

# Plants Are the Drivers of Geographic Variation of Floral Scents in a Highly Specialized Pollination Mutualism: a Study of *Ficus Hirta* in China

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## Research article

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# Abstract

## Background

Floral volatiles play an important role in pollinator attraction. This is particularly true in obligate brood site pollination mutualisms. The plants generally produce inconspicuous flowers and depend on odours to attract to their inflorescences specialised pollinators that breed in their floral structures. Little is known about the processes shaping the micro-evolution of these floral odours. Here, we investigate geographic variation of floral odour in an obligate host-specific brood site pollination mutualism where plant and pollinator genetic structures are different, *Ficus hirta* and its specialised pollinators.

## Results

We evidence progressive geographic divergence of floral odours. The pattern of variation fits plant genetic structure but differs from pollinating insect structuring into species and populations. In our study system, the evolution of receptive floral odour presents a pattern that is not distinguishable from neutral drift that is not canalised by the insects.

## Conclusion

We propose that this pattern characterises obligate brood site pollination mutualisms in which pollinators are host specific and dispersal is limited. Insects with their short generation times and large population sizes track variation in host receptive inflorescence odours. Plants are the drivers and insects the followers. Strict sense plant-insect co-evolution is not involved. In contrast, stabilizing selection may be at work in more dispersive brood site pollination mutualisms, while pollinators may mediate local interspecific plant floral odour convergence when plant species share local pollinators.

## Background

A major challenge for plants is to achieve successful gamete transfers [1]. This is particularly true in species rich habitats in which plants compete for pollinators [2] and in which pollen may end up on stigmas of the wrong species [3]. Some 87.5% of the 350 000 extant species of angiosperms rely on animals to ensure their pollination, potentially allowing better control of pollen transfer than wind pollination [4]. To ensure detection by pollinators, plants rely on signalling, mainly visual and olfactory [5]. The micro-evolutionary processes underlying the evolution of signalling may vary depending on a diversity of factors such as the nature of the interaction, e.g. beneficial or parasitic, for the pollinator or for the plant [6]. The evolution of plant attractive devices may rely on pre-existing pollinator sensory bias and behavioural traits, with plants tracking pollinator traits [7–9], but also on the pollinator's evolutionary capacity to track preferred resources [10, 11]. Understanding the evolution of plant signalling and the evolution of pollinator response to plant signalling 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, causality is difficult to establish [12–16]. Specialised systems are easier to handle. They allow assessing the evolution of signalling and the role of plant-pollinator co-evolution in contexts where both plant and pollinator population structures are known. Among such specialised systems, obligate brood site pollination mutualisms provide simple systems to investigate the evolution of signalling as the specialised pollinators depend mainly or exclusively on olfactory signalling by their host-plants to locate potential oviposition sites [17].

In these mutualisms, pollinator species use one or a few plant species as hosts, and each plant species is pollinated by one or a few insect species [18, 19]. Brood site pollination mutualisms are diversified as to the resource on which pollinator offspring feed. They may develop feeding on young seeds (*Yucca* [20], Phyllanthaceae [21]), galled plant ovules (*Ficus* [22]), pollen (*Castilla* [23]), decaying stamens (Cyclanthaceae [24]), decaying male inflorescences or post anthesis male inflorescence structures (Cycadales [25], Cyclanthaceae [24], Arecaceae [17]), fungi growing on male inflorescences (*Artocarpus* [26]), or nectar produced by floral bracts (*Macaranga* [27]).

Despite their diversity, brood site pollination mutualisms share a number of ecological and evolutionary attributes. For instance, in most obligate brood site pollination mutualisms, flowers are inconspicuous, with yuccas a notable exception, and chemical signalling by the plants constitutes the main cue used by pollinators to locate receptive flowers [18]. The attractive odours may be emitted by receptive flowers (Phyllanthaceae [19]), inflorescences (*Ficus* [20], Cycadales [21], Araceae [22]), or even leaves (*Chamaropsis* [18]), providing a diversity of ontogenetic and evolutionary origins of signalling. Despite the central role played by odours in these systems, little is known about micro and macro-evolutionary patterns and processes of floral odour evolution and few predictions about their evolution have been made.

Specificity is an important factor affecting signalling evolution. Some plant species involved in obligate brood site pollination mutualism share their pollinators with other plant species of the same genus. This may facilitate year round survival of pollinators using sequentially different plant species flowering at different periods (e.g. in *Macaranga* [23], in Araceae [22], in *Ficus* [24]), or survival of pollinators of plants that flower erratically across years (e.g. African cycads [25]). In such systems, we predict local convergence of floral odours between the different plant species associated with a same set of insects, forming a pollination ring, in a process reminiscent of the formation of Müllerian mimicry rings [26].

Other plant species interact with one or a few insect species that are specialised on a single host. This is particularly the case for brood site pollination mutualisms in which offspring feed on developing flower ovules (e.g. most species of *Yucca*, Phyllantheae and *Ficus* [27–29]). In such specific systems, the selective forces underlying plant-signalling evolution could involve stabilising selection acting on plants and insects, or co-evolutionary trajectories, or plants tracking insect sensory bias, or insects tracking plant odours. How attractive odours evolve and vary geographically as a function of host plant and insect spatial genetic structure is almost totally unknown. A theoretical prediction is that stabilising selection could be at work in pairwise mutualistic interactions, limiting geographic differentiation [30].

*Ficus* provide a highly diversified brood site pollination mutualism for investigating factors affecting the evolution of signalling. Floral odours produced by receptive figs are generally species specific [31, 32] and play a central role in pollinator attraction [33]. Nevertheless, only limited data is available on geographic variation in fig floral odours, and in these cases, plant and insect present share a same spatial pattern of genetic structure [34, 35]. To establish whether plant or insect drives 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* Vahl is a widely distributed shrub growing throughout continental South-East Asia from the Himalayan foothills to Java. It presents a pattern of spatial genetic structure suggesting genetic isolation by distance without genetic discontinuity across continental South-East Asia [29, 36]. It is pollinated by a set of parapatric wasp species forming the species complex of *Valisia javana sensu lato* [29]. In China, *F. hirta* is pollinated by sp1 in the south-east and the south, from Fujian province to Guangxi province, while it is pollinated by sp2 westwards in Yunnan province. Throughout continental south-eastern to southern China, over more than 1000 km, sp1 forms a single population, while on Hainan island, 20 km off the coast, it is pollinated by a different population of sp1 [29, 37]. The contrasted genetic structure between pollinators and the host fig allows addressing the question of how variation in floral odour is determined. 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 or a different one in Hainan island depending on the speed of evolution. Alternatively, if variation in receptive odours is driven by plant spatial genetic structure, then we predict a simple pattern of geographic differentiation by distance. Finally, if there is ongoing stabilizing selection then we predict no geographic variation in receptive fig odour. We investigated geographic variation of *Ficus hirta* receptive fig odours in China to answer these questions.

## Results

### Overall Odour Profile

Across nine locations, a total of 45 receptive fig odour samples were collected and analysed (Fig. 1). Thirty eight different volatile organic compounds (VOCs) were detected and identified in the odours emitted by the receptive figs (Table 1). The identified scent compounds included 3 fatty acid derivatives, 6 monoterpenes and 24 sesquiterpenes, while 5 compounds remained unidentified. Odours from locations pollinated by sp1 were mainly composed of a few sesquiterpenes while at XTBG, pollinated by sp2, the odours contained markedly higher quantities of monoterpenes, mainly (E)- $\beta$ -ocimene but also linalool (Table 1). Compounds that were present throughout all locations accounted for 84–95% of local emissions, depending on location. VOC emissions varied among locations and figs in south-eastern locations emitted higher quantities of volatiles than average (general ANOVA,  $P < 0.001$ , locations Ning, Sui and Sha different from mean, respectively  $p < 0.05$ ,  $p < 0.05$  and  $p < 0.01$ , all other locations not significantly different from mean).

Table 1

Occurrence and relative proportion (% mean  $\pm$  SD) of volatile compounds from three classes, and total amount, detected in the bouquets of scents emitted b

Compounds	RI	Ning		Sha		Sui		SCBG		DHS		Nan	
		N=5		N=5		N=5		N=5		N=5		N=7	
		0	%	0	%	0	%	0	%	0	%	0	%
<b>Fatty acid derivatives</b>													
(E)-3-Hexenyl acetate*	1005	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
Nonanal*	1102	2	0.03 $\pm$ 0.05	1	n.d.	3	0.17 $\pm$ 0.19	1	0.01 $\pm$ 0.02	0	n.d.	6	1.41 $\pm$ 1.16
Decanal*	1203	0	n.d.	0	n.d.	4	0.27 $\pm$ 0.23	1	0.07 $\pm$ 0.16	0	n.d.	0	n.d.
<i>Total percent</i>			0.03		0.00		0.44		0.08		0		1.41
<b>Monoterpene</b>													
$\alpha$ -pinene*	934	2	0.02 $\pm$ 0.03	0	n.d.	1	0.03 $\pm$ 0.06	0	n.d.	1	0.07 $\pm$ 0.15	0	n.d.
$\beta$ -myrcene	991	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.
Limonene*	1030	2	0.19 $\pm$ 0.26	3	0.32 $\pm$ 0.4	5	2.76 $\pm$ 1.75	2	0.18 $\pm$ 0.39	1	1.91 $\pm$ 4.27	3	1.43 $\pm$ 2.18
(E)- $\beta$ -ocimene*	1048	1	0.08 $\pm$ 0.17	4	1.93 $\pm$ 3.77	1	0.09 $\pm$ 0.19	1	0.04 $\pm$ 0.09	4	1.83 $\pm$ 2.21	4	0.93 $\pm$ 1.52
Linalool*	1101	0	n.d.	1	0.01 $\pm$ 0.01	0	n.d.	0	n.d.	0	n.d.	2	0.98 $\pm$ 2.41
pyranoid linalool oxide piranoid	1172	1	n.d.	0	n.d.	0	n.d.	0	n.d.	0	n.d.	1	0.06 $\pm$ 0.16
<i>Total percent</i>			0.119		2.26		2.88		0.22		3.81		3.40
<b>Sesquiterpenes</b>													
$\delta$ -elemene*	1343	5	0.94 $\pm$ 0.37	5	1.56 $\pm$ 0.7	5	1.68 $\pm$ 0.47	2	0.83 $\pm$ 1.15	4	0.67 $\pm$ 0.98	5	0.6 $\pm$ 0.68
$\alpha$ -cubebene	1355	4	3.49 $\pm$ 3.35	1	0.03 $\pm$ 0.07	5	0.81 $\pm$ 0.33	5	0.58 $\pm$ 0.59	5	0.68 $\pm$ 0.66	7	1.15 $\pm$ 1.13
cyclosativene	1375	5	1.86 $\pm$ 0.55	3	0.19 $\pm$ 0.22	4	1.85 $\pm$ 2.12	4	1.46 $\pm$ 0.89	3	0.48 $\pm$ 0.45	6	1.92 $\pm$ 2.08
$\alpha$ -copaene*	1384	4	26.44 $\pm$ 23.2	5	1.79 $\pm$ 1.08	5	6.16 $\pm$ 5.39	5	10.71 $\pm$ 8.89	5	7.69 $\pm$ 5.09	7	11.95 $\pm$ 4.11
$\beta$ -cubebene	1387	3	0.6 $\pm$ 0.79	5	0.44 $\pm$ 0.49	5	2.94 $\pm$ 2.65	3	0.16 $\pm$ 0.21	4	0.67 $\pm$ 0.77	4	0.48 $\pm$ 0.58
$\beta$ -elemene*	1398	4	1.72 $\pm$ 2.28	5	3.64 $\pm$ 1.73	5	2.32 $\pm$ 1.8	5	4.08 $\pm$ 1.3	5	2.2 $\pm$ 0.78	5	2.57 $\pm$ 2.08
$\alpha$ -cedrene	1412	2	0.07 $\pm$ 0.15	0	n.d.	0	n.d.	4	3.33 $\pm$ 4.43	4	8.83 $\pm$ 10.96	6	2.91 $\pm$ 3.03
$\alpha$ -gurjunene	1419	5	0.72 $\pm$ 0.5	5	0.53 $\pm$ 0.69	4	0.27 $\pm$ 0.35	3	0.3 $\pm$ 0.53	5	0.1 $\pm$ 0.07	5	0.3 $\pm$ 0.5
Cedrene	1425	2	0.14 $\pm$ 0.31	3	0.66 $\pm$ 1.38	2	0.02 $\pm$ 0.02	3	0.06 $\pm$ 0.06	2	0.33 $\pm$ 0.71	0	n.d.
(E)- $\beta$ -Caryophyllene*	1430	5	30.8 $\pm$ 18.69	5	46.45 $\pm$ 8.09	5	36.38 $\pm$ 5.99	5	56.89 $\pm$ 8.71	5	45.11 $\pm$ 8.94	7	56.98 $\pm$ 9.13
$\beta$ -copaene	1437	5	3 $\pm$ 1.64	5	2.75 $\pm$ 1.94	5	4.06 $\pm$ 0.77	5	0.68 $\pm$ 0.1	5	1.65 $\pm$ 2.09	7	1.26 $\pm$ 0.76
(E)- $\alpha$ -bergamotene*	1441	3	0.3 $\pm$ 0.29	5	0.36 $\pm$ 0.4	3	0.57 $\pm$ 0.58	3	0.11 $\pm$ 0.11	1	0.06 $\pm$ 0.13	1	0.23 $\pm$ 0.61
$\alpha$ -guaiene	1445	0	n.d.	0	n.d.	0	n.d.	5	1.69 $\pm$ 0.87	4	1.66 $\pm$ 1.11	4	0.57 $\pm$ 0.66
alloaromadendrene	1453	4	0.67 $\pm$ 0.53	3	0.77 $\pm$ 0.83	5	0.99 $\pm$ 0.18	0	n.d.	1	0.36 $\pm$ 0.81	1	0.1 $\pm$ 0.27
E- $\beta$ -farnesene*	1457	3	1.34 $\pm$ 1.53	5	0.68 $\pm$ 0.69	4	1.41 $\pm$ 1.41	2	2.26 $\pm$ 3.51	5	4.3 $\pm$ 4.11	2	0.22 $\pm$ 0.47
$\alpha$ -humulene*	1463	5	5.2 $\pm$ 2.99	5	7.61 $\pm$ 4.33	5	4.91 $\pm$ 1.77	5	9.02 $\pm$ 1.62	5	7.23 $\pm$ 1.22	7	6.21 $\pm$ 1.6
$\gamma$ -muurolene*	1482	5	0.79 $\pm$ 0.44	4	0.47 $\pm$ 0.35	5	0.99 $\pm$ 0.29	3	0.37 $\pm$ 0.53	3	0.3 $\pm$ 0.29	4	0.22 $\pm$ 0.26
germacrene D*	1488	4	6.41 $\pm$ 6.7	5	6.04 $\pm$ 4.33	5	13.26 $\pm$ 4.81	3	1.04 $\pm$ 1.25	5	4.86 $\pm$ 6.82	6	3.85 $\pm$ 3.34
$\alpha$ -selinene	1494	3	0.44 $\pm$ 0.66	4	1.56 $\pm$ 2.64	2	1.75 $\pm$ 3.78	5	0.96 $\pm$ 0.39	5	1.48 $\pm$ 1.57	4	0.26 $\pm$ 0.28
$\beta$ -guaiene	1500	4	0.1 $\pm$ 0.15	0	n.d.	2	0.12 $\pm$ 0.25	0	n.d.	0	n.d.	2	0.06 $\pm$ 0.11
$\alpha$ -bulnesene	1503	5	1.74 $\pm$ 2.46	5	3.32 $\pm$ 1.56	3	1.25 $\pm$ 1.74	5	1.58 $\pm$ 0.81	5	0.92 $\pm$ 0.48	2	0.04 $\pm$ 0.08
$\alpha$ -muurolene*	1505	5	1.94 $\pm$ 0.7	5	1.64 $\pm$ 0.8	5	2.99 $\pm$ 1.11	5	1.75 $\pm$ 1.18	4	4.15 $\pm$ 4.04	7	1.6 $\pm$ 1.01
$\gamma$ -cadinene	1520	5	0.75 $\pm$ 0.98	5	1.3 $\pm$ 0.53	5	0.68 $\pm$ 0.31	5	0.77 $\pm$ 0.94	5	1.04 $\pm$ 0.87	5	0.19 $\pm$ 0.23

δ-cadinene*	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
<i>Total percent</i>			92.4		82.96		86.94		99.69		96.02		94.87
<b>Unknown</b>													
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.
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.
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.
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.
<b>Unknown5</b>	<b>1476</b>	<b>5</b>	<b>3.76±2.63</b>	<b>5</b>	<b>9.57±4.15</b>	<b>4</b>	<b>7.81±4.57</b>	<b>0</b>	<b>n.d.</b>	<b>4</b>	<b>0.1±0.09</b>	<b>5</b>	<b>0.33±0.37</b>
<i>Total percent</i>			7.28		14.79		9.74				0.15		0.33
<b>Total amount (ng/fig/hr)</b>			<b>4.48±2.8</b>		<b>5.2±4.36</b>		<b>2.04±0.32</b>		<b>1.16±0.62</b>		<b>1.2±0.8</b>		<b>0.76±0.68</b>
<b>mean diameters of figs (mm)</b>			<b>23.2 ± 4.9</b>		<b>21.4 ± 2.6</b>		<b>20.0 ± 2.6</b>		<b>14.3 ± 2.7</b>		<b>13.4 ± 1.7</b>		<b>16.78± 2.0</b>

\* compound identification confirmed by comparison of mass spectra and RI with those of authentic standards; N= number of individuals sampled; O = number of individuals in which that compounds was found; RI = Kowat 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.

## Geographic variation in floral scents

The scatterplot obtained by NMDS ordination based on the Bray-Curtis dissimilarity index (stress = 0.172) is shown in Fig. 2. Overall there was significant variation among locations in the relative proportions of the different compound in odours emitted by receptive figs (PERMANOVA,  $F_{8,45} = 5.0198$ ,  $P = 0.001$ ). The combined results of pairwise comparisons (Table 2) and the NMDS plot suggest that samples of floral odours can be grouped into three geographic clusters, 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 cluster is ambiguous as it is not significantly different from location Ning and from location Wan in the PERMANOVA analysis, and it has an intermediate position between the south-eastern and the southern group in the NMDS plot. Analysis of the similarity percentage (simper) reveals that the quantities of 4 to 6 compounds explained more than 30% of the dissimilarity between locations and relative quantities of both main and minor compounds, involving four to six compounds, explained more than 30% of the dissimilarity between locations.

Table 2

Significance of the differences between locations in the relative proportions of the different VOCs in the floral odours. 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	<b>0.079</b>							
Sui	<b>0.103</b>	0.046						
SCBG	0.022	0.02	0.02					
DHS	0.02	0.02	0.02	<b>0.344</b>				
Nan	0.012	0.02	0.014	0.045	0.02			
Ding	<b>0.081</b>	0.036	0.028	0.022	0.04	0.02		
Wan	0.023	0.012	0.02	<b>0.21</b>	<b>0.588</b>	0.014	<b>0.071</b>	
XTBG	0.022	0.021	0.02	0.02	0.02	0.012	0.03	0.03

## 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 sp1, removing location XTBG, was also significant (Mantel test without XTBG,  $r = 0.4069$ ,  $p < 0.001$ ). A third test performed including only all samples from the locations pollinated by 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 on a single odour 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 (Mantel statistic  $r = 0.3423$ ,

$p = 0.028$ ) and when removing location XTBG (Mantel statistic  $r: 0.3525, p = 0.043$ ). When including only southern and south-eastern continental locations (6 data points) the test became marginally non-significant (Mantel statistic  $r = 0.45, p = 0.072$ ).

## Discussion

The results of the present study provide new insights into the possible drivers of micro-evolution of flower signalling in highly specialized mutualistic plant-pollinator interactions. The geographic pattern of increasing receptive fig odour differentiation with distance is analogous to the pattern of genetic isolation by distance exhibited by the plant. It is strikingly different from the pattern of genetic structuring of the pollinating insects into species and populations [29]. Hence, there was no evidence in favour of the stabilising selection predicted by theoretical models [30], and no evidence in favour of strict sense plant-insect co-evolution for floral odour composition and its perception by the insects. These results demonstrate that, in *F. hirta*, the plant is the driver of floral odour evolution.

Structuring occurs at much larger spatial scales in more dispersive systems such as the one represented by *F. racemosa*. In this species, the plant is structured into large gene pools, covering huge surfaces, and presenting no or almost no spatial genetic structure. Each gene pool is pollinated by a wasp species forming a single population [38]. In that situation, no differences in receptive fig odours were observed between two locations, 900 km apart, corresponding to a same gene pool [34]. Large gene flow could limit geographic variation in floral odour either by limiting drift or by limiting adaptive differentiation of floral odours. A direct consequence of the lack of geographic differentiation of floral odours is that pollinators drifting in the wind above the canopy and dispersing over large distances [39] will still recognise receptive figs: long distance plant gene flow facilitates long distance insect colonisation and reciprocally in a feedback loop. The opposite may be true for the lower dispersal system of *F. hirta*. Differentiation of local floral odours may select for reduced pollinator dispersal, and this will result in stronger spatial genetic structure in the plants, in a self-reinforced process. Such self-reinforced processes may be at the origin of the divergent dispersal strategies of fig-pollinating wasps. Pollinators of monoecious *Ficus* species generally disperse by drifting in the wind above the canopy while pollinators of the spatially more structured dioecious *Ficus* species, including *F. hirta*, are generally not observed in the aerial plankton [39].

The evolutionary stability observed in the monoecious *F. racemosa* could be present in other systems such as some species of yuccas. Indeed, *Yucca filamentosa* floral odours do not vary across its range [40, 41]. Both *Y. filamentosa* and its pollinator *Tegeticula yuccasella* presents limited genetic differentiation across their ranges suggesting strong gene flow, reminiscent of the situation in *F. racemosa* [40, 42]. A broader study of receptive floral odour variation among *Yucca* species is required to uncover patterns. Indeed, the floral odours of *Y. elata* are undistinguishable of those of *Y. filamentosa* while other *Yucca* species produce distinctive odours [43]. However, *Yucca* moth and pollinating fig wasp population dynamics are not comparable. Individual *Yucca* moths may survive in the soil in diapause for 30 years [44], while fig wasps only survive one or two days outside figs [45]. This short life span leads to local pollinating wasp population extinctions during climatic accidents. The distinctive genetic signature of such dynamics is lack of spatial genetic structure combined with small effective population size in species presenting very large populations [46].

*Ficus septica* in the Philippines and Taiwan provides complementary information on patterns of geographic variation [35]. *Ficus septica* is structured into at least three gene pools (Taiwan / Luzon-Negros / Mindanao) and is pollinated by a different black coloured wasp species belonging to the *Ceratosolen bisulcatus* species group in each of the three regions. Nevertheless, a fourth wasp species, belonging to the same species group, *C. jucundus*, has colonised the whole region, bridging the odour differences [35]. This observation suggests that, given sufficient time, receptive fig odour differentiation within host-species does not preclude wasps from expanding their range.

Geographic variation in floral odours has been investigated throughout numerous populations in the facultative brood site pollination mutualism between *Lithophragma spp.* and *Greya* moths. As in *Ficus hirta*, floral odours varied among locations within species, and the difference in floral odours increased with geographic distance (Fig. 4 in [47]). Further, odour distance between populations of two sympatric but not syntopic clades of *Lithophragma* did not correlate with geographic distance, demonstrating that there was no concerted odour evolution between the two clades [47]. These results suggest that in this example too, the plants are the drivers of floral odour evolution and the insects are the followers, despite strong spatial genetic structure in the *Greya* pollinators [48].

Models of mutualism predict the occurrence of stabilising selection, especially when individuals of one species need to interact with many mutualistic individuals of another species [49]. *Lithophragma spp.* individuals, and even more *Ficus spp.* trees, need to interact with many individuals of their insect pollinator species, but the evolution of their floral odours do not conform to prediction. However, because of the short generation time of pollinators comparatively to the plants, the theoretical models do not apply. We suggest that in such systems, local populations of pollinators may rapidly track the slow evolution of local floral odours.

In the case of plant-species sharing pollinators, a simple prediction could be local convergence of floral odours between the different plant species associated with a same set of insects. This has been shown in cases of plants sharing pollinators in Cycads [50], in *Ficus* [51, 52] and in *Glochidion* [53, 54]. We may further predict that plant species involved in different pollination rings in different localities may evolve different attractive odours among localities as suggested by results on Cycads [50]. Results on Cycads suggest within pollinator-species evolution of divergent responses to floral odours, adjusted to the odours produced locally by their hosts [50], as suggested above for *C. jucundus*. In such systems, the pollinators are the mediators of the selection for odour convergence among co-occurring plant species.

## Conclusion

While brood site pollination mutualisms are highly diversified in terms of resources used by the pollinator offspring and in terms of the organs emitting floral odours, their olfactory signalling seems to follow a common set of evolutionary rules. We propose a general framework to investigate the evolution of floral odours within plant species involved in obligate brood site pollination mutualisms. When pollinators are host specific, plants are the drivers of the evolution of floral odours, and pollinating insects, with their large population sizes and short generation times, are the followers. The presence and the intensity of

geographic differentiation of floral odours will depend on spatial genetic structure of the host-plant. When plant species share pollinators, their floral odours converge, through a mimicry process among plants, mediated by the pollinators. In all cases, pollinators have the potential to expand their range by evolving the capacity to recognise floral odours of their host species from new locations. This framework will allow testing predictions on floral odour evolution.

## Methods

### Study system and collection sites

In a previous study samples collected from all the sites investigated here had been included in a broader genetic study of *F. hirta* and its pollinating wasps throughout China to Java. All the plants were shown to belong to a single species presenting clinal genetic variation while the pollinators belonged to a single species group [29]. Reference herbarium samples for that study were deposited at IBSC under numbers 817854–817899. FK formally identified the specimens as *Ficus hirta*, by comparing live plants from locations SCBG, XTBG and the voucher specimens collected by YH throughout the sampling range, with descriptions and with reference herbarium samples, mainly at P, identified by EJH Corner and/or CC Berg. While *F. triloba* and *F. hirta* may sometimes be tricky to distinguish in herbarium material, they are easily distinguished in the field. In this study, sample identification in the field was done either by XD and HY or by XD and FK.

*Ficus hirta* Vahl. (section *Ficus*) is a shrub or small tree approximately 1–3 m high. Figs are produced year-round [55]. Figs develop asynchronously within the tree, and a few plants are sufficient to produce pollinators throughout the year [55, 56]. The production of receptive figs peaks in May–June [55]. In June–July 2019, we collected floral odours from receptive figs in 9 locations distributed across China, with 3 south-eastern locations (Ning, Sha and Sui), 5 southern locations including 2 in Hainan, and 1 south-western location (XTBG) in South Yunnan. (Table 3, Fig. 1). Collections were made on wild-growing plants and we attempted to sample at least 5 individuals per location. All the odour samples collected came from the same season.

Table 3  
Sampling site, corresponding pollinating wasps, GPS coordinates, and No. of samples

Sampling site	Pollinating wasp	GPS coordinates	No. of samples
Ning	sp1 pop1	119.73 E, 26.63 N	5
Sha	sp1 pop1	117.73 E, 26.39 N	5
Sui	sp1 pop 1	114.24 E, 26.41 N	5
SCBG	sp1 pop1	113.35 E, 23.17 N	5
DHS	sp1 pop1	112.54 E, 23.16 N	4
Nan	sp1 pop1	108.39 E, 22.79 N	7
Ding	sp1 pop2	110.36 E, 19.54N	3
Wan	sp1 pop2	110.20 E, 18.77 N	6
XTBG	sp2	101.27 E, 21.92 N	5

### Floral Odour Collection

We used the head-space technique following methods initially developed for *Silene* [57] and that have been successfully used in several *Ficus* species [34, 51, 58, 59]. As the size of receptive figs varied geographically [60], 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  $13 \pm 4$ , for southern locations  $17 \pm 4$ , and for the south-western location  $19 \pm 10$ . Odour collection was performed under natural light between 10:00 am and 5:00 pm, corresponding to the insects' period of maximum activity during our field season.

Receptive figs were enclosed in a polyethylene terephthalate (Nalophan®, Kalle Nalo GmbH, Wursthüllen, Germany) bag for 30 min. Then, air was pulled out of the bag (flow rate:  $200 \text{ mL min}^{-1}$ ) through a Chomatoprobe filter (filled with 1.5 mg of Carbotrap 20–40 and 1.5 mg of Tenax 60–80) in which the volatile organic compounds (VOCs) were trapped. Each collection lasted 5 min. 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 microlitre of a solution of internal standards (n-Nonane and n-Dodecane, 110 ng/ $\mu\text{l}$  of each) was added to each filter, before odour extraction, so that we could control for VOC loss during storage and transport, and estimate the total amount of VOCs emitted by figs. The samples were stored at  $-20^\circ\text{C}$  until VOC analysis.

### 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 ( $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ , 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 Unity 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 minutes, increased from 40 °C to 210 °C at a rate of 5 °C/min and from 220 to 250 °C at 10 °C/min, 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 38 m/z to 350 m/z, and the ionization energy is 70 eV. The 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 (Alkanes standard solution, 04070, Sigma Aldrich®) were used to convert retention times into retention index. VOCs were identified based on matching of the mass spectra with the NIST 98 MS and Adams 2007 libraries, and on confirmation by comparison of their retention index (RI) with libraries and published data [61]. Identification of some compounds was confirmed by comparison of both mass spectra and RI with those of authentic standards (see Table 2).

## Data analysis

Only VOCs that appeared in at least two different odour samples were retained to determine odour profiles. From this VOC set, we calculated the emission rate and the relative composition of each odour profile. The emission rates were the sum of emission rates of all VOCs detected in a given sample, calculated as ng/fig/hour. Relative odour composition was the relative contribution of each VOC to the odour profile, expressed as a percentage.

All statistical analyses were performed with R version 3.5.1 [62]. Emission rate variation among locations were analyzed globally in an ANOVA and testing for deviations from mean value. Divergence in chemical profiles across locations was estimated with non-metric multidimensional scaling (NMDS) in two dimensions, based on a Bray-Curtis similarity matrix, using the package vegan [63]. We used the relative proportions of all the compounds emitted by figs (semiquantitative data). Data were standardized prior to the analysis. 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). To test if the overall variation in chemical composition between groups was significantly different, we carried out permutational multivariate analysis of variance tests (PERMANOVA) based on a Bray-Curtis distance matrix. The chemical distance matrices were calculated with the function "vegdist" after data standardization with "decostand" function, and PERMANOVA were performed using the function adonis in the vegan package [63]. We performed pairwise comparisons after detecting significant interactions with PERMANOVA with the "pair-wise.perm.manova" function in the RVAideMemoire package [64], and we used the false discovery rate method for multiple test p-value correction. Similarity percentage, simper [65], 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 potential relationships between chemical distance and geographic distance, we performed Mantel tests. We used the chemical matrices generated above. The Mantel test requires that the matrices being tested have the same samples, so we calculated geographic distances using our GPS dataset that were represented in the floral dataset. Geographic distances were calculated using our GPS data. 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 [47]. This method has been used as a drastic way to avoid the risk of pseudo-replication associated with using several data points from a single location as independent points [47].

## Abbreviations

VOCs: volatile organic compounds; XTBG: Xishuangbanna Tropical Garden, the Chinese Academy of Sciences; ANOVA: Analysis of Variance; NMDS: Non-metric multi-dimensional scaling; PERMANOVA: Permutational Multivariate Analysis of Variance; PACE: Platform for Chemical Analyses in Ecology; GC: gas chromatograph; FID: Flame Ionization Detector; CIS: Cold Injection System; RI: retention index; GPS: Global Position System

## Declarations

### Ethics approval and consent to participate

The work carried out at South China Botanical Garden, Chinese Academy of Sciences, and Xishuangbanna Tropical Garden, Chinese Academy of Sciences, was under the direction of the co-authors belonging to the staff of these gardens. All the other sampling sites were not privately owned or protected, and field sampling did not involve protected species. Therefore, sampling was not subject to authorisation.

### Consent of publication

Not applicable.

### Availability of data and material

All data generated or analysed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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#### Author's contributions

XD collected samples, analysed the data and wrote the manuscript; BB analysed the data; YQP and YC helped collecting samples; HY, KF and MP organized the work and wrote manuscript; all authors have read and approved the manuscript

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Not applicable.

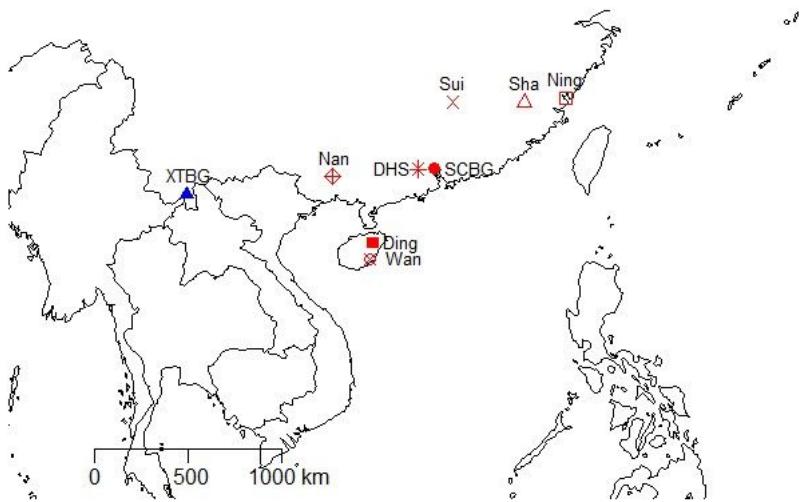
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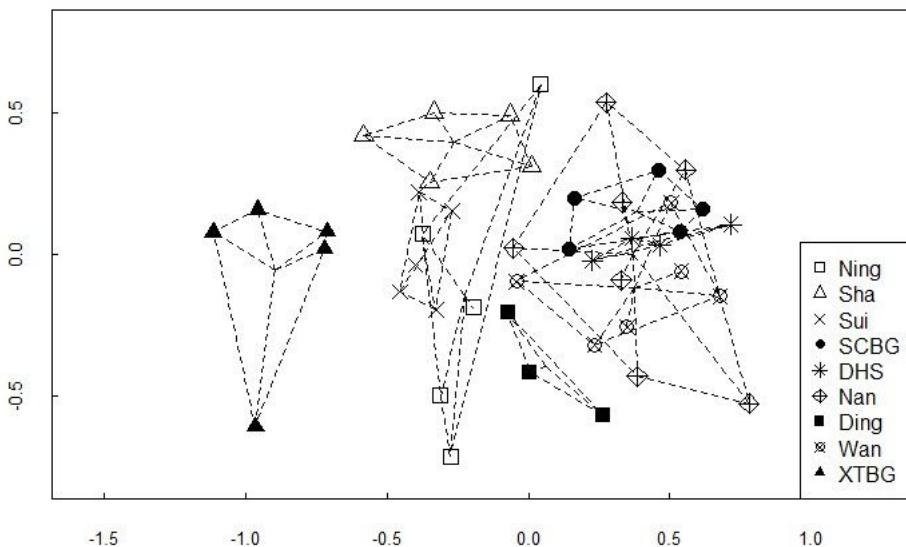
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## Figures



**Figure 1**

Receptive fig odour collection 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. Map drawn with the package 'rworldmap' using the public domain Natural Earth map dataset <https://naturalearthdata.com>. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



**Figure 2**

Non-metric multi-dimensional scaling representation 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 visualised: 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.