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Plants are the drivers of geographic variation of floral odours in brood site pollination mutualisms: A case study of *Ficus hirta*

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ABSTRACT

Plant odours are central for pollinator attraction. This is particularly true in obligate brood site pollination mutualisms. However, we know little about the evolution of olfactory signalling in these mutualisms. Here, we investigate geographic variation of floral odour in the obligate host-specific brood site pollination mutualism between *Ficus hirta* and its specialised pollinators. Floral scent samples from nine locations in China were collected using head-space adsorption and were analysed by gas chromatography-mass spectrometry. We evidence progressive geographic divergence of floral odours. The pattern of variation fits plant genetic structure for neutral genes but differs from pollinating insect structuring into species and populations. In our study system, the geographic variation of receptive floral odour presents a pattern that is not distinguishable from neutral drift. The variation is not canalised by the insects. We propose that this pattern characterises obligate brood site pollination mutualisms in which pollinators are host specific and dispersal of plant and insect is limited. Insects with their short generation times and large population sizes rapidly track any chance variation in host receptive inflorescence odours. Plants are the drivers and insects the followers. The source of the geographic variation in floral odours can be genetic or phenotypic in response to local conditions. Strict sense plant-insect co-evolution is not involved. In contrast, previous results on another *Ficus*-pollinating wasp association suggest that stabilising selection could be at work in more dispersive systems.

1. Introduction

Achieving successful gamete transfer is a major challenge for plants (Boavida et al., 2005). This is particularly true in species rich habitats in which plant species compete for pollinators (Vamosi et al., 2006) and in which pollen may end up on stigmas of the wrong species (Morales and Traveset, 2008). About 300 000 extant species of flowering plants (Ollerton et al., 2011) and two of the four extant phyla of gymnosperms (Toon et al., 2020) rely on animals to ensure their pollination, potentially allowing better control of pollen transfer than wind pollination. Odour is an important plant trait, ensuring detection by pollinators and mediating plant-pollinator interactions (Segar et al., 2019; van der Kooi et al., 2021). The evolution of the olfactory signal may rely on pre-existing pollinator sensory bias, with plants adjusting to pollinator traits (Sayers et al., 2020; Schiestl and Dötterl, 2012). Alternatively, it may depend on the pollinator's evolutionary capacity to track the odours produced by plants producing preferred resources (Kula et al., 2013). Understanding the interplay between the evolution of the emitted odour and the evolution of pollinator response to this odour is challenging in generalist interactions when plants depend on a diversity of pollinators and when pollinators use a diversity of plant species. In such diffuse systems, the determinant selective pressures are difficult to establish (Johnson and Stinchcombe, 2007; Rosas-Guerrero et al., 2014). Specialised systems are easier to handle. They can allow the assessment of the driving forces behind evolution of olfactory signalling in contexts where population structures of the interacting plant and pollinator are known.

Among such specialised systems, obligate brood site pollination mutualisms provide simple systems to investigate the evolution of olfactory signalling. Indeed, the specialised pollinators depend mainly or

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exclusively on olfactory signalling by their host-plants to locate potential oviposition sites (Hossaert-McKey et al., 2010). In a number of cases, the plants interact with one or a few insect species that are specialised on a single host (Kawakita and Kato, 2009; Pellmyr, 2003; Yu et al., 2019). In such specific systems, the selective forces underlying odour signal evolution can involve 1) stabilising selection acting on plants and insects (Hossaert-McKey et al., 2010), or 2) co-evolutionary trajectories (plant and insect tracking each other's evolution) (Bain et al., 2016; Soler et al., 2011), or 3) plants tracking insect sensory bias (Ramírez et al., 2011), or 4) insects tracking plant odour variation (Suinyuy et al., 2015). In such systems, the pattern of geographic variation of attractive odours as a function of host plant and insect spatial genetic structure should enable to establish which one of these evolutionary processes is at work. A theoretical prediction is that stabilising selection could limit geographic differentiation in such mutualisms (Yoder and Nuismer, 2010; Raimundo et al., 2014).

The association between the over 750 species of *Ficus* and their pollinating Agaonid wasps (Agaonidae, Chalcidoidea) provides a highly diversified brood site pollination mutualism for investigating factors affecting the evolution of the olfactory signal. The wasps breed within the closed urnshaped inflorescences of *Ficus* called figs. The inside of the fig is lined by uniovulate pistillate flowers and by staminate flowers. At female anthesis, the wasps are attracted to the figs by species-specific odours (Souto-Vilarós et al., 2018; Proffit et al., 2009, 2020). The wasps enter the figs, and oviposit in the pistillate flowers. Several weeks later, the wasp offspring emerge from the galled pistillate flowers at the time of fig male anthesis, become pollen loaded and leave the fig in search of a fig at female anthesis (a receptive fig). In half of *Ficus* species, all figs produce both wasps and seeds. These species are monoecious. The other *Ficus* species are functionally dioecious. Functionally male trees bear figs that produce pollen in their staminate flowers, and wasps but no seeds in their pistillate flowers. The other trees are female. They produce seeds but no wasps in their pistillate flowers.

In the high dispersal monoecious *Ficus racemosa*, plant and insect present almost no spatial genetic structure from South China to Thailand (Bain et al., 2016). Comparison of the odours emitted by receptive figs between a locality in southern China and one in the North-Eastern province of Thailand did not detect any significant difference in the composition of volatile organic compounds (VOCs) between these two localities (Soler et al., 2011). Conversely, a study of the dioecious *Ficus septica* uncovered similar plant spatial structuring into distinct populations and pollinator structuring into species, each corresponding to an island or a group of islands in the Philippines. Distinct plant populations produced different receptive fig odours. However, receptive fig odours were more structured than plants and pollinators as odour differences were observed between two locations on different islands (Luzon and Negros) that presented no plant genetic differentiation for neutral genes and that shared the same species of pollinators (Rodriguez et al., 2017). In these two case studies, it was not possible to establish whether plant or insect were the driving force responsible for receptive fig odour geographic differentiation because plant and insect presented similar spatial genetic structure. To establish whether plants or insects drive the evolution of floral odours, it is necessary to analyse spatial variation of floral odours in a system in which insect and plant present contrasted spatial genetic structures.

This is the case for *Ficus hirta* and its pollinators. *Ficus hirta* is a widely distributed understory shrub of secondary habitats growing throughout continental South-East Asia from the Himalayan foothills to Java. It presents a pattern of spatial genetic structure without genetic discontinuity across continental South-East Asia, suggesting genetic isolation by distance (Yu and Nason, 2013; Yu et al., 2019). It is pollinated by a set of parapatric wasp species forming the species complex of *Valisia javana sensu lato* (Yu et al., 2019). In China, *F. hirta* is pollinated by *V. javana* sp1 in the south-east and the south, from Fujian province to Guangxi province, while it is pollinated by *V. javana* sp2 westwards in Yunnan province. Throughout continental south-eastern China to southern China, over more than 1000 km, *V. javana* sp1 forms a single population, with almost no spatial genetic structure for neutral genes, while on Hainan Island, 20 km off the coast, it is pollinated by a different population of *V. javana* sp1 (Tian et al., 2015; Yu et al., 2019). The contrasted genetic structure between pollinators and the host fig allows addressing the question of what are the evolutionary forces affecting floral odour composition. If the insects are driving the selection for receptive fig odour variation then we expect to observe two or three groups of receptive fig odours: one in Yunnan, one in south and south-east China and the same as on the continent or a different one in Hainan Island depending on the speed of evolution. Alternatively, if variation in receptive odours is driven by the plant, either by spatial genetic structure for neutral genes or by plant phenotypic variation in response to variation in ecological conditions, then we predict a simple pattern of geographic differentiation by distance. Finally, if there is ongoing stabilising selection then we predict no geographic variation in receptive fig odour. By analysing and comparing the VOC composition of receptive figs of *Ficus hirta* in nine localities in China, we investigated geographic variation of *Ficus hirta* receptive fig odours in China to answer these questions.

2. Materials and methods

2.1. Study system and collection sites

Ficus hirta is a functionally dioecious understorey shrub or small tree 1–3 m high. Figs are produced year-round (Yu et al., 2006). Figs develop asynchronously within the shrub, and a few plants are sufficient to produce pollinators throughout the year (Yu et al., 2006, 2008). The production of receptive figs peaks in May–June (Yu et al., 2006). In *Ficus*, when figs are produced at the same time on functionally male and female plants, their odour at receptivity is identical (Hossaert-McKey et al., 2010). Nevertheless, we chose to restrict the collections to figs on male trees to reduce that potential source of variation. We sampled similar habitats in all locations, namely secondary understorey vegetation. Sampling was concentrated over a short period from July 13 to August 4 (Supplementary Table S1) and from 10:00 to14:18 with a large spread of time of collection within location and a large overlap in time of collection among locations (Supplementary Table S1), as odour composition varies during the day in *Ficus* (Conchou et al., 2014). In order to establish whether daily variation depending on collection timing could result in overlap with geographic variation, we took care to sample odours at various times of the day at each location.

We collected floral odours from male receptive figs in nine locations distributed across China, with three south-eastern locations (Ning, Sha and Sui), five southern locations including two in Hainan, and one south-western location (XTBG) in South Yunnan. (Table 1, Fig. 1).

Some of the results presented here were used in a study comparing *Ficus hirta* receptive fig odours and those of the closely related *F. triloba* (Deng et al., 2022). However, the study protocol, data analysis methods, raw data and statistical results were neither published nor discussed.

All the sites in which odours were collected were also part of a broader study on the genetics of *F. hirta* and its pollinating wasps throughout China and Indochina to Java. All the plants belonged to a single species presenting clinal genetic variation while the pollinators belonged to several parapatric species (Yu et al., 2019). Reference herbarium samples for that study were deposited at herbarium IBSC. FK formally identified the specimens as *Ficus hirta*, by comparing live plants from locations South China Botanical Garden (SCBG), Guangzhou, China, Xishuangbanna Tropical Botanical Garden (XTBG), Mengla, China and the voucher specimens collected by YH throughout the sampling range, with descriptions and with reference herbarium samples, mainly at the Paris herbarium (P), identified by EJH Corner and CC Berg. In this study, sample identification in the field was done either by XD and HY or by XD and FK.

Table 1
Sampling site, Sampling date, corresponding pollinating wasps, GPS coordinates, and No. of samples.

Sampling site	Sampling date	Pollinating wasp	GPS coordinates	No. of samples
Ning	24/07/2019	sp1 pop1	119.73 E, 26.63 N	5
Sha	02/08/2019	sp1 pop1	117.73 E, 26.39 N	5
Sui	31/07/2019	sp1 pop 1	114.24 E, 26.41 N	5
SCBG	16/07/2019	sp1 pop1	113.35 E, 23.17 N	5
DHS	18/07/2019	sp1 pop1	112.54 E, 23.16 N	4
Nan	13/07/2019	sp1 pop1	108.39 E, 22.79 N	7
Ding	July 04, 2017	sp1 pop2	110.36 E, 19.54N	3
Wan	07/07/2019	sp1 pop2	110.20 E, 18.77 N	6
XTBG	24/07/2019	sp2	101.27 E, 21.92 N	5

2.2. Floral odour collection

We used the head-space technique following methods initially developed for *Silene* (Dötterl et al., 2005) and that have been successfully used in several *Ficus* species (Cornille et al., 2012; Hossaert-McKey et al., 2016; Soler et al., 2011; Souto-Vilarós et al., 2018). As the size of receptive figs varied geographically (Yu et al., 2018), in order to collect sufficient quantities of odour for the analysis, the number of figs used in each bag was adjusted according to fig diameter: for south-eastern locations 13 ± 4, for southern locations 17 ± 4, and for the south-western location 19 ± 10. Odour collection was performed under natural light between 10:00 a.m. and 14:18 p.m., corresponding to the period of maximum insect activity during our field season.

Receptive figs were enclosed in a polyethylene terephtalate (Nalophan®, Kalle Nalo GmbH, Wursthüllen, Germany) bag for 30 min. Then, air was pulled out of the bag for 5 min (flow rate: 200 mL min^{-1}) through a Chomatoprobe filter (filled with 1.5 mg of Carbotrap 20–40 and 1.5 mg of Tenax 60–80) in which the VOCs were trapped. Because *Ficus hirta* figs are small, to increase the quantity of odour trapped, we repeated the above operation three times for each bag. In parallel, for every collection we made a 'blank' extraction from a bag that contained no fig, using the same protocol. One microliter of a solution of internal standards (n- Nonane and n-Dodecane, 110 ng μl^{-1} of each) was added to each filter, before odour extraction, so that we could control for VOC loss during storage and transport. The samples were stored at −20 °C until VOC analysis.

2.3. VOC analysis

Samples were analysed at the "Platform for Chemical Analyses in Ecology" (PACE), technical facilities of the LabEx CeMEB (Centre Méditerranéen pour l'Environnement et la Biodiversité, Montpellier, France), using a gas chromatograph (GC, Trace™ 1310, Thermo Scientific™ Milan, Italy) coupled to a mass spectrometer (ISQ™ QD Single Quadrupole, Thermo Scientific™ Milan, Italy). The gas chromatograph was equipped with an OPTIMA® 5-MS capillary column (30 m × 0.25 mm × 0.25 μm, Macherey-Nagel, Düren, Germany). Filters were handled with a Multi Purpose Sampler (Gerstell, Mülheim an der Ruhr, Germany) and desorbed with a double stage desorption system, composed by a Thermal Desorption Unit and a Cold Injection System (CIS) (Gerstell, Mülheim an der Ruhr, Germany). The instrumentation and temperature programs were as follows. First, the filters were desorbed splitless with a temperature of 250 °C on the CIS trap cooled at −80 °C by liquid nitrogen. Then, the CIS trap was heated to 250 °C with a 1:4 split ratio to inject the compounds in the column. Oven temperature was held at 40 °C for 3 min, increased from 40 °C to 210 °C at a rate of 5 °C.min^{-1} and from 220 to 250 °C at 10 °C.min^{-1}, and finally held for 2 min. The temperature of the transfer line and the ion source of the mass spectrometer were 250 °C and 200 °C respectively. The acquisition was from 38m/z to 350m/z, and the ionization energy is 70 eV. The flame ionization detector (FID) was heated to 250 °C. The Xcalibur™ software (Thermo Scientific™, Milan, Italy) was used for data processing. Retention times of a series of n-alkanes (Alcanes standard solution, 04070, Sigma Aldrich®) were used to convert retention times into retention index. Peak identification of VOCs was based on mass spectral interpretation and on the standard library NIST 98 and Adams (2007), and on confirmation by comparison of their retention index (RI) with libraries and published data (Adams, 2007). Identification of some compounds was confirmed by comparison of both mass spectra and RI with those of authentic standards (see Table 2). By comparing samples to the controls collected on the corresponding days of collection, potential contaminant compounds were subtracted from the samples prior to statistical analysis. Only VOCs that appeared in at least three different odour samples were retained to determine odour profiles.

2.4. Data analysis

All statistical analyses were performed with R version 3.5.1 (R Core Team, 2013). Divergence in chemical profiles within and among locations was visualised with non-metric multidimensional scaling (NMDS) in two dimensions, based on a Bray-Curtis similarity matrix, using the package vegan (Oksanen et al., 2013). We used the relative proportions

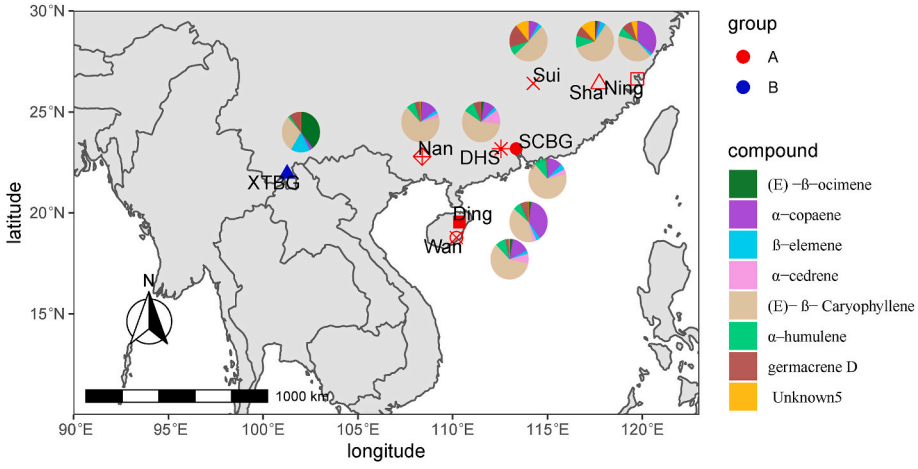

Fig. 1. Receptive fig odour collection sites and Geographical variation of volatile compounds produced by receptive figs of *Ficus hirta* in the nine study sites. In red, locations where *F. hirta* is pollinated by *Valisia javana* sp1, in blue locations where it is pollinated by *V. javana* sp2. Locations Ding and Wan are pollinated by a distinct population of *V. javana* sp1. The pie charts depict the percentage in local receptive fig odours of the compounds that represent more than 5% in the average bouquet of scents in at least one site.

Table 2
Occurrence and relative proportion (% mean ± SD) of volatile compounds from three classes, and total amount, detected in the bouquets of scents emitted by receptive figs of *Ficus hirta* from the studied locations.

Compounds	RI		Ning		Sha		Sui		SCBG		DHS		Nan		Ding		Wan		XTBG
			N = 5		N = 5		N = 5		N = 5		N = 5		N = 7		N = 3		N = 6		N = 6
		O	%	O	%	O	%	O	%	O	%	O	%	O	%	O	%	O	%
Fatty acid derivatives																			
(E)-3-Hexenyl acetate[a]	1005	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	6	3.14 ± 2.38
Nonanal[a]	1102	2	0.03 ± 0.05	1	n.d.	3	0.17 ± 0.19	1	0.01 ± 0.02	0	n.d.	6	1.41 ± 1.16	1	0.14 ± 0.24	0	n.d.	0	n.d.
Decanal[a]	1203	0	n.d.	0	n.d.	4	0.27 ± 0.23	1	0.07 ± 0.16	0	n.d.	0	n.d.	1	0.45 ± 0.79	0	n.d.	0	n.d.
Total percent			0.03		0.00		0.44		0.08		0		1.41		0.59		0.00		3.14
Monoterpenes																			
α-pinene[a]	934	2	0.02 ± 0.03	0	n.d.	1	0.03 ± 0.06	0	n.d.	1	0.07 ± 0.15	0	n.d.	0	n.d.	0	n.d.	2	0.2 ± 0.34
β -myrcene	991	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	5	0.26 ± 0.36
Limonene[a]	1030	2	0.19 ± 0.26	3	0.32 ± 0.4	5	2.76 ± 1.75	2	0.18 ± 0.39	1	1.91 ± 4.27	3	1.43 ± 2.18	1	0.42 ± 0.73	4	4.29 ± 8.69	4	2.37 ± 3.14
(E) -β-ocimene*	**1048**	**1**	**0.08 ± 0.17**	**4**	**1.93 ± 3.77**	**1**	**0.09 ± 0.19**	**1**	**0.04 ± 0.09**	**4**	**1.83 ± 2.21**	**4**	**0.93 ± 1.52**	**2**	**1.89 ± 1.76**	**3**	**2.29 ± 4.99**	**6**	**27.5 ± 24.0**
Linalool[a]	1101	0	n.d.	1	0.01 ± 0.01	0	n.d.	0	n.d.	0	n.d.	2	0.98 ± 2.41	0	n.d.	0	n.d.	6	4.66 ± 7.18
pyranoid linalool oxide piranoid	1172	1	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	1	0.06 ± 0.16	0	n.d.	0	n.d.	2	0.04 ± 0.07
Total percent			0.119		2.26		2.88		0.22		3.81		3.40		2.31		6.58		35.03
Sesquiterpenes																			
δ-elemene[a]	1343	5	0.94 ± 0.37	5	1.56 ± 0.7	5	1.68 ± 0.47	2	0.83 ± 1.15	4	0.67 ± 0.98	5	0.6 ± 0.68	2	1.17 ± 1.03	2	0.39 ± 0.79	5	1.28 ± 0.94
				1	0.03 ± 0.07	5	0.81 ± 0.33	5	0.58 ± 0.59	5	0.68 ± 0.66	7	1.15 ± 1.13	3	4.99 ± 0.57	6	0.78 ± 0.49	6	0.14 ± 0.13
cyclosativene	1375	5	1.86 ± 0.55	3	0.19 ± 0.22	4	1.85 ± 2.12	4	1.46 ± 0.89	3	0.48 ± 0.45	6	1.92 ± 2.08	1	0.42 ± 0.73	4	1.45 ± 1.42	5	0.64 ± 0.44
α-copaene*	**1384**	**4**	**26.44 ± 23.2**	**5**	**1.79 ± 1.08**	**5**	**6.16 ± 5.39**	**5**	**10.71 ± 8.89**	**5**	**7.69 ± 5.09**	**7**	**11.95 ± 4.11**	**3**	**28.67 ± 2.31**	**6**	**11.82 ± 6.34**	**6**	**1.94 ± 2.05**
β-cubebene	1387	3	0.6 ± 0.79	5	0.44 ± 0.49	5	2.94 ± 2.65	3	0.16 ± 0.21	4	0.67 ± 0.77	4	0.48 ± 0.58	2	0.53 ± 0.73	3	0.23 ± 0.32	3	0.29 ± 0.64
β-elemene*	**1398**	**4**	**1.72 ± 2.28**	**5**	**3.64 ± 1.73**	**5**	**2.32 ± 1.8**	**5**	**4.08 ± 1.3**	**5**	**2.2 ± 0.78**	**5**	**2.57 ± 2.08**	**3**	**2.63 ± 0.78**	**6**	**2.08 ± 1.01**	**6**	**11.43 ± 10.78**
α-cedrene	**1412**	**2**	**0.07 ± 0.15**	**0**	**n.d.**	**0**	**n.d.**	**4**	**3.33 ± 4.43**	**4**	**8.83 ± 10.96**	**6**	**2.91 ± 3.03**	**3**	**2.23 ± 0.21**	**6**	**6.05 ± 5.54**	**0**	**n.d.**
α-gurjunene	1419	5	0.72 ± 0.5	5	0.53 ± 0.69	4	0.27 ± 0.35	3	0.3 ± 0.53	5	0.1 ± 0.07	5	0.3 ± 0.5	1	0.13 ± 0.23	4	0.53 ± 0.77	5	0.11 ± 0.09
Cedrene	1425	2	0.14 ± 0.31	3	0.66 ± 1.38	2	0.02 ± 0.02	3	0.06 ± 0.06	2	0.33 ± 0.71	0	n.d.	0	n.d.	0	n.d.	4	0.53 ± 0.65
(E)- β- caryophyllene*	**1430**	**5**	**30.8 ± 18.69**	**5**	**46.45 ± 8.09**	**5**	**36.38 ± 5.99**	**5**	**56.89 ± 8.71**	**5**	**45.11 ± 8.94**	**7**	**56.98 ± 9.13**	**3**	**31 ± 2.4**	**6**	**46.6 ± 15.58**	**6**	**20.57 ± 9.87**
β-copaene	1437	5	3 ± 1.64	5	2.75 ± 1.94	5	4.06 ± 0.77	5	0.68 ± 0.1	5	1.65 ± 2.09	7	1.26 ± 0.76	2	1.33 ± 1.22	5	0.86 ± 0.58	6	2.76 ± 1.05
(E)- α- bergamotene[a]	1441	3	0.3 ± 0.29	5	0.36 ± 0.4	3	0.57 ± 0.58	3	0.11 ± 0.11	1	0.06 ± 0.13	1	0.23 ± 0.61	0	n.d.	0	n.d.	6	1.37 ± 1.32
α-guaiene	1445	0	n.d.	0	n.d.	0	n.d.	5	1.69 ± 0.87	4	1.66 ± 1.11	4	0.57 ± 0.66	3	1.43 ± 0.46	6	1.8 ± 0.59	0	n.d.
alloaromadendrene	1453	4	0.67 ± 0.53	3	0.77 ± 0.83	5	0.99 ± 0.18	0	n.d.	1	0.36 ± 0.81	1	0.1 ± 0.27	1	0.18 ± 0.32	1	0.07 ± 0.17	4	0.78 ± 0.64
E-β-farnesene[a]	1457	3	1.34 ± 1.53	5	0.68 ± 0.69	4	1.41 ± 1.41	2	2.26 ± 3.51	5	4.3 ± 4.11	2	0.22 ± 0.47	3	0.42 ± 0.31	5	3.09 ± 2.98	6	1.56 ± 1.72
α –humulene*	**1463**	**5**	**5.2 ± 2.99**	**5**	**7.61 ± 4.33**	**5**	**4.91 ± 1.77**	**5**	**9.02 ± 1.62**	**5**	**7.23 ± 1.22**	**7**	**6.21 ± 1.6**	**3**	**5.01 ± 1.25**	**6**	**6.93 ± 2.28**	**3**	**1.34 ± 2.18**
γ –muurolene[a]	1482	5	0.79 ± 0.44	4	0.47 ± 0.35	5	0.99 ± 0.29	3	0.37 ± 0.53	3	0.3 ± 0.29	4	0.22 ± 0.26	3	1.24 ± 0.65	5	0.79 ± 0.82	3	0.44 ± 0.5
germacrene D*	**1488**	**4**	**6.41 ± 6.7**	**5**	**6.04 ± 4.33**	**5**	**13.26 ± 4.81**	**3**	**1.04 ± 1.25**	**5**	**4.86 ± 6.82**	**6**	**3.85 ± 3.34**	**3**	**5.18 ± 1.67**	**5**	**2.35 ± 2.23**	**6**	**6.85 ± 2.61**
α-selinene	1494	3	0.44 ± 0.66	4	1.56 ± 2.64	2	1.75 ± 3.78	5	0.96 ± 0.39	5	1.48 ± 1.57	4	0.26 ± 0.28	3	0.98 ± 0.54	6	0.54 ± 0.3	4	2 ± 2.5
β-guaiene	1500	4	0.1 ± 0.15	0	n.d.	2	0.12 ± 0.25	0	n.d.	0	n.d.	2	0.06 ± 0.11	2	0.31 ± 0.27	2	0.11 ± 0.18	2	0.02 ± 0.03
α-bulnesene	1503	5	1.74 ± 2.46	5	3.32 ± 1.56	3	1.25 ± 1.74	5	1.58 ± 0.81	5	0.92 ± 0.48	2	0.04 ± 0.08	1	0.22 ± 0.38	3	0.37 ± 0.57	4	2.82 ± 2.5
α-muurolene[a]	1505	5	1.94 ± 0.7	5	1.64 ± 0.8	5	2.99 ± 1.11	5	1.75 ± 1.18	4	4.15 ± 4.04	7	1.6 ± 1.01	3	3.71 ± 0.48	6	2.01 ± 0.62	6	3.77 ± 2.95
γ -cadinene	1520	5	0.75 ± 0.98	5	1.3 ± 0.53	5	0.68 ± 0.31	5	0.77 ± 0.94	5	1.04 ± 0.87	5	0.19 ± 0.23	3	0.89 ± 0.65	6	1.38 ± 0.87	6	0.13 ± 0.15
δ-cadinene[a]	1528	5	2.94 ± 1.89	5	1.17 ± 0.23	5	1.53 ± 0.52	5	1.06 ± 0.44	5	1.29 ± 0.34	7	1.2 ± 0.44	3	4.23 ± 1.29	6	2.85 ± 1.55	6	1.01 ± 0.54
Total percent			92.4		82.96		86.94		99.69		96.02		94.87		96.9		93.08		61.78
Unknown																			
Unknown1	1339	5	2.01 ± 4.38	5	0.66 ± 1.23	4	0.08 ± 0.06	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
Unknown2	1359	5	0.19 ± 0.12	5	1.66 ± 1.16	4	0.53 ± 0.56	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
Unknown3	1360	3	0.16 ± 0.31	5	0.09 ± 0.04	2	0.03 ± 0.04	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
Unknown4	1379	4	1.16 ± 1.1	5	2.81 ± 1.53	5	1.29 ± 1.08	0	n.d.	1	0.05 ± 0.11	0	n.d.	0	n.d.	0	n.d.	4	0.06 ± 0.07
Unknown5	**1476**	**5**	**3.76 ± 2.63**	**5**	**9.57 ± 4.15**	**4**	**7.81 ± 4.57**	**0**	**n.d.**	**4**	**0.1 ± 0.09**	**5**	**0.33 ± 0.37**	**1**	**0.2 ± 0.34**	**4**	**0.34 ± 0.5**	**2**	**0.01 ± 0.01**
Total percent			7.28		14.79		9.74				0.15		0.33		0.2		0.34		0.07
Total amount (ng/fig/hr)			**4.48 ± 2.8**		**5.2 ± 4.36**		**2.04 ± 0.32**		**1.16 ± 0.62**		**1.2 ± 0.8**		**0.76 ± 0.68**		**0.8 ± 0.28**		**0.66 ± 0.5**		**0.53 ± 0.27**
mean diameters of figs (mm)			**23.2 ± 4.9**		**21.4 ± 2.6**		**20.0 ± 2.6**		**14.3 ± 2.7**		**13.4 ± 1.7**		**16.78 ± 2.0**		**15.5 ± 1.6**		**15.3 ± 2.3**		**NA**

[a] Compound identification confirmed by comparison of mass spectra and retention time with those of authentic standards; N = number of individuals sampled; O = number of individuals in which that compounds was found; RI = Kovat retention index; n. d. = compound not detected; in bold compounds that represent more than 5% in the average bouquet of scents in at least one site.

of all the VOCs emitted by figs (semiquantitative data). Data for each VOC was standardized prior to the analysis to range from 0 to 1 using the R package vegan (Oksanen et al., 2013) Two-dimensional plots were constructed using the "metaMDS" function algorithm. Pairwise distance between individuals for relative proportions of VOCs was calculated using the Bray–Curtis dissimilarity index, which ranges between 0 and 1. A stress value is given, indicating how well the particular configuration represents the distance matrix (stress values < 0.2 are desirable). We performed a Principal Component Analysis (PCA) on the NMDS coordinates (function metaMDS, option pc = TRUE in the Vegan package). As a result, after analysis, axis one contained the greatest variance.

To test if the overall difference in chemical composition between locations was significant, we carried out permutational multivariate analysis of variance tests (PERMANOVA) based on a Bray-Curtis distance matrix (with 99 999 random iterations). The chemical distance matrices were calculated with the function "vegdist" after data standardization for each VOC to range from 0 to 1 (Oksanen et al., 2013). We performed pairwise comparisons after detecting significant interactions with PERMANOVA with the "pair-wise.perm.manova" function in the RVAideMemoire package (Hervé and Hervé, 2020), and we used the false discovery rate (fdr) method for multiple test p-value correction. Similarity percentage (SIMPER) (Clarke, 1993), was used to identify the compounds that contributed most to dissimilarities among locations. The simper function performs pairwise comparisons of locations and finds the average contributions of each compound to the average overall Bray-Curtis dissimilarity. The function displays the compounds that contribute most to the differences between locations.

To investigate relationships between chemical distance and geographic distance, we performed Mantel tests. We used the chemical distance matrices generated above and geographic distances based on GPS coordinates. Mantel tests (with 99 999 random iterations) were performed for the entire data set and for data subsets.

In a second step, a reduced data set was constituted with a single value per location by averaging across samples the mean peak area of each compound. The mean peak area of each compound for all the samples of a location then became the consensus sample used in all further analyses (Friberg et al., 2019). This method is a conservative way to avoid pseudo-replication associated with the use of several data points from a single location as independent points (Friberg et al., 2019).

3. Results

3.1. Overall odour profile

Across the nine locations, a total of 45 receptive fig odour samples were collected and analysed (Fig. 1). Thirty eight different VOCs were detected and identified in the odours emitted by the receptive figs (Table 1). The identified scent compounds included three fatty acid derivatives, six monoterpenes and 24 sesquiterpenes, while five compounds remained unidentified. Odours from locations pollinated by *V. javana* sp1 were mainly composed of a few sesquiterpenes while at XTBG, pollinated by *V. javana* sp2, the odours contained markedly higher quantities of monoterpenes, mainly (E)-β-ocimene (Table 1 and Fig. 1). Compounds that were present throughout all locations accounted for 84–95% of local emissions, depending on location.

3.2. Geographic variation in floral scents

The NMDS plot (Fig. 2, stress = 0.172) groups the floral odour samples into three geographic clusters separated on axis one of the PCA, namely a south-eastern cluster (3 locations: Ning, Sha and Sui), a southern cluster (4 location: SCBG, DHS, Nan and Wan) and a south-western cluster (XTBG). The assignment of location Ding to a cluster is ambiguous. The PERMANOVA results confirm that there was significant variation among locations in the relative proportions of the different compound in odours emitted by receptive figs ($F_{8,44} = 5.02$, $P = 0.001$). Pairwise comparisons between locations belonging to different clusters always gave significant differences while comparisons within cluster gave part significant part non-significant differences (Table 3). Location Ding was significantly different from all south-eastern locations, but not significantly different from two southern locations (Table 3).

Analysis of the similarity percentage (SIMPER) reveals that four to six VOCs were mainly responsible for the dissimilarity between locations, involving both main and minor compounds (Supplementary Table S2). Eight compounds represented at least 5% of the extracts in at least one location. Out of these, only two were not observed in all locations, and they were not detected in respectively one and two locations (Table 2 and Supplementary Table S2). Hence, the main difference between groups of locations is quantitative and not qualitative referring to the relative abundance of VOCs in the odour composition.

3.3. Correlation between floral odour differences and geographic distance

There was a significant correlation between chemical distance and geographic distance including all the samples at all locations (Mantel statistic $r = 0.4897$, $p < 0.001$). A second test performed for all samples at all locations pollinated by *V. javana* sp1, removing location XTBG, was also significant (Mantel test without XTBG, $r = 0.4069$, $p < 0.001$). A third test performed including only samples from the locations pollinated by *V. javana* sp1 pop1, *i.e.* the continental south and south-eastern populations was also significant (Mantel test on continental locations without XTBG, $r = 0.49$, $p < 0.001$). Hence, at all scales, we observed a correlation between chemical distance and geographic distance.

A second set of correlations were examined using a single odour

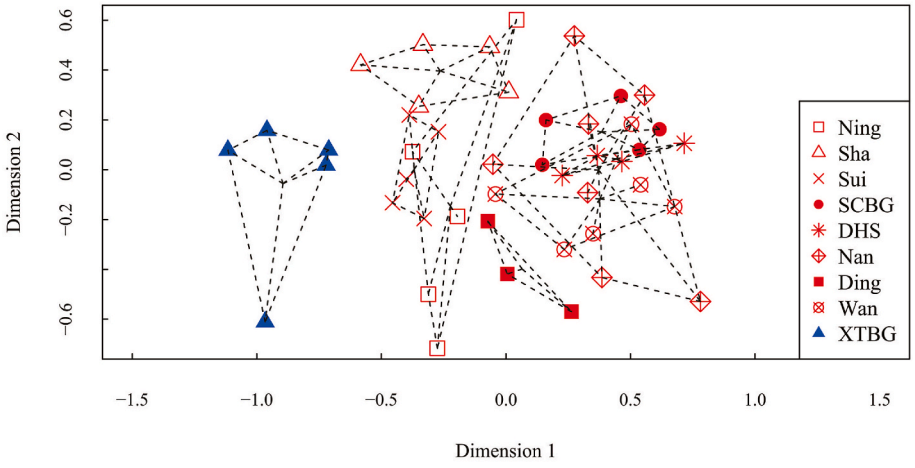

Fig. 2. First and second axis of the Principal Component Analysis on the two-dimensional non-metric multi-dimensional scaling coordinates of the relative proportions of VOCs in the odours emitted by individual plants based on the Bray-Curtis dissimilarity Index (stress = 0.173). Three geographic groups are separated on axis 1: a south-eastern one (Ning-Sha-Sui), a southern one (SCBG-DHS-Nan-Wan) and a south-western one (XTBG). Location Ding occupies an intermediate location between the south-eastern and the southern group.

Table 3
Significance of the differences between locations in the relative proportions of the different VOCs. Significance was estimated with a permutational analysis of variance (PERMANOVA). Non-significant p-values ($p < 0.05$) indicated in bold.

	Ning	Sha	Sui	SCBG	DHS	Nan	Ding	Wan	XTBG
Ning									
Sha	0.067								
Sui	0.105	**0.036**							
SCBG	**0.036**	**0.022**	**0.022**						
DHS	**0.022**	**0.025**	**0.022**	0.344					
Nan	**0.018**	**0.018**	**0.018**	**0.022**	**0.022**				
Ding	0.07	**0.027**	**0.028**	0.053	**0.042**	**0.022**			
Wan	**0.022**	**0.022**	**0.022**	**0.022**	0.611	**0.018**	0.072		
XTBG	**0.022**	**0.022**	**0.022**	**0.025**	**0.022**	**0.022**	**0.033**	**0.022**	

composition value for each location in order to avoid potential pseudo-replication problems. There was a significant correlation between chemical distance and geographic distance when including all the locations (Fig. 3, Mantel statistic $r = 0.3423$, $p = 0.028$) and when removing location XTBG (Mantel statistic r: 0.3525, $p = 0.043$). When including only continental southern and south-eastern locations (6 data points) the test became non-significant (Mantel statistic $r = 0.45$, $p = 0.072$).

4. Discussion

We show that the main source of variation of receptive fig odour in *Ficus hirta* is in the relative proportion of constitutive VOCs. This variation is geographically structured as shown by the Mantel tests on chemical distances. In agreement, the first axis of the PCA on NMDS coordinates groups samples according to the part of the range from which they originate. This remains true when restricting the study to locations pollinated by *V. javana* sp.1. The second axis of the PCA represents variation within location and variation between geographically close locations. Receptive fig odours generally change over the day (Conchou et al., 2014). Despite deliberately introducing this variation in the data, most of the information on the first axis was geographic. Hence, geographic variation was stronger than variation due to the time of collection during the day. Further, varying time of odour collection also introduced temperature variation within site. Still geographic variation remained strongest. Hence, the geographic variation in receptive fig odours is probably not an artefact due to local conditions at the time of odour collection.

We observe a pattern of increasing receptive fig odour differentiation with distance. This pattern is analogous to the pattern of genetic isolation by distance for neutral genes exhibited by the plant (Yu et al., 2019). The variation may result from genetic differentiation or from phenotypic response to local conditions. The pattern is markedly different from the pattern of genetic structuring of the pollinating insects into species and populations (Yu et al., 2019). Hence, there was no evidence in favour of the stabilising selection predicted by theoretical models (Yoder and Nuismer, 2010; Raimundo et al., 2014) and no evidence in favour of strict sense plant-insect co-evolution for floral odour composition and its perception by the insects. The results show that in *F. hirta* plant genetics or plant phenotypic response to local conditions is the driver of geographic floral odour variation, and the variation observed in this species is probably biologically meaningful for its interaction with its pollinating wasps.

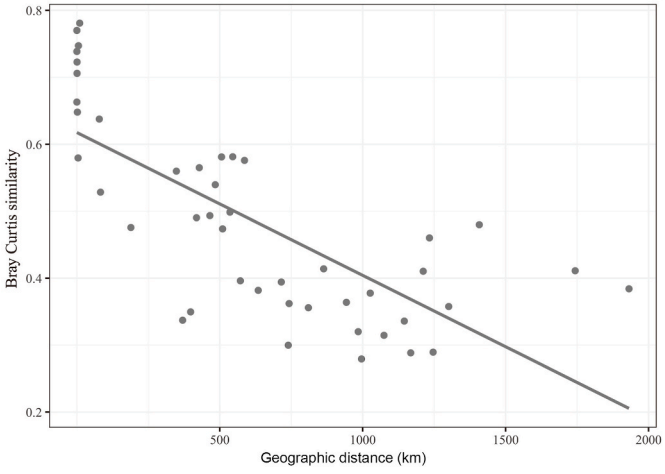

Fig. 3. The pattern of decreasing Bray Curtis similarity (1 – Bray Curtis distance) between locations with increasing geographic distance. Mantel statistic $r = 0.3423$, $p = 0.028$.

Electroantenograms show that fig pollinating wasp antennae mainly respond to a few of the VOCs constituting a fig odour at receptivity and experiments have confirmed that these VOCs attract wasps (Proffit et al., 2020; Wang et al., 2021). Nevertheless, in the two analysed situations where two *Ficus* species locally share pollinators, the VOC mixes they produced were not distinguishable, supporting convergent evolution of the complete floral odour composition (Cornille et al., 2012; Wang et al., 2016). This observation suggests that the global VOC mix has a role in the fig-pollinating wasp interaction. Given this background, the geographic variation observed in *Ficus hirta* is probably biologically meaningful for its interaction with its pollinating wasps.

Ficus septica in the Philippines and Taiwan provides complementary information on patterns of geographic variation in short distance dispersal species (Rodriguez et al., 2017). *Ficus septica* produces different odours on different islands suggesting that ruptures in gene flow may lead to odour differentiation. It is pollinated by a different black coloured wasp species belonging to the *Ceratosolen bisulcatus* species group in different groups of islands. Nevertheless, one wasp species belonging to the same species group, *C. jucundus*, has colonised the whole region, bridging the odour differences (Rodriguez et al., 2017). This observation suggests that, at the time scale of *C. jucundus* colonisation of the Philippines, receptive fig odour differentiation within host-plant species was not sufficiently large to preclude wasps from expanding their range.

Geographic variation in floral odours in the brood site pollination mutualism between *Lithophragma* spp. and *Greya* moths follows the same type of pattern as observed in *Ficus hirta*. Floral odours varied among locations within species, and the difference in floral odours increased with geographic distance (Fig. 3 in Friberg et al., 2019). The difference of floral odours among populations was genetic and not due to environmental conditions. However, odour distance between populations of two sympatric but not syntopic clades of *Lithophragma* pollinated by a same *Greya* moth species did not correlate with geographic distance, demonstrating that there was no concerted odour evolution between the two clades mediated by pollinators (Friberg et al., 2019). These results suggest that in this example too, the plants are the drivers of floral odour evolution and the insects are the followers (Thompson and Rich, 2011).

As indicated in the introduction, the geographic variation of receptive fig odours observed in *Ficus hirta* was not present in the more

dispersive *Ficus racemosa* (Soler et al., 2011). Evolutionary stability is even more pronounced in some species of yuccas. Indeed, in *Yucca filamentosa* floral odours do not vary across its range (Leebens-Mack, 2004; Svensson et al., 2005) and the floral odours of the allopatric *Yucca elata*, *Y. filamentosa, Y. glauca* and *Y. palida* are almost identical, showing remarkable evolutionary stability (Svensson et al., 2016; Tröger et al., 2021).

Hence, some brood site pollination mutualisms follow a pattern of stabilising selection, while other examples may follow a pattern in which plants are the leaders and the pollinators are the followers, but further experimental study is required. The origin of these differences needs exploring.

Author contributions

X. X. D. and B. B. collected samples, analysed the data and wrote the manuscript. Y. Q. P. help collecting samples. Y. F. C. analysed the data. H.Y.,X.J,G, K. F. and M. P. organized the work and wrote manuscript.

Declaration of competing interest

The author declares that they have no conflict of interest statement.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actao.2023.103952.

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