinated) of these two species. The 108 identified VOCs belong to four different classes of compounds, including 25 monoterpenes, 59 sesquiterpenes, 10 benzenoids and 14 fatty acid derivatives. In the bouquets of *Ficus racemosa* individuals, we found 57 different VOCs, and in those of *Ficus hispida* 94 compounds (37 VOCs were common to both species; see major compounds for both species in Table 1). Most of these volatile compounds are quite common in floral scents (Knudsen et al., 2006). Only 51 VOCs had a mean relative proportion in the bouquet higher than 1% (respectively 10 VOCs for *F. racemosa* and 45 for *F. hispida*).

2.1. Interspecific variation in floral scents

To test if each species had a particular blend of VOCs allowing reproductive isolation of sympatric species in our highly specific system, we performed multivariate analyses on our dataset of relative abundance of each compound for each individual of the two species. The PCA (Principal Component Analysis) showed a separation of the scents emitted by receptive figs of the two species (Fig. 1a). This difference between the two species was confirmed by the MANOVA performed on the coordinates (two first components) of the PCA ($F_{(15;91)} = 11.42$; $p < 0.0001$). Furthermore, the species effect was highly significant in the MANOVA performed on the relative abundance dataset (Wilks's Lambda ["species effect"]: $F_{(19;17)} = 7.95$; $p < 0.0001$). Eleven different VOCs are responsible for this interspecific variation of scents (see Table 1). These results confirm previous studies showing that the chemical signals emitted by different *Ficus* species are sufficiently distinct to allow specific attraction of their own pollinating wasps (Chen et al., 2009; Grison-Pigé et al., 2002b; Grison et al., 1999; Proffitt and Johnson, 2009). Indeed, in the case of sympatric species, the differences between species bouquets may be reinforced by selection pressure reducing the frequency of mistakes by the pollinating wasps and scents may act as a barrier to host shifts (Chen et al., 2009; Proffitt et al., 2009). Moreover, our results are in agreement with previous studies on odour extracts of receptive figs of *F. hispida* (Proffitt et al., 2008, 2009) where similar VOCs have been identified. However, our results differ from those presented in Song et al. (2001). That study used solvent extraction, a technique that recovers not only volatile compounds emitted by figs, but in addition chemicals present in the superficial cell layers of the plant. Our study, as well as those of Proffitt et al. (2008, 2009) based on dynamic headspace extraction, reported only volatile compounds, which are potentially detectable by pollinators and other animals at distance.

2.2. Intraspecific variation in floral scents

In this very specialized pollination system, we examined whether the blends of VOCs emitted by receptive figs of these two *Ficus* species vary over a large geographic scale. We first performed MANOVAs on our global dataset on relative abundance of each compound in different populations of each species (VOCs > 1% for each species), testing for a population effect. There was significant variation among populations of a single species in scents emitted by receptive figs (Wilks' Lambda ["population within species effect"]: $F_{(76;69)} = 5.62$; $p < 0.0001$). Most of the compounds contributing to interpopulation differentiation are monoterpenes and sesquiterpenes (Tables 2 and 3). In fact, previous studies on fig/fig wasp interactions showed that volatiles from these two

Table 1 Relative abundances (mean ± standard deviation) of main VOCs (>1%) found in bouquets of scents emitted by *Ficus racemosa* and *Ficus hispida* at receptivity for each studied population.

Compound	S_inj	R.I.	Ficus racemosa India		Ficus racemosa China		Ficus racemosa Thailand		Ficus hispida China		Ficus hispida India A		Ficus hispida India B		
			Occ	(N = 6)	Occ	(N = 5)	Occ	(N = 11)	Occ	(N = 6)	Occ	(N = 6)	Occ	(N = 7)	
Monoterpenes															
α -Pinenene	X	932	36	3.05 ± 2.76	4	0.67 ± 0.81	11	2.71 ± 1.58	4	1.99 ± 2.11	5	5.41 ± 2.52	7	2.52 ± 1.28	
Sabinene	X	965	29	2.77 ± 3.24	5	2.77 ± 3.24	9	1.13 ± 0.89	6	5.69 ± 4.39	3	2.8 ± 4.1	6	0.56 ± 0.97	
β -Pinenene	X	977	24	1.21 ± 2.14	2	1.21 ± 2.14	6	0.14 ± 0.18	4	2.63 ± 3.53	5	3.13 ± 1.18	7	0.7 ± 0.62	
β -Cymene	X	1026	19	0	0	0	0.37 ± 0.25	2	0.35 ± 0.66	4	2.38 ± 1.86	4	0.92 ± 1.99	4	0.55 ± 1.20
1,8-Cineole*	X	1030	18	0	0	0	0.49 ± 0.56	4	2.55 ± 3.09	3	3.17 ± 5.06	3	0.48 ± 0.48	5	1.18 ± 1.11
Limonene	X	1031	31	2.83 ± 0.02	5	7.21 ± 3.98	11	6.04 ± 4.01	5	3.18 ± 3.67	5	11.57 ± 9.77	7	31.57 ± 24.44	
<i>cis</i> - β -Ocimene*	X	1036	31	0.71 ± 0.03	5	5.99 ± 1.74	11	5.99 ± 1.74	2	0.33 ± 0.53	5	1.12 ± 1.1	7	1.18 ± 1.11	
<i>trans</i> - β -Ocimene*	X	1047	39	34.59 ± 0.04	5	72.54 ± 15.5	11	59.24 ± 11.6	5	3.33 ± 2.9	5	19.36 ± 14.9	7	1.18 ± 1.11	
<i>cis</i> -Linalool oxide furan	X	1074	13	0.08 ± 0.05	0	0	0.12 ± 0.19	1	0.18 ± 0.44	1	0.03 ± 0.07	5	4.22 ± 5.40		
Linalool	X	1099	37	1.66 ± 0.06	5	7.13 ± 4.89	10	3.96 ± 3.51	6	13.33 ± 10.3	5	1.27 ± 1.16	7	0.12 ± 0.20	
Perillene	X	1103	23	0.64 ± 0.69	2	1.2 ± 2.41	5	0.25 ± 0.34	5	2.48 ± 2.93	3	0.45 ± 0.49	4	3.51 ± 3.89	
Unknown a		1114	9	0	0	0	0.04 ± 0.09	2	0	0	0	50.69	7	47.52	
Sum				43.6		92.7	80.5		36		32.86		45.6		
Sesquiterpenes															
α -Ylangene		1375	15	0	0	0	0.36 ± 0.67	4	0	0	0.17 ± 0.12	7	3.64 ± 4.64		
α -Copaene*	X	1377	30	2.94 ± 3.87	0	0	4.07 ± 3.26	2	0.68 ± 1.23	5	6.94 ± 2.91	7	10.31 ± 7.28		
Daucene		1381	10	0	0	0	0	2	0.93 ± 1.53	1	0.30 ± 0.68	7	1.13 ± 1.07		
β -Elemene		1392	19	0	0	0	0.16 ± 0.37	4	2.28 ± 2.14	3	0.39 ± 0.37	6	0.65 ± 0.49		
Longifolene		1407	7	2	0	0	0	3	2.53 ± 2.38	2	0.20 ± 0.46	2	0.20 ± 0.46		
<i>cis</i> - α -Bergamotene		1414	14	0	3	0.18 ± 0.20	4	0.08 ± 0.13	3	1.35 ± 1.64	4	1.43 ± 1.57	0	7.50 ± 4.18	
β -Caryophyllene*	X	1421	24	2.20 ± 4.17	0	0	0.63 ± 1.29	6	4.79 ± 3.90	5	4.05 ± 1.83	7	3.71 ± 2.50		
<i>trans</i> - α -Bergamotene*		1440	26	2.98 ± 5.02	0	0	0.26 ± 0.50	4	3.02 ± 3.03	5	5.77 ± 6.82	7	0		
Zizaene		1453	7	3	4	1.94 ± 1.66	0	0	0	0	0	0	0		
<i>trans</i> - β -Farnesene*	X	1458	15	4	0	0	0	6	16.12 ± 8.49	0	0	5	0.38 ± 0.50		
α -Humulene*	X	1462	20	1	0	0	0.10 ± 0.20	4	1.81 ± 1.88	5	1.42 ± 0.93	7	2.57 ± 1.83		
Selina-4,11-diene		1471	13	0	0	0	0.59 ± 1.04	2	0.59 ± 1.04	4	3.45 ± 4.60	7	2.19 ± 1.77		
γ -Murolene		1478	15	0	0	0	0.63 ± 0.73	5	0.63 ± 0.73	5	1.51 ± 1.15	7	2.55 ± 2.45		
Germacrene D	X	1484	22	4	1.03 ± 1.62	0	0.51 ± 1.02	2	0.38 ± 0.60	4	0.59 ± 0.55	7	3.49 ± 4.77		
β -Selinene		1492	9	0	0	0	0	2	1.51 ± 3.40	2	0.39 ± 0.88	7	0.48 ± 0.30		
α -Murolene		1497	8	0	0	0	4.86 ± 4.70	2	0.37 ± 0.57	3	0.75 ± 0.87	7	1.53 ± 1.95		
Bicyclogermacrene		1499	20	0	0	0	1.09 ± 1.82	6	2.67 ± 0.86	0	0.11 ± 0.24	2	0.17 ± 0.44		
(<i>cis,cis</i>)- α -Farnesene	X	1506	20	4	9.89 ± 18.99	3	0.81 ± 0.93	5	4.02 ± 3.14	1	0.11 ± 0.24	5	0.41 ± 0.55		
Germacrene A		1509	21	0	0	0	1.29 ± 1.58	10	1.06 ± 2.59	5	0.12 ± 0.28	5	1.11 ± 1.76		
7-Epi- α -cedrene		1516	11	0	0	0	0.51 ± 0.67	1	3.11 ± 1.36	1	2.41 ± 1.33	7	2.63 ± 1.68		
β -Sesquiphellandrene		1518	7	0	0	0	0	2	0.26 ± 0.43	5	0.52 ± 0.31	0	0		
δ -Cadinene*		1521	24	4	0.82 ± 1.13	0	0	4	45.57	4	32.86	0	0		
Unknown b		1434	4	0	0	0	13.92	0	0	0	0	0	0		
Sum				21.69		2.94		45.57		45.57		32.86			
Fatty acid derivatives															
<i>cis</i> -3-Hexenyl acetate	X	1006	14	14.99 ± 17.09	0	0	0.55 ± 0.79	3	1.75 ± 2.62	2	0.85 ± 1.35	0	0		
Tridecane	X	1300	9	0	3	0.39 ± 0.37	0	3	1.47 ± 2.28	0	0	3	0.10 ± 0.18		
Tetradecane	X	1400	11	0	0	0	0	3	1.01 ± 1.71	0	0	8	0.65 ± 0.61		
Sum				14.99		0.39	0.55	4.23		0.85		0.75			
Shikimic compounds															
Benzonitrile		1142	5	0	5	1.08 ± 0.63	0	0	0	0	0	0	0		
Methyl salicylate	X	1195	10	4	2.21 ± 2.01	3	0.48 ± 0.62	3	1.65 ± 3.16	5	6.13 ± 6.60	6	2.73 ± 5.98		
Indole*	X	1295	14	0	2.21	1.57	0.11	1.65	6.13	6.13	2.73	2.73			
Sum				2.21	1.57	0.11	1.65	6.13	2.73						

RI = Retention Index; Occ = occurrence of each VOC, total and by population; N = number of samples; S_inj = VOCs for which identification has been confirmed by standards injection. * VOCs with significantly different relative proportions between the two species.

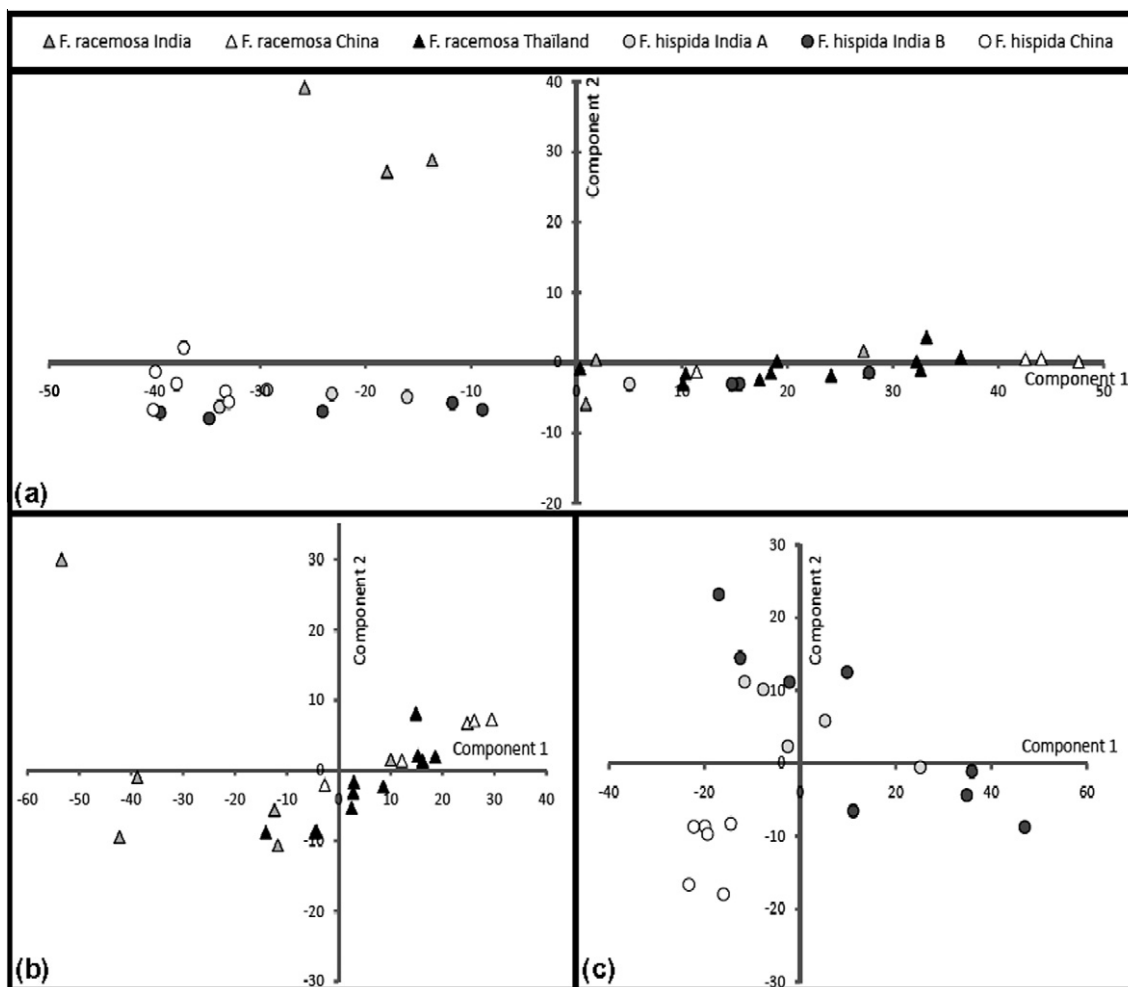


Fig. 1. PCA analysis on relative abundance of VOCs produced by receptive figs of: (a) *Ficus racemosa* and *F. hispida* together (first component explains 68% and second component 28% of the total variation observed). (b) *Ficus racemosa* from three populations (first component explains 68%, and second component 11% of the total variation observed). (c) *Ficus hispida* from three populations (first component explains 55% and second component 15% of the total variation observed).

Table 2

Relative abundances of VOCs from the bouquet of *Ficus racemosa* receptive figs for which interpopulation variation was significant (LS means comparisons).

	trans- β -Ocimene	cis-3-Hexenyl acetate	cis- β -Ocimene	Bicyclogermacrene
China	72.54 \pm 15.51 (a)	0.00 \pm 0.00 (a)	7.21 \pm 3.98 (a)	0.00 \pm 0.00 (a)
Thailand	59.24 \pm 11.57 (a)	0.55 \pm 0.79 (a)	5.99 \pm 1.74 (a)	0.11 \pm 0.24 (b)
India	34.59 \pm 18.40 (b)	14.99 \pm 17.09 (b)	0.71 \pm 1.74 (b)	0.00 \pm 0.00 (a)

Different letters in parantheses within a column indicate significant differences between populations at $p < 0.05$.

Table 3

Relative abundances of VOCs from the bouquet of *Ficus hispida* receptive figs for which interpopulation variation was significant (LS means comparisons).

	trans- β -Farnesene	β -Sesquiphellandrene	δ -Cadinene	Germacrene A
China	16.12 \pm 8.49 (a)	3.11 \pm 1.36 (a)	0.26 \pm 0.43 (a)	4.02 \pm 3.14 (a)
India A	0.00 \pm 0.00 (b)	0.12 \pm 0.28 (b)	2.41 \pm 1.33 (b)	0.11 \pm 0.24 (b)
India B	0.38 \pm 0.5 (b)	0.00 \pm 0.00 (b)	2.63 \pm 1.68 (b)	0.41 \pm 0.55 (b)
	α -Pinene	γ -Terpinene	Limonene	
China	1.99 \pm 2.11 (a)	0.00 \pm 0.00 (a)	3.18 \pm 3.67 (a)	
India A	5.41 \pm 2.52 (b)	4.60 \pm 3.85 (b)	11.57 \pm 9.77 (b)	
India B	2.52 \pm 1.28 (a)	0.18 \pm 0.36 (a)	0.48 \pm 0.48 (a)	

Different letters in parantheses within a column indicate significant differences between populations at $p < 0.05$.

chemical classes are the major compounds in scents of most *Ficus* species (Gibernau et al., 1998; Grison-Pigé et al., 2001; Proffit et al., 2008), even if some systems are mediated by benzenoids (Chen et al., 2009). In Fig. 2, which illustrates variation of different classes of volatile compounds among populations of each species, some interpopulation differences appear clearly. The most striking is the significant variation of mean proportions of monoterpenes among *F. racemosa* populations. Indeed, for differences between mean proportions of monoterpenes in Indian and Chinese populations, Indian and Thai populations and Chinese and Thai populations, the results of Wilcoxon tests were respectively ($S = 45.00$; $p = 0.0043$), ($S = 27.00$; $p = 0.0048$) and ($S = 66.00$; $p = 0.0055$).

To study interpopulation variation in greater detail, the two *Ficus* species were then examined independently. The PCA performed on relative abundance of VOCs emitted only by *F. racemosa* (Fig. 1b) did not show clear differentiation among populations. Nevertheless, the scents produced by individuals from southern India were relatively distinct compared to those produced by the two other populations (northern Thailand and southern China). A MANOVA performed on the same *F. racemosa* VOCs dataset (but including only those with mean proportion >1%) showed that the population effect was significant (Wilks' Lambda: $F_{(18;22)} = 3.95$; $p = 0.0014$). Four VOCs contributed to this interpopulation difference: bicyclogermacrene, for which relative proportions were significantly different in bouquets of the Thai population compared to those of both Chinese and Indian populations, and *trans*- β -ocimene, *cis*- β -ocimene and *cis*-3-hexenyl acetate, for all of which relative proportions were significantly different between scents of Indian figs and those of both Thai and Chinese populations (see Table 2). The last of these compounds was totally absent,

or present in very low quantity, in bouquets from Thai and Chinese populations but present in scents of most *F. racemosa* individuals from Indian populations. This is quite surprising since this compound, a typical green leaf volatile, is known in other plants to be induced by damage from herbivory (Pichersky and Gershenson, 2002), and to act as a repellent against herbivores or as a parasitoid attractant. For *F. racemosa*, the significant differences between scents of receptive figs from Indian populations and those of figs from populations in the two other Asian regions sampled also resulted from the variation of *trans*- β -ocimene. This volatile compound is present in lower proportion in the bouquets from the Indian population than in bouquets from populations of the two other regions. Interestingly, quantities of the isomer *cis*- β -ocimene varied in a similar manner. Consequently, selection on one of these compounds as part of the signal also acts on the presence (and quantity) of the second one. The much higher proportion of *trans*- β -ocimene in the bouquets might mean that selection is acting principally on this compound, or alternatively might simply reflect the chemical instability of *cis*- β -ocimene. In fact, most documented cases suggest a 10:1 ratio or more of *trans*:*cis* isomers in floral scents (Arimura et al., 2004; Flamini et al., 2004). Bicyclogermacrene is the only VOC showing significant differences in proportion between the fig bouquets from the Thai population and those of populations from the two other Asian regions. Despite this last difference, globally, scents of receptive figs from Chinese and Thai populations of *F. racemosa* were not significantly different (Wilks' Lambda: $F_{(9;6)} = 3.49$; $p = 0.0709$).

In the case of the second species, *F. hispida*, the PCA performed on the relative contribution of the different compounds in scents emitted by receptive figs (Fig. 1c) shows that the VOCs emitted by figs from both Indian populations were relatively similar, and could be separated from the VOCs emitted by figs of the population from South China. A MANOVA performed on the same VOCs dataset (but including only compounds with mean proportion >1%) showed that the population effect was significant (Wilks' Lambda: $F_{(22;12)} = 11.72$; $p < 0.0001$). This result is explained by the significantly different proportions of four compounds [*trans*- β -farnesene, β -sesquiphellandrene, δ -cadinene and germacrene A] between scents of Chinese *F. hispida* and those of both Indian populations (see Table 3). Surprisingly, three compounds (α -pinene, γ -terpinene and limonene) showed significantly different proportions between fig scents of one Indian population (India A, Agumbe) and those of both Indian B (Kudremukh) and Chinese populations, although scents from the Indian A population are graphically not distinct from those of the Indian B population (Fig. 1c). An interesting point here is the contribution of *trans*- β -farnesene to interpopulation differences. This compound is one of the major compounds of the bouquet (about 25% for some individuals), although at the same time, quantities of *cis*- β -farnesene and (*trans,trans*)- α -farnesene are very low. The biosynthesis of these three compounds is linked (Schnee et al., 2002), and we can thus hypothesize that the presence of *trans*- β -farnesene may be highly selected, or, as explained above for *cis*- β -ocimene, the quantity of *trans*- β -farnesene might simply reflect the chemical instability of *cis*- β -farnesene. Nevertheless, the presence of both δ -cadinene and germacrene A suggests that two different pathways (corresponding to two types of initial cyclization reactions of farnesyl cation) are engaged here for the biosynthesis of *trans*- β -farnesene (Degenhardt et al., 2009), further supporting the hypothesis that its presence may be highly selected.

2.3. Geographical variation in scents of receptive figs: incipient allopatric speciation?

These results show that even in an obligate specific mutualism, where the emitted scent is species-specific, interpopulation variation can occur at large geographic scales. The mutualistic pollinator

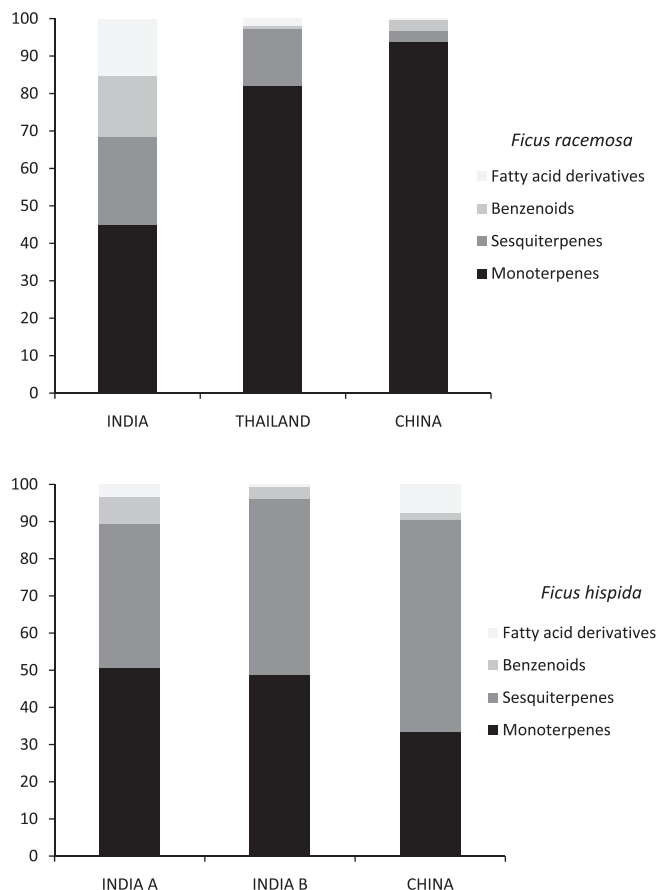


Fig. 2. Mean relative abundances by species and populations of chemical classes of volatile compounds emitted by *Ficus racemosa* and *F. hispida*.

should be able to adapt to such variation in the species-specific scent. Consequently, divergence in scent and in pollinator response to it could lead to allopatric speciation. Indeed, recent studies on spatial genetic structure of *F. racemosa* (using microsatellite markers), are consistent with such a scenario. Indian populations are genetically distinct from populations of the rest of continental Asia, whereas within India and within continental Asia, genetic differentiation was very limited (Alvarez et al., 2010). For the wasp pollinator of this species, morphological observations suggest that Indian wasps are somewhat different from all the others, in contrast to wasps from the rest of continental Asia which form one genetic entity (Kobmoo et al., 2010). Fewer genetic data are available for dioecious *Ficus*. However, recent results showed strong isolation by distance in several dioecious fig species, including *F. hispida* (Dev et al., in press), as well as the existence of different species or subspecies of the pollinating wasp of this species in India and East Asia (A. Cruaud, unpublished data). Over the wide range of our study, the Eastern Ghats, for example, might have acted as a physical barrier that initiated a breakdown in gene flow, and consequently a process of incipient allopatric speciation (Rajagopalan et al., 1997). In our case, for both studied species, the major differentiation we observed is between scents in Indian fig populations and those elsewhere in Asia. The results of genetic and phylogeographic studies suggest that geographic barriers (present or past) may have led as well to the observed discontinuities in scent variation.

Interestingly, our results are very different from those found in another nursery pollination mutualism where interpopulation variation of scent has been investigated, the *Yucca*/yucca moth interaction (Svensson et al., 2005). These authors demonstrated that the scents were similar among populations even when pollinators were different. In another study on scent variation in a nursery pollination system, the case of *Trollius* and its pollinating flies, Ibanez et al. (2010) also found some geographical variation of scent, but with large overlap in scent samples among the populations. However, in both of these studies, the geographic distances covered are much lower than in our fig study. Moreover, specificity in these other nursery pollination systems is less strict than in fig/wasp interactions. Consequently, in both of these studies, pressure for scent constancy and probability of occurrence of geographical barriers are both lower than in our case.

Alternatively, geographic variation in scent could concern only compounds not used by the pollinator to localize its host, as has been shown in *Silene latifolia* and *Salix* species (Dötterl et al., 2005a,b, 2006; Füssel et al., 2007). While genetic data thus provide strong arguments in favor of incipient speciation in our fig/wasp example, further experiments are necessary to draw conclusions. GC-EAD tests and coupled behavioral experiments with fig wasps are necessary to determine whether global fig scent and compounds used as cues by the wasps show a similar pattern of interpopulation variation.

3. Concluding remarks

Our work shows that even in a highly specific obligate mutualism, where the odour emitted by the plant is necessary and preponderant in its specific partner attraction, the scent can be constant over large geographical scales, but may vary if geographical barriers occur. Indeed, these latter may disrupt gene flow, which could lead to an incipient speciation process. However, geographic variation in scent within each of the species studied here remains less than that between species, so that in each population, specificity with respect to other sympatric fig/pollinator pairs is preserved. Coupled physiological and behavioral experiments would enable determining whether the VOCs principally contributing to geographical differentiation are compounds that actually

contribute to the olfactory signal that pollinators detect and find attractive, or whether such variation is possible due to plasticity in the olfactory response of the pollinator.

4. Experimental

4.1. Plant material

We studied the scents of two broadly sympatric Asian *Ficus* species: *F. hispida* Linnaeus (section *Sycocarpus*) and *F. racemosa* Linnaeus (section *Sycomorus*). *F. racemosa* is pollinated by *Ceratosolen fusciceps* Mayr (Chalcidoidea, Agaonidae), and *F. hispida* by *Ceratosolen solmsi marchali* Mayr (Chalcidoidea, Agaonidae), (<http://www.figweb.org>). *F. hispida*, a (gyno)dioecious species, is a short free-standing fig usually found along streams. *F. racemosa*, a monoecious species, is a large tree up to 30 m high that grows mainly in moist valleys or along rivers. All individuals used were wild, i.e. non-planted trees. For *F. racemosa*, Indian samples came from the campus of the Indian Institute of Science (12°58' N, 77°35' E), Bangalore, Karnataka State, and Thailand samples from the Khao Yai region (14°24' N, 101°22' E). Both species were sampled from wild populations in Xishuangbanna Tropical Botanical Garden (XTBG) in Yunnan, China (21°55' N, 101°15' E). For Indian samples of *F. hispida*, half were collected in Agumbe, Shimoga District (13°30' N, 75°5' E), and the other half in Kudremukh, Chikmagalur District of Karnataka state in the Western Ghats of India (13°15' N, 75°7' E). These two Indian sites are separated by 75 km. In each study site, the availability of trees with figs at the right stage was often quite low due to variable phenology and low density of fig trees. This sometimes limited the number of trees we could sample. We were able to sample the scents from 22 individuals of *F. racemosa* (6 from India, 5 from China and 11 from Thailand) and 19 individuals (males only) of *F. hispida* (6 from India A (Agumbe), 7 from India B (Kudremukh) and 6 from China).

4.2. Collection of volatiles

To collect the scent of receptive figs, pre-receptive figs were isolated from pollinators using mesh bags. Fig floral stages were estimated by their physical characteristics. For example, an open ostiole and attraction of pollinators to the outside of the bag are both signals showing that a fig is receptive.

The scent of receptive figs of both *F. hispida* and *F. racemosa* was collected using the same dynamic headspace technique (Proffit et al., 2009; Raguso and Pellmyr, 1998). Sampling was conducted for 3 h, from 12:00 to 15:00, during the peak of odour emission. Receptive figs were enclosed in a polyethylene terephthalate (Nalophan®) bag. A glass cartridge (1/4" OD Borosilicate tubing) filled with 30 mg of Porapak® Q (or Alltech super Q) adsorbent was connected to the bag. During odour collection, air was passed through the filter at a rate of 300 ml/min. Air purified with charcoal cartridges was also blown into the bag (entrance flux: 400 ml/min). Controls with empty bags were also performed. After collection, the filter was eluted with 150 µl of dichloromethane and the extracts were stored at –18 °C.

4.3. Chemical and data analysis

The extracts were analyzed in a CP-3800 (Varian Inc., Palo Alto, CA) gas chromatograph with an FID detector coupled with a Saturn 2000 mass spectrometer (Varian). For each of both gas chromatography (GC) and mass spectrometry (MS), a CP-SIL 8CB low bleed MS Varian column (30 m, ID 0.25 mm, film thickness 0.25 µm), helium carrier gas (at 1 ml min⁻¹), and injection peak in a split mode at 1:4 ratio were used. The temperature of the two injectors was 200 °C.

The oven temperature programme for the analysis was: injection at 50 °C and maintained at that temperature for 3 min, then ramped by 3 °C min⁻¹ to 100 °C, by 2.7 °C min⁻¹ to 140 °C, by 2.4 °C min⁻¹ to 180 °C and by 6 °C min⁻¹ to 250 °C. The temperatures of the different parts of the mass spectrometer (transfer-liner, manifold and trap) were 250, 80 and 170 °C. These analyses were performed at the “Plate-Forme d'Analyses Chimiques en Ecologie” (platform for chemical analyses in ecology) of “IFR 119 Montpellier Environnement Biodiversité”.

Compound identification was based on matching of the mass spectra with NIST 98 MS and Adams (2007) and on confirmation by comparison of RI with libraries and published data (Adams, 2007). Identification of some compounds was confirmed by comparison of both mass spectrum and GC retention indices with those of standards (see Table 1). We used the MS data to identify the VOCs, and the FID data for their quantification.

For the statistical analysis of the chemical blend, we ran Principal Component Analysis (PCA, covariance matrix, Stat Box v6.3) to compare patterns of scent composition of receptive figs between the two species and among populations. For these multivariate analyses, we used the relative amounts (percentages) of the compounds which represented at least 1% of the total compound emission. This restriction is appropriate here, because in mutualisms such as fig/fig wasp interactions that have undergone long periods of coevolution, selection pressures should have led to high levels of signal compounds to facilitate encounter as suggested by Hossaert-McKey et al. (2010). To confirm or invalidate the results of the PCAs, we then performed on the same dataset a multivariate analysis of variance followed by a multiple comparison of means (SAS v9: manova, proc glm, and LSMEANS with Tukey–Kramer multiple comparison tests; predictor “population within species”). The MANOVA also included univariate (i.e. sequential) analyses on each dependent variable (relative amount of each VOC) to test which compounds contributed to the overall significance of the statistical analysis (Stevens, 1992). We also analyzed the variation among populations of mean proportions of compound classes, comparing populations two by two, by using the nonparametric Wilcoxon test.

For this study, we worked on the relative amounts of VOCs only. As previously reported (Proffit et al., 2008), even a single species in a single site may emit a different global amount at different periods of the year. Variable abiotic constraints among geographical scales, and at different periods of the year, mean that comparisons between global amounts of VOCs produced by figs in different sites and at different seasons would not have been relevant for this study.

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References

- Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy, fourth ed. Allured Publishing, Carol Stream, IL, USA.
- Ahmed, S., Compton, S., Butlin, R., Gilmartin, P., 2009. Wind-borne insects mediate directional pollen transfer between desert fig trees 160 km apart. *Proc. Natl. Acad. Sci. USA* 106, 20342.
- Alvarez, N., McKey, D., Kjellberg, F., Hossaert-McKey, M., 2010. Phylogeography and historical biogeography of obligate specific mutualisms. In: Morand, S., Krasnov, B. (Eds.), *The Biogeography of Host–Parasite Interactions*. Oxford University Press, pp. 31–39.
- Arimura, G., Ozawa, R., Kugimiya, S., Takabayashi, J., Bohlmann, J., 2004. Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (*E*)- β -ocimene and transcript accumulation of (*E*)- β -ocimene synthase in *Lotus japonicus*. *Plant Physiol.* 135, 1976–1983.
- Azuma, H., Toyota, M., Asakawa, Y., 2001. Intraspecific variation of floral scent chemistry in *Magnolia kobus* DC. (*Magnoliaceae*). *J. Plant Res.* 114, 411–422.
- Borges, R.M., Bessiere, J.M., Hossaert-McKey, M., 2008. The chemical ecology of seed dispersal in monoecious and dioecious figs. *Funct. Ecol.* 22, 484–493.
- Chen, C., Song, Q.S., Proffit, M., Bessiere, J.M., Li, Z.B., Hossaert-McKey, M., 2009. Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*. *Funct. Ecol.* 23, 941–950.
- Cook, J., Rasplus, J., 2003. Mutualists with attitude: coevolving fig wasps and figs. *Trends Ecol. Evol.* 18, 241–248.
- Degenhardt, J., Köllner, T.G., Gershenzon, J., 2009. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* 70, 1621–1637.
- Dev, S.A., Kjellberg, F., Hossaert-McKey, M., Borges, R.M., in press. Fine-scale population genetic structure of two dioecious Indian keystone species, *Ficus hispida* and *F. exasperata* (*Moraceae*). *Biotropica* doi:10.1111/j.1744-7429.2010.00704.x.
- Dormont, L., Delle-Vedove, R., Bessiere, J.M., Hossaert-McKey, M., Schatz, B., 2010. Rare white-flowered morphs increase the reproductive success of common purple morphs in a food-deceptive orchid. *New Phytol.* 185, 300–310.
- Dötterl, S., Füssel, U., Jürgens, A., Aas, G., 2005a. 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. *J. Chem. Ecol.* 31, 2993–2998.
- Dötterl, S., Wolfe, L.M., Jürgens, A., 2005b. Qualitative and quantitative analyses of flower scent in *Silene latifolia*. *Phytochemistry* 66, 203–213.
- Dötterl, S., Jürgens, A., Seifert, K., Laube, T., Weissbecker, B., Schütz, S., 2006. Nursery pollination by a moth in *Silene latifolia*: the role of odours in eliciting antennal and behavioural responses. *New Phytol.* 169, 707–718.
- Ellis, A.G., Johnson, S.D., 2009. The evolution of floral variation without pollinator shifts in *Gorteria diffusa* (*Asteraceae*). *Acta Oecol.* 96, 793–801.
- Flamini, G., Bader, A., Cioni, P.L., Katbeh-Bader, A., Morelli, I., 2004. Composition of the essential oil of leaves, galls, and ripe and unripe fruits of Jordanian *Pistacia palaestina* Boiss. *J. Agric. Food Chem.* 52, 572–576.
- Füssel, U., Dötterl, S., Jürgens, A., Aas, G., 2007. Inter- and intraspecific variation in floral scent in the genus *Salix* and its implication for pollination. *J. Chem. Ecol.* 33, 749–765.
- Gibernau, M., Hossaert-McKey, M., Frey, J., Kjellberg, F., 1998. Are olfactory signals sufficient to attract fig pollinators? *Ecoscience* 5, 306–311.
- Grison, L., Edwards, A.A., Hossaert-McKey, M., 1999. Interspecific variation in floral fragrances emitted by tropical *Ficus* species. *Phytochemistry* 52, 1293–1299.
- Grison-Pigé, L., Bessiere, J.M., Turlings, T.C.J., Kjellberg, F., Roy, J., Hossaert-McKey, M.M., 2001. Limited intersex mimicry of floral odour in *Ficus carica*. *Funct. Ecol.* 15, 551–558.
- Grison-Pigé, L., Bessière, J.-M., Hossaert-McKey, M., 2002a. Specific attraction of fig-pollinating wasps: role of volatile compounds released by tropical figs. *J. Chem. Ecol.* 28, 283–295.
- Grison-Pigé, L., Hossaert-McKey, M., Greeff, J.M., Bessiere, J.M., 2002b. Fig volatile compounds – a first comparative study. *Phytochemistry* 61, 61–71.
- Hodgkison, R., Ayasse, M., Kalko, E.K.V., Haeblerlein, C., Schulz, S., Mustapha, W.A.W., Zubaid, A., Kunz, T.H., 2007. Chemical ecology of fruit bat foraging behavior in relation to the fruit odors of two species of paleotropical bat-dispersed figs (*Ficus hispida* and *Ficus scortechnii*). *J. Chem. Ecol.* 33, 2097–2110.
- Hossaert-McKey, M., Gibernau, M., Frey, J.E., 1994. Chemosensory attraction of fig wasps to substances produced by receptive figs. *Entomol. Exp. Appl.* 70, 185–191.
- Hossaert-McKey, M., Soler, C., Schatz, B., Proffit, M., 2010. Floral scents: their roles in nursery pollination mutualisms. *Chemoecology* 20, 75–88.
- Ibanez, S., Dötterl, S., Anstett, M., Baudino, S., Caissard, J., Gallet, C., Després, L., 2010. The role of volatile organic compounds, morphology and pigments of globe-flowers in the attraction of their specific pollinating flies. *New Phytol.* 188, 451–463.
- Janzen, D.H., 1979. How to be a fig. *Annu. Rev. Ecol. Syst.* 10, 13–51.
- Knudsen, J.T., 2002. Variation in floral scent composition within and between populations of *Geonoma macrostachys* (*Arecaceae*) in the western Amazon. *Am. J. Bot.* 89, 1772–1778.
- Knudsen, J.T., Eriksson, R., Gershenzon, J., Stahl, B., 2006. Diversity and distribution of floral scent. *Bot. Rev.* 72, 1–120.
- Kobmoo, N., Hossaert-McKey, M., Rasplus, J.Y., Kjellberg, F., 2010. *Ficus racemosa* is pollinated by a single population of a single agaonid wasp species in continental South-East Asia. *Mol. Ecol.* 19, 2700–2712.
- Levin, R.A., Raguso, R.A., McDade, L.A., 2001. Fragrance chemistry and pollinator affinities in *Nyctaginaceae*. *Phytochemistry* 58, 429–440.
- Majetic, C.J., Raguso, R.A., Ashman, T.-L., 2009. Sources of floral scent variation: can environment define floral scent phenotype? *Plant Signal. Behav.* 4, 129–131.
- Mant, J., Peakall, R., Schiestl, F., 2005. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? *Evolution* 59, 1449–1463.
- Pichersky, E., Gershenzon, J., 2002. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* 5, 237–243.

- Proffit, M., Johnson, S.D., 2009. Specificity of the signal emitted by figs to attract their pollinating wasps: comparison of volatile organic compounds emitted by receptive syconia of *Ficus sur* and *F. sycomorus* in Southern Africa. *S. Afr. J. Bot.* 75, 771–777.
- Proffit, M., Schatz, B., Bessiere, J.M., Chen, C., Soler, C., Hossaert-McKey, M., 2008. Signalling receptivity: comparison of the emission of volatile compounds by figs of *Ficus hispida* before, during and after the phase of receptivity to pollinators. *Symbiosis* 45, 15–24.
- Proffit, M., Chen, C., Soler, C., Bessiere, J.M., Schatz, B., Hossaert-McKey, M., 2009. Can chemical signals, responsible for mutualistic partner encounter, promote the specific exploitation of nursery pollination mutualisms? The case of figs and fig wasps. *Entomol. Exp. Appl.* 131, 46–57.
- Raguso, R.A., 2008. Wake up and smell the roses: the ecology and evolution of floral scent. *Annu. Rev. Ecol. Evol. Syst.* 39, 549–569.
- Raguso, R.A., Pellmyr, O., 1998. Dynamic headspace analysis of floral volatiles: a comparison of methods. *Oikos* 81, 238–254.
- Rajagopalan, G., Sukumar, R., Ramesh, R., Pant, R.K., 1997. Late Quaternary vegetational and climatic changes from tropical peats in southern India – an extended record up to 40,000 years BP. *Curr. Sci.* 73, 60–63.
- Schatz, B., Djieto-Lordon, C., Dormont, L., Bessiere, J.M., McKey, D., Blatrix, R., 2009. A simple nonspecific chemical signal mediates defence behaviour in a specialised ant-plant mutualism. *Curr. Biol.* 19, R361–R362.
- Schlumpberger, B.O., Raguso, R.A., 2008. Geographic variation in floral scent of *Echinopsis ancistrophora* (Cactaceae); evidence for constraints on hawkmoth attraction. *Oikos* 117, 801–814.
- Schnee, C., Kollner, T.G., Gershenzon, J., Degenhardt, J., 2002. The maize gene terpene synthase 1 encodes a sesquiterpene synthase catalyzing the formation of (E)-beta-farnesene, (E)-nerolidol, and (E,E)-farnesol after herbivore damage. *Plant Physiol.* 130, 2049–2060.
- Song, Q.S., Yang, D.R., Zhang, G.M., Yang, C.R., 2001. Volatiles from *Ficus hispida* and their attractiveness to fig wasps. *J. Chem. Ecol.* 27, 1929–1942.
- Stevens, J., 1992. *Applied Multivariate Statistics for the Social Sciences*. Lawrence Erlbaum Associates, Hillsdale, New Jersey.
- Svensson, G.P., Hickman, M.O., Bartram, S., Boland, W., Pellmyr, O., Raguso, R.A., 2005. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). *Am. J. Bot.* 92, 1624–1631.
- Wiebes, J.T., 1979. Co-evolution of figs and their insect pollinators. *Annu. Rev. Ecol. Syst.* 10, 1–12.
- Zavodna, M., Arens, P., Van Dijk, P., Partomihardjo, T., Vosman, B., Van Damme, J., 2005. Pollinating fig wasps: genetic consequences of island recolonization. *J. Evol. Biol.* 18, 1234–1243.