Spécificité des pollinisateurs et des odeurs des figues, variation et conséquences pour la diversification dans le mutualisme Ficus-pollinisateurs

Xiaoxia Deng

THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Écologie et Biodiversité

École doctorale GAIA

Unité de recherche CEFE

Spécificité des pollinisateurs et des odeurs des figues, variation et conséquences pour la diversification dans le mutualisme *Ficus*-pollinisateurs

Présentée par Xiaoxia DENG
Le 22/09/2021

Sous la direction de Finn KJELLBERG

Devant le jury composé de

Thibaud DECAËNS, professeur, Université de Montpellier	**président du jury**
Catherine FERNANDEZ, professeur, Aix Marseille Université	**Member du jury**
Finn KJELLBERG, directeur de recherches CNRS, Université de Montpellier	**directeur de thèse**
Marc GIBERNAU, chargé de recherches CNRS, Université de Corse	**rapporteur**
Rodrigo Augusto Santinelo PEREIRA, professeur associé, Universidade de São Paulo	**rapporteur**
Magali PROFFIT, chargée de recherche CNRS, Université de Montpellier	**Member du jury**

UNIVERSITÉ DE MONTPELLIER

Acknowledgments/ Remerciements

Life is like a journey, in a different journey, and we will meet different people. I am so lucky to meet you on this part of the journey. Many thanks to all those wonderful people that made the past four-year journey a difference.

I would like to express my heartfelt thanks to my two supervisors. Without their assistance and dedicated involvement in every step throughout the process, the work would have never been accomplished. I am very grateful for your support and understanding in the past four years. Prof. Finn Kjellberg, for his continual support, advice, and expertise throughout this study. No matter what difficulties I have, as long as I ask him for help, he will give a prompt response, which makes me feel relieved. To my co-supervisor Dr. Magali Proffit for her technical knowledge and expertise on chemical analysis. From the sample collection, chemical analysis, insect behavior experiment, and data analysis. She assisted me with the statistical analysis in the past four years and was very patient with my knowledge gaps in the area. She was very patient to teach everything, and thank you again for being so thoughtful.

I would like to take this opportunity to thank all laboratory members for their help and support in daily life and the study. I also thank my parents for the unceasing encouragement, support, and attention. I am also grateful to my partner who supported me through this venture.

I am very grateful to the China Scholarship Committee (CSC) for its financial support during my study at CEFE-CNRS (Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), UMR CNRS). I also would like to thank the members of the Thesis Defense Committee,

Prof. Jean-Yves Rasplus, Prof. Patrice DAVID, Prof. Mathilde Dufaÿ, and Prof. Simon T. Segar. Thank you for your advice on my work.

Contributions of collaborators

I would like to thank Dr. Ting Zhang, Yufen Cheng,Yaolin Liao,Juncheng Guo,Guiyuan Wu,Prof. Gang Wang, Prof. Yan-Qiong Peng, Prof. Hui Yu, who helped me in field sampling.

Résumé

L'un des plus grands défis de l'étude de la coévolution, voire de la biologie en général, est de comprendre comment des traits complexes aux fonctions multiples se sont diversifiés et co- diversifiés à travers les aires géographiques chez des lignées en interaction. Le mutualisme de pollinisation entre les *Ficus* et leurs guêpe pollinisatrices offre une opportunité unique d'examiner les processus fondamentaux de la coévolution, à savoir l'adaptation réciproque où les partenaires en interaction sont les agents de sélection. Dans cette thèse, nous explorons les forces qui gouvernent la variation géographique de la communication plante-insecte et leurs conséquences biologiques, en nous concentrant sur la relation entre les odeurs de figues réceptives, la structure génétique et le comportement de la plante hôte et de la guêpe pollinisatrice. Nous combinons des résultats sur les odeurs des figues, l'écologie de la pollinisation et la génétique des populations chez trois espèces de *Ficus* largement distribuées, à savoir *Ficus hirta*, *Ficus hispida* et *Ficus triloba.* Les résultats montrent que la variation géographique des odeurs réceptives des figues correspond à la structure génétique de la plante et non à celle du pollinisateur. La variation géographique des odeurs de figues réceptives affecte la capacité de l'insecte à localiser des figues réceptives non locales car les insectes sont adaptés aux odeurs locales. Néanmoins, les signaux olfactifs émis par des espèces de *Ficus* apparentées peuvent se chevaucher de sorte que dans certaines localités, les pollinisateurs peuvent être attirés par des figues réceptives de figuier non hôtes. Cette thèse explore des aspects jusqu'ici non étudiés du mutualisme entre les *Ficus* et leurs guêpes pollinisatrice et fournit de nouvelles perspectives pour l'étude de cette association emblématique et écologiquement importante. De manière plus générale, les résultats donnent un aperçu des processus évolutifs et coévolutifs qui régissent la communication plante-insecte dans les mutualismes de pollinisation des sites de ponte, un système de pollinisation dont la fréquence chez les plantes est largement sous- estimée.

Abstract

One of the greatest challenges in the study of coevolution, indeed, for biology in general, is to understand how complex traits of multiple functions have diversified and codiversified across interacting lineages and geographic ranges. The fig-wasp pollination mutualism provides a unique opportunity to examine fundamental processes of coevolution, namely, reciprocal adaptation where interacting partners are the agents of selection. In this thesis, we explore the forces governing geographic variation in plant-insect communication and their biological consequences, focusing on the relationship between receptive fig odor and host plant and pollinating fig wasp genetic structure and behaviour. Integrating inflorescence odors, pollination ecology and population genetics in three widely distributed *Ficus* species, namely *Ficus hirta*, *Ficus hispida*, and *Ficus triloba*, the results show that geographic variation in receptive fig odors corresponds to plant genetic structure and not to pollinator genetic structure. Geographic variation of receptive fig odors affects the insect's capacity to locate non-local receptive figs as the insects are adapted to the local odors. Nevertheless, the olfactive signaling of related *Ficus* species may overlap so that in some locations, pollinators may be attracted by non-host receptive figs. This dissertation explores previously unstudied aspects of the fig and fig wasp mutualism and can provide new perspectives for future study of this iconic and ecologically important association. More generally, the results provide insights into co-evolutionary processes governing plant-insect communication in brood site pollination mutualisms, a pollination system whose frequency in plants is largely underestimated.

Table des matières / Table of Contents

Introduction générale

Un défi majeur en biologie évolutive est de comprendre comment des traits complexes aux fonctions multiples se sont diversifiés et ont codiversifiés à travers des lignées et des aires géographiques en interaction (Friberg et al. 2019). Il existe plus de 300 000 espèces de plantes vasculaires dans le monde, dont 88,7 % sont des angiospermes (Christenhusz et Byng 2016). Les interactions entre les plantes et les insectes phytophages sont parmi les plus diverses sur Terre (Thompson et al. 2005, 2013, 2017). Les interactions plantes-insectes pollinisateurs revêtent une importance particulière car 90 % des angiospermes sont pollinisées par des insectes, tels que des abeilles, des coléoptères, des papillons de jour et de nuit et des mouches (Ollerton et al. 2011). Bien que de nombreux traits complexes façonnent ces interactions, l'odeur florale joue généralement un rôle clé dans l'établissement du flux génétique chez les plantes et dans la spécialisation de nombreux insectes (Raguso 2008, 2009, Schiestl 2015).

La signalisation florale en général est essentielle dans toute interaction plante-pollinisateur, et consiste souvent en une combinaison d'indices visuels (par exemple, la couleur et la forme) et de stimuli olfactifs (odeurs, émises par exemple par les pétales ou le pollen) qui suscitent des réponses chez les pollinisateurs (Lunau 1995, Dobson et Bergström 2000, Chittka et Raine 2006, Raguso 2008). Les fleurs pollinisées par les insectes émettent des quantités bien plus élevées de composés organiques volatils (COV) et présentent une plus grande diversité de ces composés que les fleurs pollinisées par le vent (Farré-Armengol et al. 2015). Bien que les indices visuels soient importants pour attirer les pollinisateurs, ils ne sont souvent pas aussi spécifiques que les indices olfactifs (Kunze et Gumbert 2001, Wright et al. 2009). Par exemple, des tests de terrain ont montré que les signaux olfactifs émis par les figues, en l'absence de tout indice visuel, sont suffisants pour attirer leurs pollinisateurs spécifiques (Hossaert-McKey et al. 1994). Les substances volatiles des plantes sont les acteurs invisibles

de la course aux armements entre plantes et insectes (Binyameen et al. 2021). Des volatiles particuliers sont les médiateurs de certaines interactions entre les plantes et les pollinisateurs (Schiest et al. 2003, Chen et al. 2009), mais les bouquets d'odeurs florales complexes sont plus courants (Knudsen et al. 2006). Les odeurs florales peuvent être impliquées dans l'attraction des pollinisateurs, mais aussi dans l'incitation à l'atterrissage, à l'alimentation et, dans certains cas, à l'accouplement et à la ponte sur la fleur, selon l'association fleur-insecte (Dobson 2006, Bouwmeester et al. 2019). Malgré leur importance, le rôle des odeurs florales dans la spéciation et l'évolution des plantes reste mal compris (Whitehead et Peakall 2009).

Systèmes de pollinisation généralistes mutualistes

Parmi les exemples typiques de familles dont les espèces relèvent d'un syndrome de pollinisation généraliste figurent les Rosaceae, Apiaceae, Arecaceae et Ranunculaceae, où les fleurs sont pollinisées par une diversité d'insectes (coléoptères, mouches, abeilles, papillons) qui se nourrissent du nectar et du pollen, présentés dans des fleurs ouvertes et généralement en forme de bol, d'ombelle ou d'inflorescences cylindriques ou globulaires (Dobson 2006). Bien que souvent de nombreux insectes sont des pollinisateurs, certains d'entre eux peuvent être plus importants et donc influencer la sélection sur la chimie des odeurs bien plus fortement que d'autres. Des études détaillées sur la pollinisation sont nécessaires pour découvrir la contribution relative des différents pollinisateurs (Pelletier et al. 2001, Kandori 2002, Zych 2002, Jürgens 2004). En général, les odeurs florales ne présentent pas de modèle de patron de composition particulier, si ce n'est qu'elles consistent en six classes chimiques , à savoir les dérivés d'acides gras, les terpénoïdes, les phénylpropanoïdes/benzénoïdes, une classe prédominant souvent sur les autres dans une odeur florale donnée (Borg-Karlson et al. 1993, Robertson et al. 1993, Tollsten et Bvstedal 1994, Tollsten et al. 1994, Robertson et al. 1995, Knudsen et al. 2001, 2006). La causalité est difficile à établir pour l'évolution des odeurs florales chez les plantes à pollinisation généraliste. Les

variations des odeurs florales entre les populations suggèrent une adaptation au cortège local de pollinisateurs (Dobson 2006, Breitkopf et al. 2013).

Mutualismes de pollinisation des sites de ponte

Les mutualismes de pollinisation des sites de ponte (également appelés mutualismes de pollinisation de nurserie) sont des interactions dans lesquelles des insectes spécialisés pollinisent en tant qu'adultes et se nourrissent en tant que larves de structures florales de leurs plantes hôtes. Ils ont été des systèmes d'étude influents pour l'étude de la co-évolution bien que les auteurs restreignent souvent la définition au sous-ensemble d'interactions dans lesquelles les larves des pollinisateurs se nourrissent de graines (Hembry et Althoff 2016).

Les mutualismes de pollinisation des sites de pontes sont très répandus. Ils sont présents chez de nombreuses espèces, par exemple dans le genre *Macaranga* (20, probablement 40 espèces ; Yamasaki et al. 2015), dans le genre *Yucca* (environ 50 espèces ; Pellmyr 2003), dans le genre *Ficus* (environ 820 espèces), dans les Cyclanthaceae (230 espèces ; Franz 2007), dans les Cycadales (beaucoup des 353 espèces ; Suinyuy et al. 2010, Toon et al. 2020), dans les Araceae pollinisées par les Drosophilidae (environ 540 espèces ; Gibernau 2016, Kawakita et al. 2019), chez les Phyllantheae pollinisées par les papillons *Epicephala* (quelque 750 espèces, dont des espèces de l'ancien et du nouveau monde ; Kawakita 2010, Kawakita et al.
2019), chez certaines Arecaceae (palmiers), mais probablement aussi chez de nombreuses Moraceae dont la plupart des espèces de Castillae (59 espèces ; Sakai 2001), Dorsteniae (138 espèces ; Araújo et al. 2017) et Artocarpeae (70 espèces ; Sakai et al. 2000). Des nombreuses données montrent que les odeurs émises à l'anthèse des fleurs jouent un rôle central pour attirer les pollinisateurs dans la plupart des mutualismes de pollinisation des sites de ponte (Miyake et Yafuso 2005, Dotterl et al. 2006, Ibanez et al. 2010, Svensson et al. 2010, Svensson et al. 2011, Proffit et al. 2020).

Ces odeurs peuvent être produites par les fleurs mais aussi par divers organes comme les

bractées et les bractéoles des inflorescences (Souza et al. 2015, Yamasaki et al. 2015, Hu et al. 2020) ou même par les feuilles (Dufaÿ et al. 2003).

Que savons-nous de la variation intra-spécifique de l'odeur émise à l'anthèse par les inflorescences dans de tels systèmes ? Que peut-elle nous apprendre sur les processus évolutifs impliqués ? Dans le système du yucca et du papillon du yucca, la différenciation génétique entre les populations de *Tegeticula yuccasella* sur des hôtes alternatifs est faible (Leebens-Mack 2004), et aucune différenciation dans l'odeur des fleurs n'a été observée (Svensson et al. 2005). Ce système suggère un flux génétique élevé médié par les papillons entre les sites et la conservation de la composition de l'odeur entre les populations ayant des pollinisateurs différents. Chez les Cycadales, il existe une forte variation entre populations dans l'odeur des cônes d'*Encephalartos villosus*, mais les assemblages de pollinisateurs ne diffèrent pas entre les différentes populations, ce qui suggère que ces modèles ont été produits par coévolution ou dérive, plutôt que par des déplacements de pollinisateurs (Suinyuy et al. 2012). En outre, dans un contexte de forte variation des odeurs florales entre les populations au sein des espèces de *Lithophragma* (Saxifragaceae), il y avait une forte relation négative entre la similarité entre paires de populations (distance 1-Bray-Curtis) et leur distance géographique au sein de la large aire de distribution de *L. parviflorum* (Thompson et Cunningham 2002, Friberg et al. 2019). Par conséquent, les mutualismes obligatoires de pollinisation des sites de ponte fournissent des systèmes modèles très contraints, dans lesquels nous pouvons étudier comment les plantes, leurs insectes associés et leurs odeurs florales se sont diversifiés ou codiversifiés. Dans ce travail, nous explorons ces questions en profitant du système bien connu des figuiers-guêpes des figues comme système d'étude.

Mutualisme entre figues et guêpes des figues

Il existe quelque 820 espèces de *Ficus* réparties dans les régions tropicales et subtropicales du monde. Elles ont une relation de pollinisation réciproquement obligatoire avec des guêpes des

figues (Chalcidoidea, Agaonidae), qui sont petites (1-2 mm) et de courte durée de vie (1-2 jours de vie adulte). En général, une espèce d'agaonidé est associée à une seule espèce de *Ficus*, alors que souvent un complexe d'espèces de guêpes Agaonidae pollinise une espèce de *Ficus* (Darwell et al. 2014, Bain et al. 2016, Rodriguez et al. 2017, Yu et al. 2019, Cook et Rasplus 2003, Cornille et al. 2012, Wang et al. 2016, Rodriguez et al. 2017).

L'inflorescence des *Ficus*, la figue, est une inflorescence refermée en urne dont l'intérieur est tapissé de fleurs femelles uniovulées et de fleurs mâles. Les guêpes déposent leur ponte dans les fleurs femelles, pondant un œuf par fleur, et assurent la pollinisation. La larve de guêpe se développe à l'intérieur de la fleur transformée en galle en se nourrissant d'albumen dont le développement a été induit par la pollinisation ou par la guêpe. Les guêpes mâles émergent d'abord de leurs galles et s'accouplent avec les femelles. Ensuite, les femelles émergent dans la cavité de la figue, se chargent de pollen et partent à la recherche d'un arbre portant des figues réceptives. Chez les espèces de *Ficus* monoïques, c'est-à-dire chez environ la moitié des espèces, les guêpes et les graines se développent côte à côte dans les figues. Chez les espèces fonctionnellement dioïques, les guêpes qui pénètrent dans les figues des arbres femelles, pollinisent, mais ne parviennent pas à pondre. Cela est dû au fait que les longs styles par lesquels les guêpes introduisent leur ovipositeur ne lui permettent pas d'atteindre la limite entre le tégument interne et le nucellus où l'œuf est normalement pondu. Par conséquent, les figues des arbres femelles produisent des graines mais pas de guêpes. Lorsqu'une guêpe pénètre dans une figue d'un arbre fonctionnellement mâle, elle pondra dans les fleurs car les styles sont beaucoup plus courts. Chez les espèces de guêpes à pollinisation active, le pollen est déposé précisément dans les stigmates dans lesquels les guêpes pondent, tandis que chez les espèces à pollinisation passive, le pollen ne germe pas dans les arbres fonctionnellement mâles. Par conséquent, les arbres mâles produisent des guêpes, du pollen mais pas de graines.

En général, les espèces de *Ficus* monoïques sont des arbres hémipiphytes (ils germent en tant qu'épiphytes, et leurs racines se développent jusqu'à atteindre le sol) ou de grands arbres autoportants. À l'inverse, les espèces de *Ficus* dioïques sont principalement des lianes, des arbustes et des petits arbres.

Le développement des figues est généralement synchrone sur toute la couronne d'un arbre, ce qui implique que les guêpes émergeant d'un arbre volent vers un nouvel arbre localisé par olfaction (Grison et al. 1999). Les données phénologiques et la diversité des donneurs de pollen montrent que les pollinisateurs des espèces monoïques de *Ficus* se dispersent couramment à 5-14 km (Bronstein et al. 1990, Nason et al. 1998, Harrison 2003). Les guêpes non pollinisatrices qui se développent également dans les ovules des figues mais sans fournir de services de pollinisation sont également attirées olfactivement par les figues (Bronstein et al. 1990, Proffit et al. 2007). Il s'agit notamment d'insectes galligènes et de parasitoïdes.

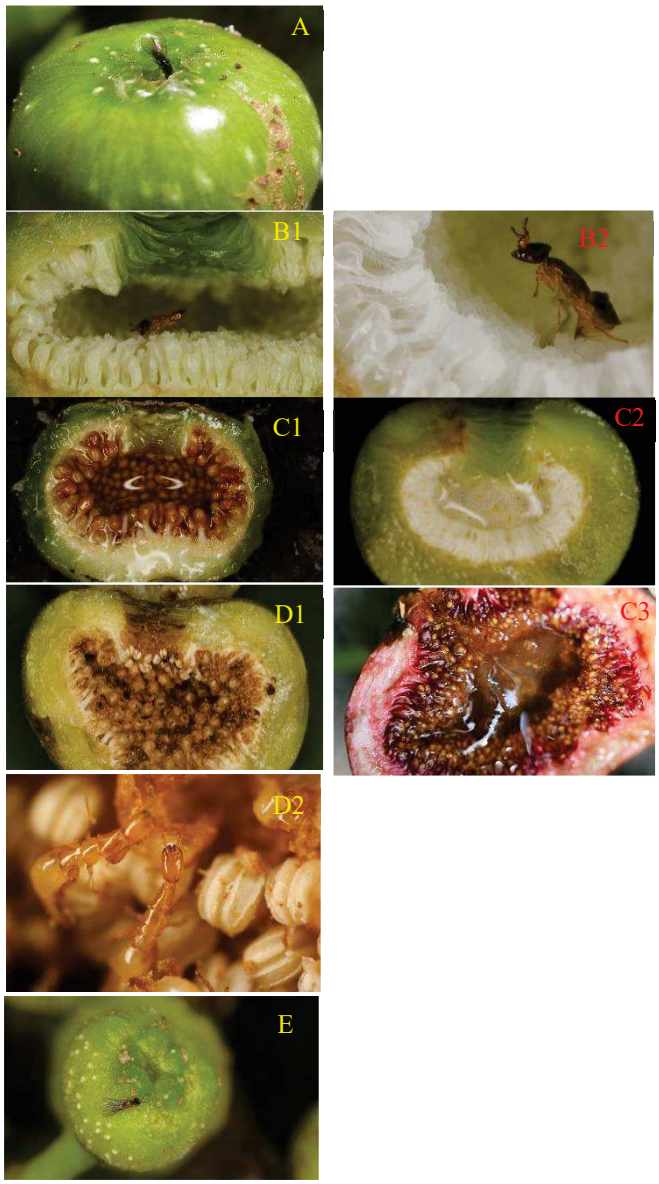


Figure 1.1 Cycle de vie des guêpes chez une espèce de Ficus fonctionnellement dioïque. (A) Une fondatrice pénétrant l'ostiole d'une figue réceptive. (B1) En entrant, les fondatrices pondent des œufs dans la figue mâle. (B2) Les fondatrices essaient de pondre des œufs dans la figue femelle. Le stigmate forme un tube dont l'extérieur est recouvert de papilles permettant une germination facile du pollen. (C1) Dans la figue mâle, les larves de guêpes se développent dans les fleurs femelles transformées en galles et les autres fleurs restent stériles. (C2) Les graines se développent dans la figue femelle. (D1) Les guêpes émergent de leurs galles. (D2) Les mâles sortent en premier et s'accouplent avec les femelles qui sont encore dans leurs galles. (D3) Les figues femelles, qui ne contiennent pas de guêpes, n'ont pas de phase mâle et murissent directement. Ces figues mûres deviennent molles et charnues. E. Une guêpe femelle porteuse de pollen qui s'est dispersée depuis son arbre natal, sur le point de pénétrer dans une figue réceptive sur un autre arbre.

Figues et odeurs de figues

Les composés organiques volatils (COVs) émis par les figues réceptives ont été étudiés chez des espèces de *Ficus* appartenant principalement à quatre sous-genres sur six, à savoir *Urostigma*, *Sycomorus*, *Sycidium* et *Ficus* (Gibernau et al. 1997, Grison et al. 1999, Hossaert- McKey et al. 2016, Zhang et al. 2017). Le rôle des COVs dans l'attraction des pollinisateurs a été démontré chez certaines espèces (Chen et al. 2009, Proffit et al. 2009, Chen et Wu 2010, Wang et al. 2013, Wang et al. 2016, Zhang et al. 2016, Souto-Vilarós et al. 2018). Des études

sur la composition des odeurs réceptives des figues ont maintenant été menées chez plus de 30 espèces de *Ficus* (Gibernau et al. 1997, Grison et al. 1999, Delle-Vedove et al. 2011, Cornille et al. 2012, Soler et al. 2012, Hossaert-McKey et al. 2016, Zhang et al. 2017, Souto-Vilarós et al. 2018). Dans ces études, généralement seuls quelques composés ont été détectés dans chaque espèce (1 à 50 composés communs pour chaque espèce), et il y avait généralement 1 à 3 composés dominants qui étaient beaucoup plus abondants que les autres COV. Les COV se composent principalement de terpènes (monoterpènes et sesquiterpènes), suivis de benzénoïdes, de dérivés d'acides gras et de composés shikimiques. Les composés dominants, cependant, sont le plus souvent un ou plusieurs des suivants : (E)-β-ocimène, β-caryophyllène, 1,8-cinéole, linalol, alcool benzylique, α-copaène et limonène. À cette liste, on peut ajouter certains composés constituants majoritaires d'une odeur, mais qui ont été détectés chez une seule espèce, comme les dérivés oxygénés du linalol, le 6-méthyl-5-heptène-2-ol et le 4-méthylanisole (Gibernau et al. 1997, Gu et al. 2012, Hossaert-McKey et al. 2016).

Les études précédentes indiquent que la médiation chimique joue un rôle clé dans la rencontre des partenaires, permettant aux guêpes de localiser les plantes hôtes et de trouver des récompenses florales, c'est-à-dire des sites de reproduction (Grison-Pigé et al. 2002, Proffit et al. 2007, Proffit et al. 2009, Hossaert-McKey et al. 2010). Une perspective complémentaire est que la spécialisation dans l'association figue-guêpe des figues peut résulter de l'intégration de plusieurs filtres, dont les odeurs spécifiques à l'espèce, les composés non-volatiles présents sur la cuticule de la figue et la taille des ostioles, dans une série de filtres sensorio-mécaniques qui excluent les visiteurs non adaptés (Noort et Compton 1996, Gibernau et al. 1998, Raguso 2008). Certaines données montrent que les odeurs émises par des espèces de figues étroitement apparentées peuvent être très similaires (Grison et al. 1999, Wang et al. 2016). De plus, un cas d'évolution convergente de l'odeur florale a été rapporté dans le système figuier- guêpe des figues. En Afrique du Sud, deux lignées sœurs de guêpes, *Elisabethiella*

stuckenbergi et *E. socotrensis*, pollinisent *Ficus natalensis* mais *E. stuckenbergi* pollinise aussi régulièrement *Ficus burkei*. Les deux espèces de guêpes sont présentes ensemble sur des individus de *F. natalensis* dans tout le KwaZulu-Natal. Les mélanges volatils floraux émis par *F. natalensis* au KwaZulu-Natal étaient similaires à ceux émis par *F. burkei* et différents de ceux produits par d'autres espèces de *Ficus* africains sympatriques. La similitude des odeurs de figue suggère une convergence évolutive pour attirer la même espèce de guêpe (Cornille et al. 2012).

Odeurs de figues et coévolution plante-insecte

Dans le système hautement spécialisé de la figue et de la guêpe des figues, les données montrent que la communication spécifique à l'espèce est assurée soit par un canal privé de composés peu communs ou uniques (Chen et al. 2009), soit, plus fréquemment, par un mélange de quelques composés dans lequel leurs proportions relatives sont déterminantes (Gibernau 1997, Proffit et al. 2020). La signature olfactive spécifique est spécifiquement ciblée par les pollinisateurs. Ainsi, la reproduction (et, par conséquent, la survie des espèces) de la guêpe et du figuier sont intimement liées. Si les odeurs florales et les réponses des insectes sont localement coadaptées, ces indices chimiques pourraient fonctionner comme d'importants traits d'isolement entre les populations, et donc comme des moteurs de diversification (Suinyuy et al. 2015).

La variation géographique de l'odeur florale a été documentée dans le système figuier-guêpe des figues (Soler et al. 2011, Rodriguez et al. 2017) ainsi que dans d'autres mutualismes de pollinisation des sites de ponte (Suinyuy et al. 2012, 2015, Cruaud et al. 2017). Par exemple, *Ficus racemosa*, une espèce de *Ficus* extrêmement répandue, et ses guêpes pollinisatrices étaient structurées en quatre grandes populations vicariantes d’arbres qui correspondent à des espèces différentes chez les insectes, situées en Inde, en Chine-Thaïlande, à Bornéo et en Australie (Bain et al. 2016). L'analyse chimique a révélé qu'elle produit une

même odeur de la Chine à la Thaïlande mais une odeur différente en Inde (Soler et al. 2011). Cette structuration géographique correspond à une co-structuration entre espèces pollinisatrices et génétique végétale, mais aussi à des barrières climatiques et géographiques. À l'inverse, chez *F. septica*, un petit arbre distribué dans toute l'Asie du Sud-Est insulaire, l'odeur de la figue réceptive varie selon les îles (Rodriguez et al. 2017). Ce schéma est similaire à la co-structuration des pools génétiques des plantes et des espèces pollinisatrices. Cependant, les odeurs réceptives des figues dans les Negros différaient de celles de Luzon, alors que les plantes appartenaient au même pool génétique et étaient pollinisées par la même espèce de guêpe pollinisatrice. Par conséquent, avons-nous une évolution au niveau local ou au niveau d'une ile de la communication plante-insecte ? De même, nous n'avons aucun indice sur les mécanismes évolutifs responsables de la diversification des odeurs. Comment comprendre les forces qui régissent la variation géographique de la communication plante-insecte et leurs conséquences biologiques ?

Dans les deux cas évoqués ci-dessus, *F. racemosa* et ses pollinisateurs sont des espèces à forte dispersion, limitant la différenciation (Bain et al. 2016), tandis que *F. septica* et ses pollinisateurs présentent un flux génétique réduit entre îles (Rodriguez et al. 2017).

Par conséquent, nos hypothèses sont les suivantes :

Lorsque le flux génétique est élevé, il n'y a pas de réponse à la sélection locale, les odeurs florales restent homogènes sur de longues distances (chez les *Yucca*, *F. racemosa*).

Lorsque les espèces végétales présentent un flux génétique réduit et partagent des pollinisateurs, leurs odeurs florales convergent avec celles des autres plantes avec lesquelles elles partagent des pollinisateurs (chez les cycadales, *Encephalartos*, chez les *Ficus*, *F. natalensis-F. burkei*, *F. auriculata-F. oligodon*).

Lorsque les pollinisateurs sont spécialisés sur un seul hôte, et que la dispersion des gènes est limitée, à la fois chez les plantes et chez les insectes, l'évolution des inflorescences réceptives peut impliquer :

une coévolution locale plante-insecte,

ou l'évolution de l'odeur de la plante, les pollinisateurs s'adaptant à cette variation,

ou l'évolution de biais sensoriels des insectes, les odeurs des plantes évoluant en réponse à ces biais.

Pour faire la part des choses entre les options 1, 2 et 3, nous étudions, dans un cadre continental, trois espèces asiatiques de *Ficus* dioïques très répandues et présentant un flux génétique faible à intermédiaire : *Ficus hispida*, *Ficus hirta* et *Ficus triloba*. *Ficus hispida* appartient au sous genre *Sycomorus*, alors que *F. hirta* et *F. triloba* sont deux espèces sœurs, mais de taille différente, appartenant au sous genre *Ficus*, section *Eriosycea*.

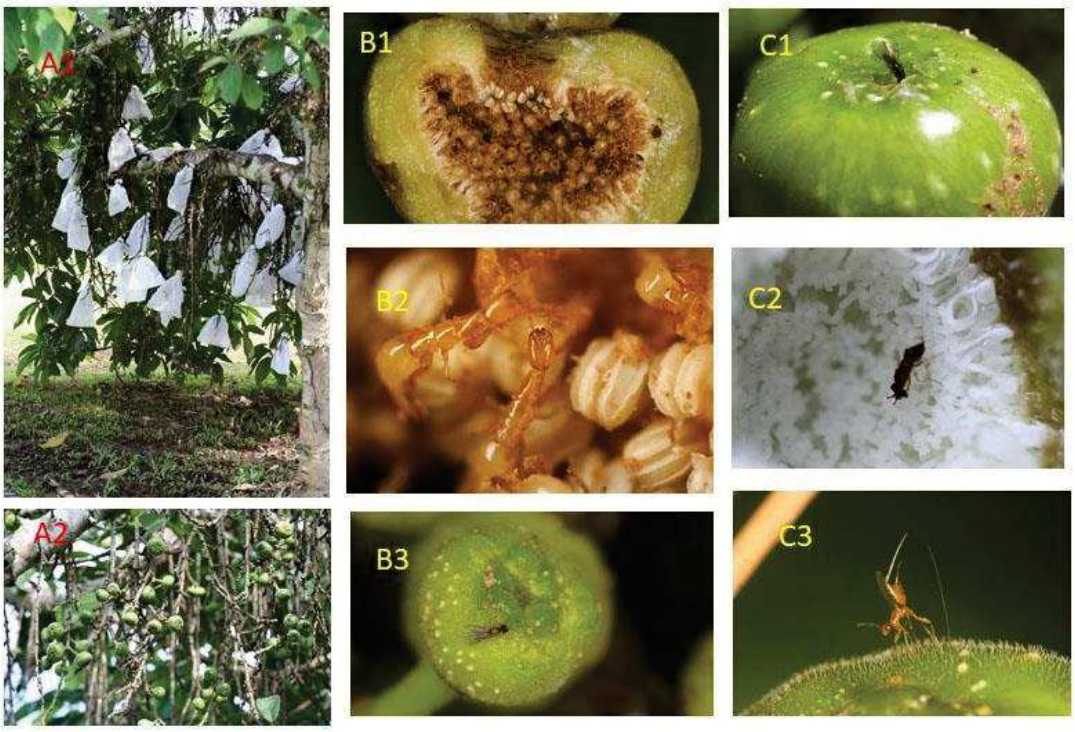


Figure 1.2 *Ficus hispida* et ses guêpes pollinisatrices en Chine (A1) Un arbre de *Ficus hispida* (A2) Branches chargées de figues. (B1) Les guêpes sortent de leurs galles. (B2) Guêpes mâles. (B3) Guêpe femelle. (C1) Une guêpe femelle pénètre dans une figue réceptive par l'ostiole. (C2) Une guêpe femelle pollinisant tout en essayant de pondre des œufs dans une figue femelle. (C3) Une guêpe femelle non pollinisatrice pondant à travers la paroi de la figue.

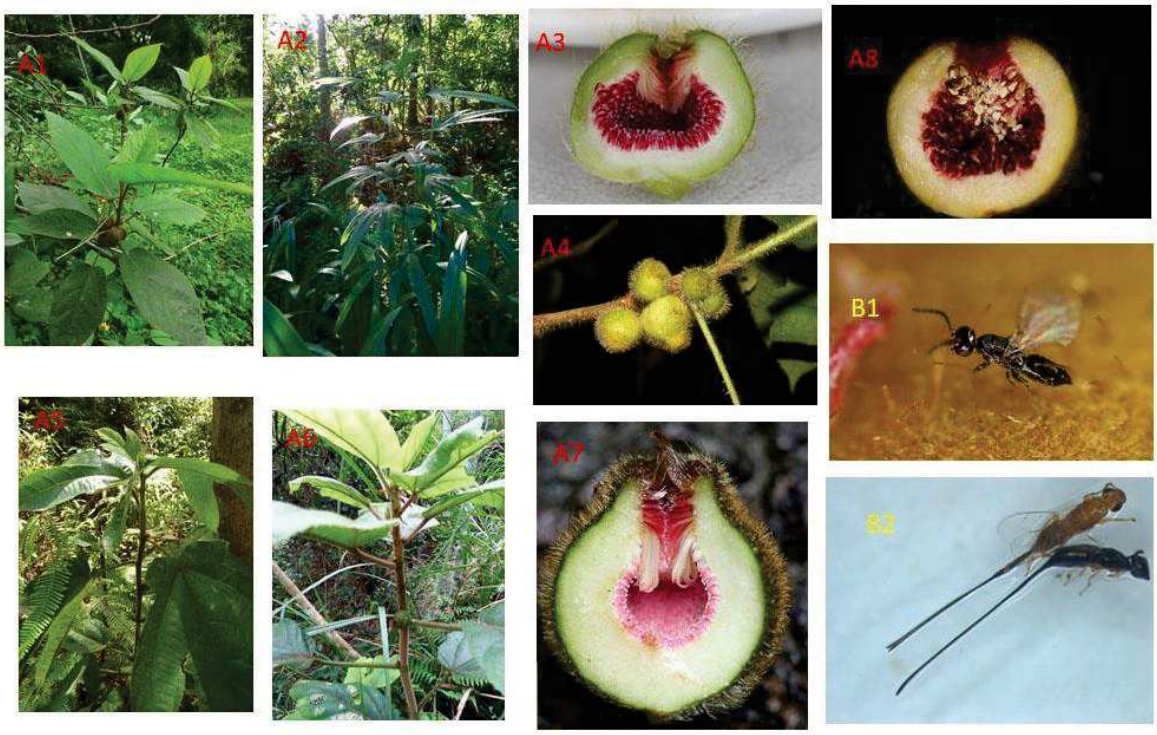


Figure 1.3 *Ficus* hirta et ses guêpes pollinisatrices en Chine. (A1) *Ficus hirta* poussant à Guangzhou. (A2) *Ficus hirta* poussant à Hainan. (A5) *Ficus hirta* poussant dans la province de Jiangxi. (A6) *Ficus hirta* poussant dans la province de Fujian. (A3) Figue réceptive dans le sud de la répartition de *F. hirta*. (A7) Figue réceptive dans le nord de sa distribution. (A4) Une branche portant des figues. (A8) Les guêpes sortent de leurs galles. (B1) Une guêpe femelle pollinisatrice. (B2) Guêpes femelles non pollinisatrices.

Figure 1.4 *Ficus triloba* et sa guêpe pollinisatrice (A1) Un arbre de *Ficus triloba* (A2) Une branche chargée de figues (B1) Stigmate dans une figue femelle réceptive (B2) Une guêpe pollinisatrice femelle.

II Présentation synthétique des questions abordées et des résultats

Les résultats détaillés, les références et les discussions spécifiques sont fournis dans les manuscrits des articles. L'objectif de ce chapitre est de présenter un aperçu général de la manière dont les différents résultats obtenus et/ou analysés au cours de mon doctorat forment un ensemble cohérent. Ils apportent des réponses sur la façon dont les odeurs de figues réceptives varient géographiquement au sein d'une même espèce et entre espèces étroitement apparentées, sur l'origine évolutive de cette variation et sur ses conséquences biologiques.

Afin de comprendre les forces qui régissent la variation géographique de la communication plante-insecte et leurs conséquences biologiques, nous devons établir la structure génétique spatiale de la plante hôte et de ses insectes pollinisateurs. Nous l'avons fait pour *Ficus hirta* et ses guêpes pollinisatrices dans Hui Yu, Enwei Tian, Linna Zheng, Xiaoxia Deng, Yufen Cheng, Lianfu Chen, Wei Wu et al. "*"Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation." Molecular Ecology* 28, no 9 (2019) : 2391-2405.

Des données antérieures ont montré que *Ficus hirta* Vahl, une espèce largement distribuée, présente un modèle de structure génétique spatiale suggérant un isolement génétique par la distance à travers l'Asie du Sud-Est continentale. Cependant, on peut s'attendre à un taux de spéciation plus rapide chez les guêpes du figuier, par rapport à leurs hôtes, étant donné leur temps de génération beaucoup plus court. Deux morphotypes de guêpes pollinisatrices du figuier morphologiquement distincts, l'un à Java et l'autre à Hong Kong, ont été décrits à partir des figues de *F. hirta*, suggérant plus d'une espèce de guêpe pollinisatrice. Nous avons donc testé l'hypothèse que *F. hirta* supporte plusieurs espèces de pollinisateurs dans son aire de répartition. Nos résultats confirment qu'à travers son aire de répartition, *F. hirta* présente une variation génétique clinale. Il est pollinisé par neuf espèces de guêpes parapatriques, avec un

pollinisateur accidentel à Singapour et huit espèces appartenant au complexe d'espèces de *Valisia javana*. La distribution parapatrique des pollinisateurs suggère une exclusion compétitive entre espèces de pollinisateurs.

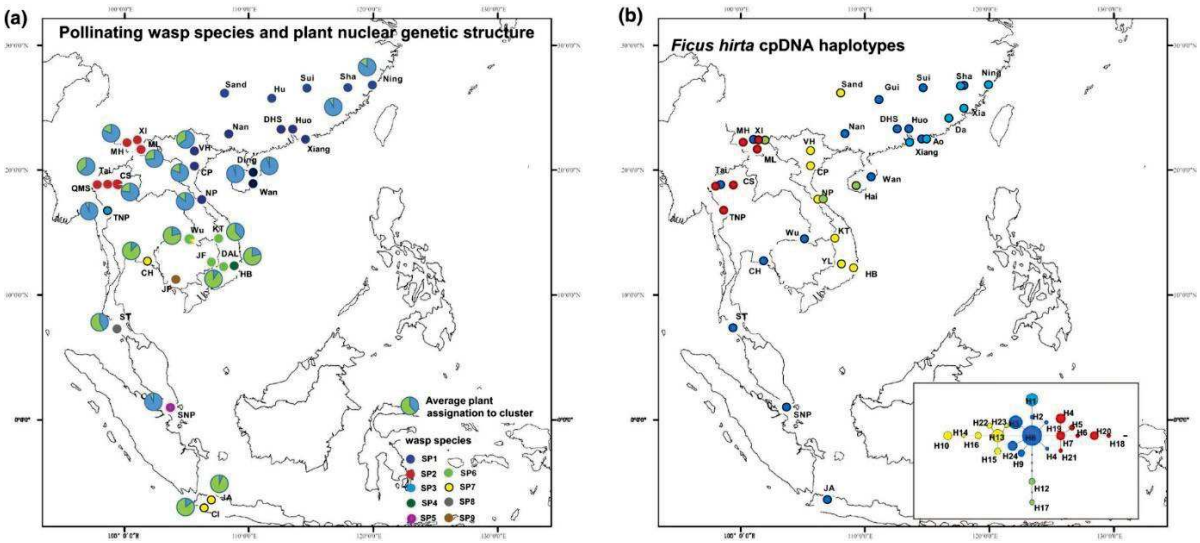


Figure 2.1. Structure spatiale de *Ficus hirta* et de ses guêpes pollinisatrices. Panneau (a) Distributions des espèces de guêpes pollinisatrices associées à *Ficus hirta* et variation génétique de *F. hirta*. Panneau (b) distribution des haplotypes d'ADNc de *F. hirta*. Notez la distribution disjointe de sp7, la distribution allopatrique des sp2 et sp9 étroitement apparentés (selon les données COI) et la distribution parapatrique des sp4, sp6 et sp7 étroitement apparentés (selon les données COI). Pour l'ADNc, des couleurs sont utilisées pour montrer la distribution localisée de l'haplotype H1 et des différentes branches du réseau d'haplotypes.

De ce fait une population locale d'arbres n'est capable d'entretenir qu'une espèce locale de pollinisateur. La diversité des pollinisateurs locaux est limitée par l'habitat et la compétition et non par la spéciation. La répartition géographique des espèces de pollinisateurs n'est pas corrélée avec la structure génétique de la plante hôte. Il sera donc possible d'établir si la structure spatiale des plantes ou celle des insectes est à l'origine de la variation spatiale de la signalisation des plantes, c'est-à- dire des odeurs des figues réceptives.

Pour confirmer que le renouvellement des espèces de pollinisateurs est contrôlé par la compétition entre les espèces de pollinisateurs et non par un trait de la plante que nous n'avons pas détecté, nous avons étudié la structure spatiale des guêpes non pollinisatrices du figuier associées à *F. hirta* dans Xiaoxia Deng, Lianfu Chen, Enwei Tian, Dayong Zhang, Tanming

Wattana, Hui Yu, Finn Kjellberg et Simon T. Segar. *"Low host specificity and broad geographic ranges in a community of parasitic non-pollinating fig wasps (Sycoryctinae; Chalcidoidea)." Journal of Animal Ecology* (2021).

Les guêpes non pollinisatrices du figuier (NPFW, appartenant à plusieurs familles de Chalcidoidea) comprennent des galligènes des fleurs, des cleptoparasites et des parasitoïdes. Une seule larve de guêpe se développe par ovule de fleur. Les NPFW sont généralement associés à une seule espèce de *Ficus*. Certaines données biologiques suggèrent qu'elles se dispersent plus efficacement que les pollinisateurs, tandis que d'autres données, dont les nôtres, suggèrent qu'elles sont moins dispersives. La composition locale de la communauté de guêpes associée aux figues *Ficus hirta* est constituée dans toute son aire de répartition, sauf à Java, par une espèce locale de *Philotrypesis* (un cleptoparasite du pollinisateur) et une espèce locale de *Sycoscapter* (un parasitoïde du pollinisateur) et tous deux pondent plus d'une semaine après les pollinisateurs. Les NPFW sont également attirés par l'odeur des figues, mais seuls ceux qui pondent au moment de la réceptivité des figues sont attirés par les odeurs de figues réceptives.

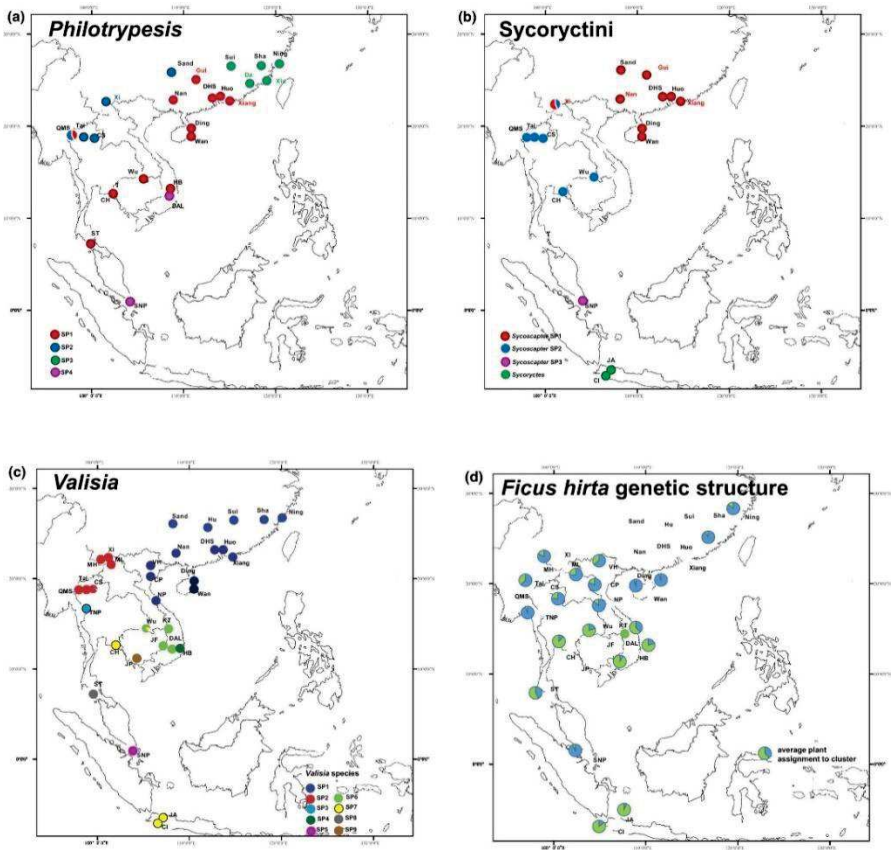


Figure 2.2. Distribution géographique des trois ensembles de guêpes du figuier associées à *Ficus hirta* et variation génétique de la plante. Panneau (a) distribution des espèces de guêpes cleptoparasites, genre *Philotrypesis* ; panneau (b) distribution des espèces de guêpes parasitoïdes, tribu des Sycoryctini ; panneau (c) distribution des espèces de guêpes pollinisatrices, genre *Valisia* ; panneau (d) structure génétique spatiale de la plante hôte. Pour la plante, la variation génétique est clinale et est illustrée ici par le pourcentage d'affectation à deux pools génétiques extrêmes.

Nous documentons 3 espèces parapatriques de *Sycoscapter* et 4 espèces parapatriques de *Philotrypesis*. Sur Java, *Philotrypesis* est absent et *Sycoscapter* est remplacé par une espèce de *Sycoryctes*. Les limites de distribution des espèces de *Valisia*, *Philotrypesis* et *Sycoscapter* diffèrent. Par conséquent, la structure de la communauté locale de guêpes est constante dans la majeure partie de l'aire de répartition de *Ficus hirta*, tandis que la composition des espèces locales varie indépendamment pour les différents membres de la communauté, et indépendamment de la structure génétique de la plante. Il n'y a pas de structure cachée chez *Ficus hirta* qui imposerait la distribution de ces espèces pollinisatrices vicariantes.

En Chine, *Ficus hirta* est pollinisé par une population de *Valisia javana hilli* (sp1) dans le sud de la Chine continentale jusqu'à la province de Fujian, il est pollinisé par une autre population de *V. javana hilli* dans l'île de Hainan et il est pollinisé par *Valisia* sp2 dans le Yunnan alors que *F. hirta* présente une variation génétique clinale dans toute son aire de répartition. Cela permet de déterminer si les odeurs réceptives des figues varient géographiquement en fonction de la variation génétique des plantes ou en fonction de la structuration des guêpes pollinisatrices en espèces ou en populations. C'est le sujet de Xiaoxia Deng, Bruno Buatois, Yan-Qiong Peng, Hui Yu, Yufen Cheng, Finn Kjellberg et Magali Proffit. "*Plants are the drivers of geographic variation of floral scents in a highly specialized pollination mutualism: a study of* Ficus hirta *in China*." (2021).

Nous montrons que les odeurs réceptives des figues ne varient pas en fonction de l'espèce ou de la population de pollinisateurs. Les odeurs réceptives des figues suivent un modèle de différenciation par la distance, similaire à la structure génétique spatiale de *Ficus hirta*. Nous concluons que la plante est le moteur de la variation des odeurs de figues réceptives et que les guêpes doivent s'y adapter localement.

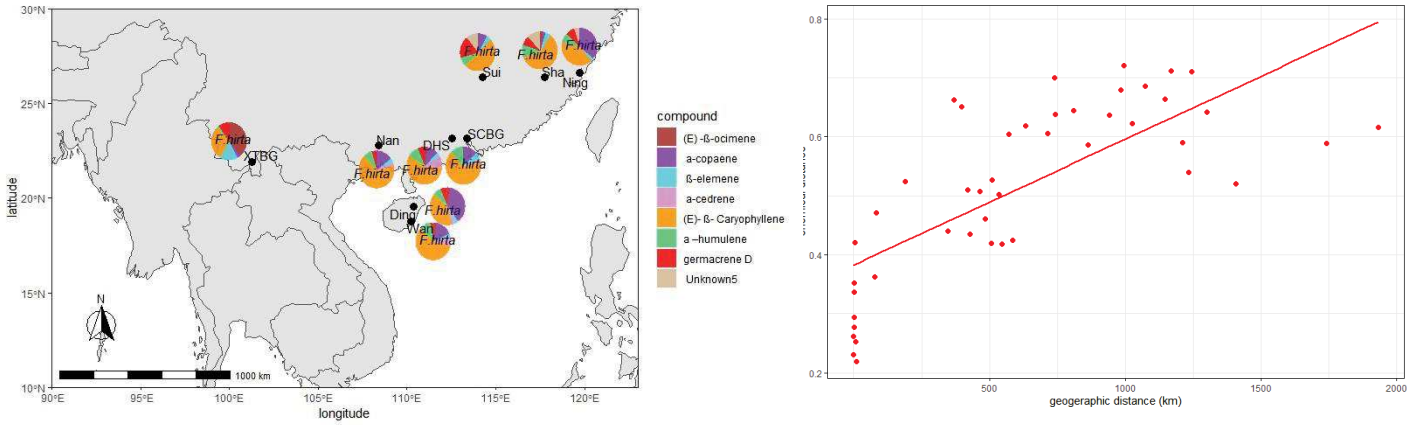


Figure 2.3. Variation géographique des composés volatils de *Ficus hirta* dans les différents sites d'étude. Panneau a) les diagrammes circulaires représentent le pourcentage dans les odeurs locales de figues réceptives des composés qui représentent plus de 5% dans le bouquet moyen d'odeurs dans au moins un site. Panneau b) corrélation entre la distance chimique et la distance géographique incluant tous les échantillons sur tous les sites (statistique de Mantel r=0.4897, p<0.001).

Les guêpes pollinisatrices localisent les figues réceptives de leurs espèces hôtes, malgré la variation de l'odeur des figues réceptives (Deng et al. 2021). Cette variation affecte principalement les proportions relatives des composés organiques volatils (COV) constitutifs plutôt que la présence ou l'absence de certains composés. L'attraction des guêpes pollinisatrices par les figues réceptives est-elle adaptée à un mélange particulier de composés organiques volatils ou réagit-elle à une large gamme de variations des concentrations relatives ? Nous avons étudié cette question en utilisant un système modèle situé à proximité du laboratoire, l'association entre *Ficus carica*, une espèces dioïque et *Blastophaga psenes* dans Magali Proffit, Magali, Benoit Lapeyre, Bruno Buatois, Xiaoxia Deng, Pierre Arnal, Flora Gouzerh, David Carrasco, et Martine Hossaert-McKey. *"Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction." Scientific Reports* 10, no. 1 (2020) : 1-11.

Nous montrons que *Blastophaga psenes* détecte 5 COV dans les odeurs de figues réceptives, et que ces

COV constituent la majeure partie de l'odeur de figue réceptive. Les guêpes n'étaient attirées que par un mélange de COV imitant les proportions trouvées dans les figues réceptives. La variation géographique des odeurs de figues réceptives auxquelles une espèce de guêpe pollinisatrice est exposée, que nous démontrons *chez Ficus hirta*, et qui a également été démontrée précédemment chez *Ficus septica*, implique probablement que les guêpes sont adaptées au mélange local de COV.

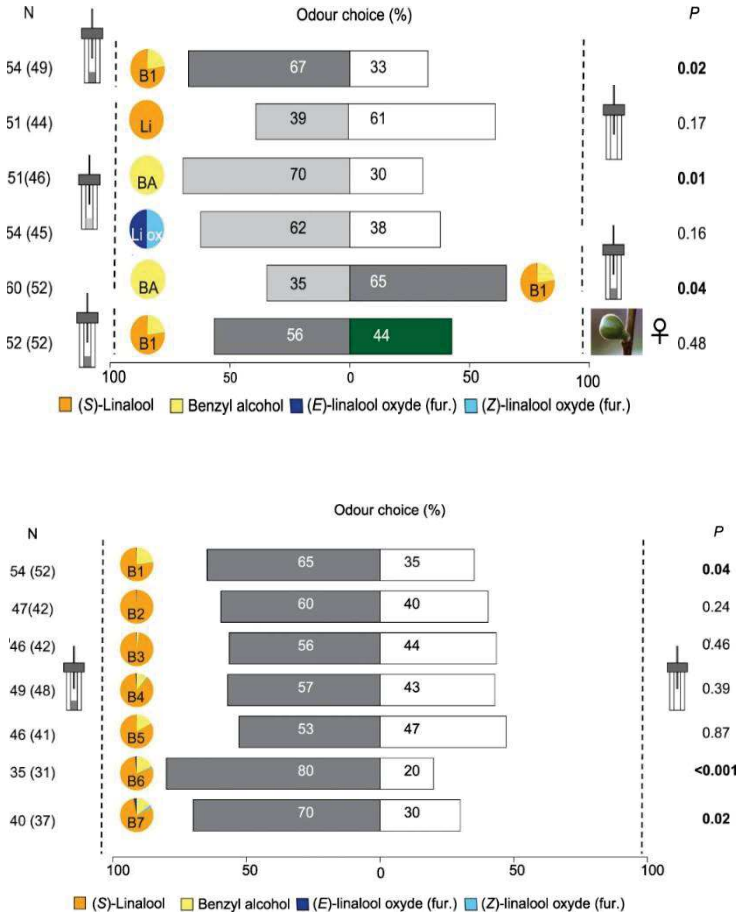


Figure 2.4. Attraction des femelles de *Blastophaga psenes* par des mélanges présentant différentes proportions de quatre COV. Les tests ont été effectués dans des olfactomètres à tube en Y dans lesquels les guêpes femelles pouvaient choisir entre des combinaisons synthétiques des quatre COV en différentes proportions, dans une branche, et une odeur témoin dans l'autre. Pour les quatre COV détectés par les femelles pollinisatrices, les proportions dans chaque source d'odeur sont indiquées dans le graphique circulaire. Les taux d'attraction les plus élevés ont été obtenus pour les mélanges B6 et B7, ceux présentant la correspondance la plus étroite des proportions avec les odeurs de figues réceptives. Le nombre de guêpes testées (N), le nombre d'individus ayant fait un choix (entre parenthèses) et les valeurs P (probabilité binomiale sous l'hypothèse de chances égales de choisir les deux branches de l'olfactomètre) sont indiqués pour chaque comparaison.

Cela suggère qu'elles ne sont pas attirées par des odeurs de figues réceptives non locales. Nous avons testé cette hypothèse en utilisant *Ficus hispida* comme système modèle dans Xiaoxia Deng, Gang Wang, Yan-Qiong Peng, Hui Yu, Aroonrat Kidyoo, Ting Zhang, Finn Kjellberg, Magali Proffit. *"Geographic variation in plant-insect signalling: The Achilles heel of the obligate fig-fig wasp mutualism when exposed to climatic accidents?"*(soumis).

Nous montrons que les odeurs réceptives de figues varient géographiquement chez *Ficus hispida*. En particulier, les odeurs réceptives des figues diffèrent entre deux sites chinois, le Jardin botanique de Chine du Sud dans la province de Guangdong et le Jardin botanique tropical de Xishuangbanna dans la province de Yunnan. *Ficus hispida* est pollinisé par la même espèce de guêpe, *Ceratosolen marchali*, dans ces deux endroits.

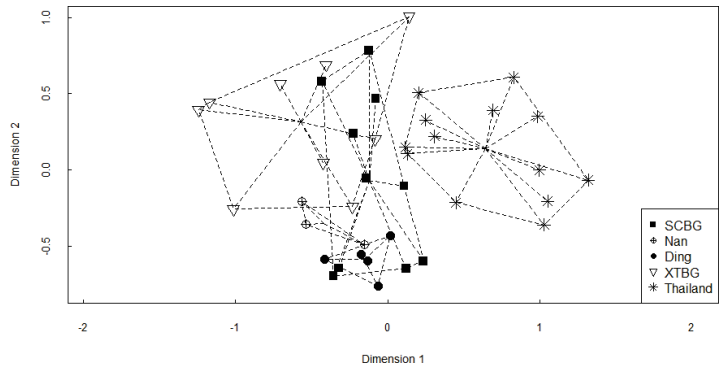


Fig. 2.5. Proportions relatives des COV dans les bouquets globaux émis par les figues réceptives de *F. hispida* dans les différentes localités sur la base de l'indice de dissimilarité de Bray-Curtis (stress=0,190). Représentation en Mise à l'échelle multidimensionnelle non métrique (NMDS). Les échantillons sont regroupés (lignes pointillées) par localité des figues et le centroïde de chaque groupe est indiqué.

Nous avons ensuite testé dans un olfactomètre à tube en Y l'attraction des guêpes *Ceratosolen marchali* de ces deux endroits par des figues réceptives locales et non-locales. Nous montrons que les guêpes appartenant à une même espèce étaient uniquement attirées par les odeurs de figues réceptives locales de leur lieu d'origine.

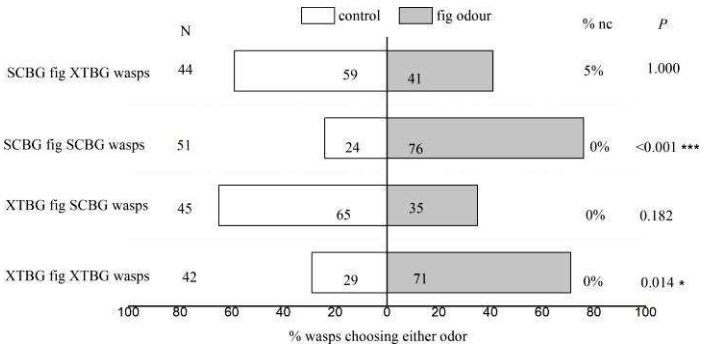


Figure 2.6. Réponses comportementales de *Ceratosolen marchali* confrontés à des odeurs de figues réceptives locales et non locales dans des expériences en tube en Y. Des figues réceptives fraîches ont été utilisées comme sources d'odeurs. Les choix de comportement en fonction des combinaisons d'odeurs dans les tests en Y sont les suivants : hôte non local contre air et hôte local contre air, (test binomial). contrôle : air purifié ; N : nombre de guêpes testées ; NC : pourcentage de guêpes n'ayant pas répondu (*P<0,05 ; ***P<0,001).

Les *Ficus* représentent donc un cas extrême de spécialisation de la pollinisation, les pollinisateurs ne reconnaissant que les figues réceptives de leur population hôte locale ou régionale. Néanmoins, il a été signalé des guêpes visitant localement des figues réceptives, ou des guêpes attirées par des figues réceptives dans des tests olfactométriques en tube Y, de proches parents de leurs espèces hôtes. Dans toute sa distribution, *Ficus hirta* coexiste avec un proche parent, *Ficus triloba*. Dans la province de Guangdong, le pollinisateur local de *Ficus* hirta, *Valisia javana* hilli, a été observé visitant les figues de F. triloba alors qu'il n'a pas encore été détecté dans les figues de F. triloba dans d'autres endroits. Nous étudions cette situation dans Xiaoxia Deng, Yufen Cheng, Hui Yu, Magali Proffit, Finn Kjellberg. "Limited interspecific divergence in olfactive signalling coupled with geographic variation may result in localised pollinator sharing between closely related *Ficus* species" (en préparation).

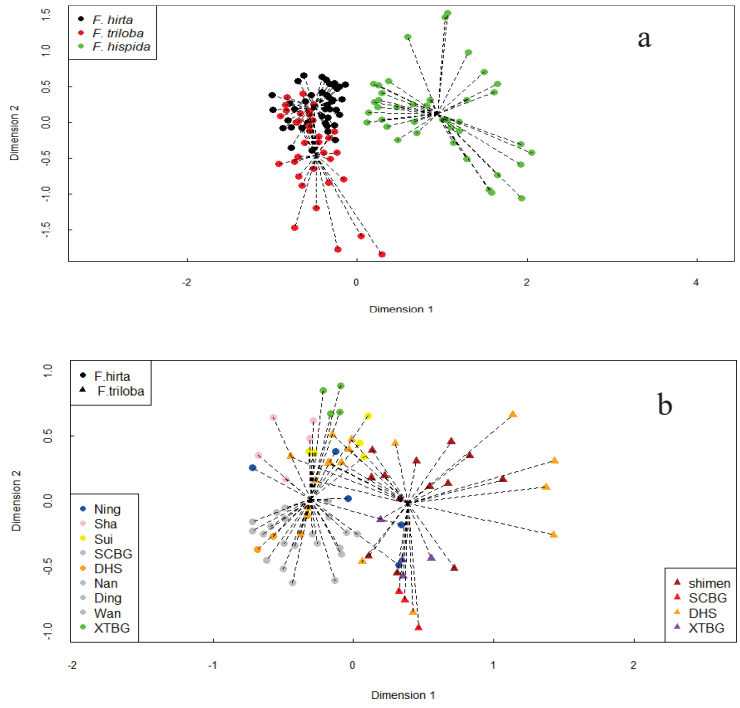


Fig. 2.7. Variation de l'odeur des figues réceptives au sein d'une espèce et entre espèces. Panneau a) comparaison entre *Ficus triloba*, *F.hirta* et *F. hispida*. Panneau b) comparaison entre stations de *F. triloba* et de *F. hirta*. Représentation non métrique à échelle multidimensionnelle des proportions relatives des COV dans les odeurs émises par les plantes individuelles, basée sur l'indice de dissimilarité de Bray-Curtis (stress=0,18 pour 2a et stress= 0,20 pour 2b).

Des espèces de *Ficus* étroitement apparentées peuvent avoir des odeurs similaires en raison de l'inertie phylogénétique. Pour tester cette hypothèse, nous avons collecté l'odeur de *F. triloba* sur l'ensemble de son aire de répartition de 1200 km dans le sud de la Chine. Nous montrons que les odeurs de figues réceptives de *Ficus triloba* et de *F. hirta*, bien que différentes, contiennent les mêmes COV principaux : tous les composés représentant plus de 5% de l'odeur de figue réceptive d'une espèce dans au moins une localité ont également été détectés dans au moins un extrait de l'autre espèce. A l'inverse, les odeurs réceptives de figues de *F. triloba* et *F. hirta* étaient très différentes de celles de *F. hispida*, une espèce phylogénétiquement éloignée.

Dans la montagne de Dinghu, on a observé que *V. javana hilli* se développait fréquemment dans les figues de *F. triloba* tandis que *V. esquirolianae* n'a pas été observé se développer dans les figues de *F. hirta*. Nous avons testé l'attraction de *Valisia javana hilli* et *V. esquirolianae* de la montagne de Dinghu, par des figues locales réceptives des deux espèces. Lorsqu'elles étaient confrontées à un choix entre de l'air pur et des odeurs de figues réceptives, les deux espèces n'étaient attirées que par les figues de leur espèce hôte habituelle. Cependant, lorsqu'on leur donnait le choix entre des odeurs de figues réceptives des deux espèces, *V. javana hilli* était également attiré par les figues des deux espèces. Dans les endroits où *V. javana hilli* est stimulé par les odeurs de figues réceptives de *F. hirta*, il pourrait également visiter *F. triloba*.

Nous concluons que la variation géographique des odeurs réceptives des figues, comme illustré ci-dessous, et la cooccurrence locale des deux espèces hôtes peuvent entraîner un chevauchement des odeurs produites par les deux espèces de *Ficus* jusqu'à un point où l'une ou l'autre espèce de pollinisateur peut localement visiter à la fois *Ficus hirta* et *F. triloba*. Cela peut se produire malgré la sensibilité des guêpes à la

proportion relative des différents COV qu'elles perçoivent. Cela ne se produira qu'entre des espèces de *Ficus* partageant une partie suffisante de leur signalisation olfactive, c'est-à-dire entre des espèces étroitement apparentées.

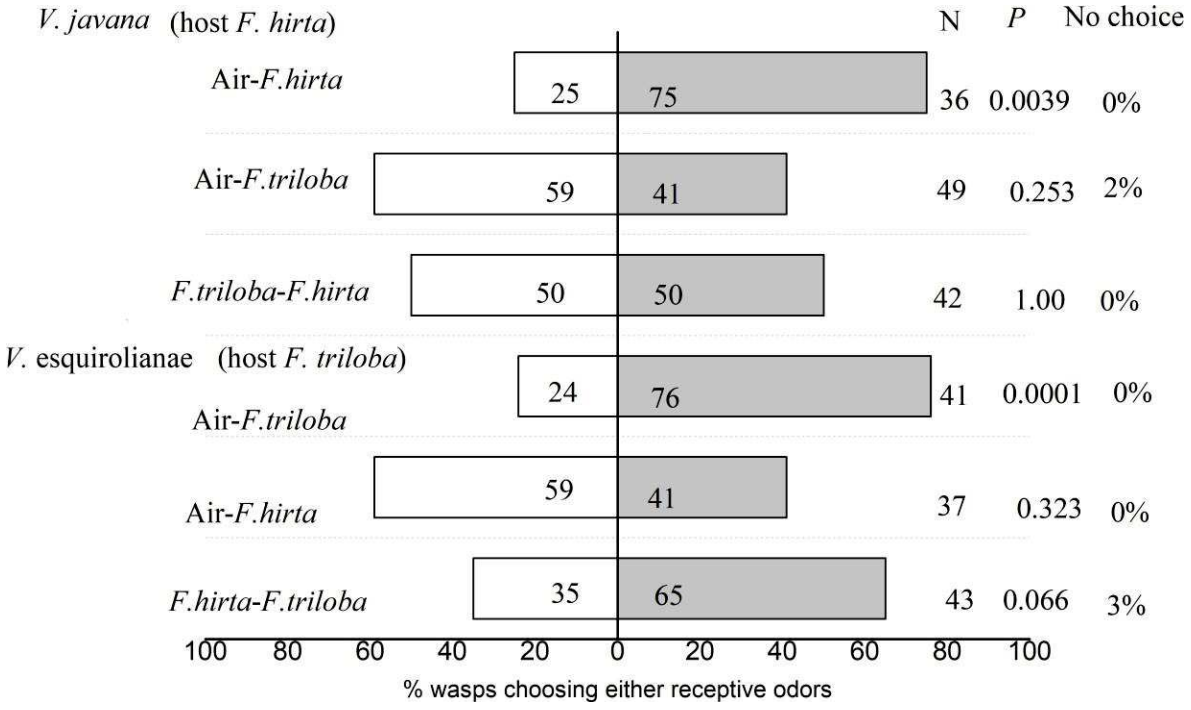


Fig. 2.8. Choix effectués par *Valisia javana hilli* et *V. esquirolianae* lorsqu'ils sont confrontés à différentes sources d'odeurs (figues réceptives) dans un olfactomètre à tube en Y. Nous avons utilisé des tests binomiaux pour les comparaisons statistiques entre le nombre de choix pour l'odeur versus l'air pur ou le choix entre les odeurs des deux espèces de *Ficus*. N : nombre de guêpes testées. P : probabilité, bilatéral. No choice : pourcentage des insectes qui n'ont pas fait de choix.

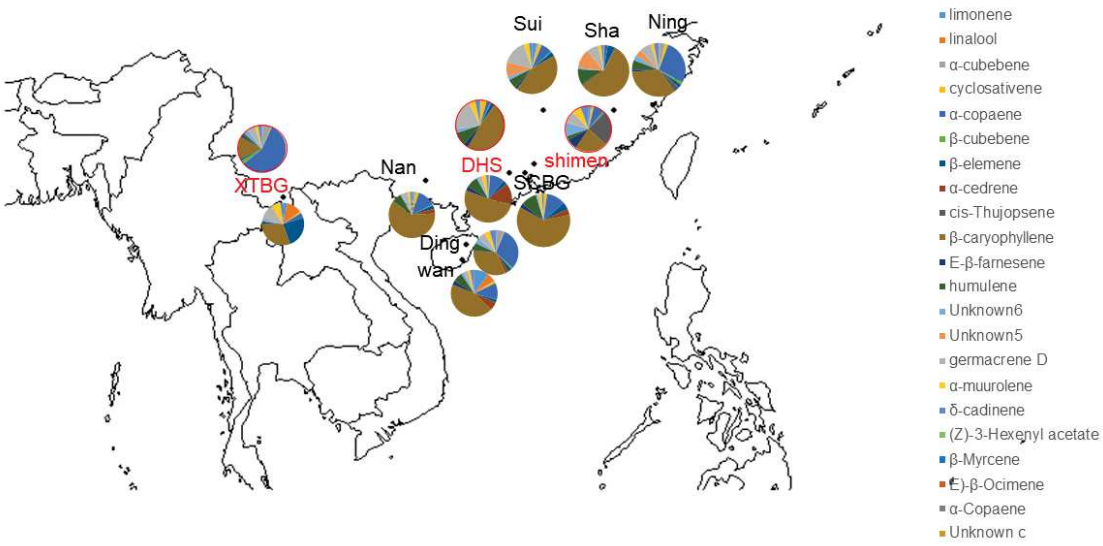


Fig. 2.9. Variation géographique de la composition de l'odeur de figue réceptive pour *Ficus hirta* et *Ficus triloba*. Les diagrammes circulaires illustrent la représentation locale dans les odeurs de figues réceptives des composés qui représentent plus de 5% dans le bouquet moyen d'odeurs d'au moins une espèce dans au moins un site.

En résumé, nous avons montré que la variation géographique des odeurs de figues réceptives correspond à la structure génétique des plantes et non à celle des pollinisateurs. La variation géographique des odeurs de figues réceptives affecte la capacité de l'insecte à localiser des figues réceptives non locales car les insectes sont adaptés aux odeurs locales. Néanmoins, la signalisation olfactive d'espèces de *Ficus* apparentées peut se chevaucher de sorte que dans certains endroits, les pollinisateurs peuvent être attirés par des figues réceptives de figuiers non hôtes.

Discussion générale, conclusions et perspectives

Ce travail explore les forces qui gouvernent la variation géographique de la communication plante-insecte et leurs conséquences biologiques, en nous concentrant sur la relation entre l'odeur de la figue réceptive, la structure génétique de la plante hôte, celle de la guêpe pollinisatrice et son comportement. Nous avons focalisé notre travail sur les processus de diversification en cours chez trois espèces de *Ficus* dioïques à large aire de distribution, à savoir *Ficus hispida*, *Ficus hirta* et *Ficus triloba*, dans un contexte continental. Les figuiers et leurs guêpes pollinisatrices (Agaonidae) constituent l'un des mutualismes de pollinisation les plus étroitement intégrés connus, dont l'origine remonte à quelque 70 millions d'années (Cruaud et al. 2012). Des études empiriques ont mis en évidence le rôle déterminant des odeurs émises à l'anthèse dans l'attraction olfactive des pollinisateurs dans les mutualismes de pollinisation de sites de reproduction (Dotterl et al. 2006, Chen et al. 2009, Hossaert-McKey et al. 2010, Svensson et al. 2010, Song et al. 2014, Proffit et al. 2020, Terry et al. 2021). Ces odeurs sont composées d'un mélange de composés organiques volatils (COV). Une approche intégrant les odeurs des inflorescence, l'écologie de la pollinisation et la génétique des populations a ouvert de nouvelles pistes pour comprendre le fonctionnement et l'évolution de ces systèmes spécialisés. Après les études menées sur les trois espèces de *Ficus* mentionnées ci-dessus, plusieurs conclusions et généralisations peuvent être proposées.

Plusieurs éléments de preuve obtenus par des comparaisons interspécifiques de mutualismes de pollinisation de sites de reproduction indiquent un rôle important pour une évolution des odeurs florales induite par les pollinisateurs. Par exemple, lorsque deux espèces de plantes interagissent avec le même groupe de pollinisateurs, la composition de l'odeur émise à l'anthèse converge (Svensson et al. 2011, Cornille et al. 2012, Huang et al. 2015, Wang et al. 2016). Il est clair que les pollinisateurs exercent des pressions sélectives sur l'évolution des odeurs florales dans la mesure où ils sont attirés par ces odeurs et où leur comportement détermine le succès de la

reproduction des plantes. Il a été suggéré que toute variation dans leur présence et/ou leur impact sur la reproduction des plantes devrait également influencer la force et la direction de la sélection sur la signalisation des plantes (Delle-Vedove et al. 2017). Cependant, des études empiriques, dont nos résultats, montrent que la sélection exercée par les pollinisateurs n'explique pas la variation géographique de la signalisation olfactive dans les mutualismes obligatoires de pollinisation de sites de ponte (Knudsen 2002, Svensson et al. 2005, Suinyuy et al. 2012, Rodriguez et al. 2017, Friberg et al. 2019, Deng et al. 2021). Par exemple, des données récentes chez *Lithophragma* montrent une forte similitude d'odeur florale entre des populations proches au sein d'une même espèce, mais pas entre des populations proches d'espèces appartenant à des lignées différentes mais partageant les pollinisateurs (Friberg et al. 2019). Ces résultats ne soutiennent pas une sélection des odeurs florales canalisée par les pollinisateurs chez *Lithophragma*. Des données sur la cycadale africaine *Encephalartos villosus* montrent également que différents chémotypes de plantes, séparés géographiquement et dont les cônes produisent des odeurs extrêmement différentes sont associés à un même pollinisateur (Donaldson 1997, Suinyuy et al. 2012). La variation intraspécifique de la composition des odeurs des inflorescences entre les populations correspond à une convergence locale des odeurs à anthèse des inflorescences entre les espèces de cycadales partageant des pollinisateurs, les pollinisateurs évoluant en réponse aux odeurs réceptives locales des inflorescences (Suinyuy et al. 2012). Cependant, nous ne pouvons pas confirmer si la variation de la composition volatile des inflorescences correspond à la structure génétique spatiale des cycadales. En effet, peu de données moléculaires sont disponibles pour *Encephalartos* en raison de la faible variation génétique au sein du genre (Treutlein et al. 2005), et parmi les cycadales en général (Ellstrand et al. 1990, González et Vovides 2002). D'autres études sont nécessaires pour explorer la structuration génétique à travers les différentes populations des deux espèces de cycadales qui peuvent être corrélées avec les différences dans les odeurs des cônes. De même, chez

Encephalartos ghellinckii Lem, une espèce présente dans des habitats présentant de grandes variations de température allant du froid glacial avec de la neige en hiver dans le Drakensberg à la chaleur extrême et aux vents desséchants en été dans les populations plus proches de la côte (Suinyuy et Johnson 2018), deux chémotypes différents ont été observés, un chémotype de montagne et un chémotype de plaine (Suinyuy et Johnson 2018, Suinyuy et Johnson 2020), qui correspondent à des différences dans les assemblages d'insectes, mais avec la présence d'espèces pollinisatrices partagées (Suinyuy et Johnson 2018).

Dans notre étude, *Ficus hirta* est pollinisé par une population de *Valisia javana hilli* (sp1) en Chine du Sud continentale s'étendant jusqu'à la province de Fujian, il est pollinisé par une autre population de *V. javana hilli* dans l'île de Hainan et il est pollinisé par une autre espèce du complexe *Valisia javana*, sp2, dans le Yunnan alors que *F. hirta* présente une variation génétique progressive à travers son aire de répartition. Nous avons mis en évidence une divergence géographique progressive des odeurs réceptives des figues chez *F. hirta*, ce qui correspond à la structure génétique de la plante. Ainsi, nos résultats montrent que la variation géographique de l'odeur de figue réceptive correspond à la génétique des plantes et non à la structure génétique des insectes. Cela suggère que les plantes évoluent d'abord et que les guêpes doivent ensuite s'adapter aux odeurs locales. Les plantes sont les moteurs de la variation géographique des odeurs florales et les insectes les suiveurs (Deng et al. 2021). Bien que la structure génétique de *F. hispida* ne soit pas encore disponible, des données moléculaires non publiées montrent que la variation génétique est clinale et des données génétiques et morphologiques sur les insectes ont confirmé qu'à SCBG et XTBG *F. hispida* est pollinisé par la même guêpe pollinisatrice, mais en Thaïlande par une espèce différente, et par ces deux espèces sur l'ile de Hainan. Nos résultats montrent que *F. hispida* émet une odeur similaire dans le sud de la Chine continentale et sur l'île de Hainan, pollinisé par un assemblage différent de guêpes figues, alors que les odeurs florales varient entre SCBG et XTBG pollinisés par la même

espèce de guêpe figue. Nos résultats montrent donc que la variation des odeurs n'est pas corrélée à la pollinisation par différentes espèces de pollinisateurs. Les variations d'odeurs de figues réceptives sont signifiantes biologiquement, ce qui correspond aux résultats obtenus sur les cycadales.

Nous supposons que chez *F. triloba*, une plante plus grande et probablement plus dispersive, nous observerons moins de variations géographiques dans les odeurs de figues réceptives que chez *Ficus hirta*. Des travaux supplémentaires sur cette espèce sont nécessaires pour vérifier cette hypothèse. En effet, la variation géographique de l'odeur de figue réceptive semble avoir une corrélation négative avec le flux génétique des plantes. Chez les plantes à fleurs, le flux génétique se produit par le mouvement du pollen et des graines, le flux de pollen contribuant souvent le plus (Campbell 1991). Des flux de pollen sur de longues distances entre figuiers (*Ficus* spp.) ont été documentés (Harrison et Rasplus 2006, Ahmed et al. 2009), tandis que la dispersion moyenne des guêpes pollinisatrices dans une forêt tropicale humide a été estimée à 6-14 km (Nason et al. 1998). Les données accumulées sur les mutualismes de pollinisation de sites de ponte montrent que les espèces les plus dispersives présentent une moindre structuration spatiale des odeurs réceptives (Svensson et al. 2005, Soler et al. 2011, Bain et al. 2016, Friberg et al. 2019), par rapport aux espèces à dispersion limitée (Suinyuy et al. 2012, Rodriguez et al. 2017, Friberg et al. 2019). Chez *Encephalartos villosus*, les pollinisateurs perdent une quantité importante de pollen quelques heures après avoir quitté les cônes mâles et les plantes isolées situées à 5 km des populations sources ne sont pas visitées par les insectes pollinisateurs (Donaldson 1997, Suinyuy et al. 2012). La dispersion du pollen semble être surtout intra station chez *E. villosus*, ce qui facilite l'évolution d'une forte variation régionale des odeurs des cônes (Suinyuy et al. 2012). L'extrême diversification des odeurs florales au sein des espèces est probablement associée à une dispersion limitée du pollen et des graines chez *Lithophragma* (Furches et al. 2008, Friberg et al. 2019) alors que les insectes pollinisateurs conservent une

L'odeur florale diffère souvent entre les espèces sympatriques étroitement apparentées, ce qui suggère que le comportement des pollinisateurs médié par leurs caractéristiques sensorielles peut être un facteur clé pour l'établissement d'un isolement reproductif en cas de sympatrie (Okamoto et al. 2007, Svensson et al. 2016, Okamoto et Su 2021). En revanche, pour des espèces à distribution parapatriques comme *Glochidion obovatum* et *G. rubrum* (Kawakita et Kato 2004), les odeurs florales peuvent ne pas différer (Okamoto et al. 2007). De même, dans le mutualisme Yucca-*Tegeticula*, les espèces de *yucca* dont les aires de répartition ne se chevauchent pas peuvent partager la même odeur florale, tandis que les espèces sympatriques, dont les phénologies de floraison se chevauchent et qui appartiennent à des sections différentes du genre *Yucca*, présentent des différences dans leurs odeurs florales (Svensson et al. 2011, Svensson et al. 2016). Ces études confirment que les pollinisateurs font souvent la distinction entre les hôtes sympatriques sur la base de signaux chimiques. Néanmoins, dans un certain

nombre de cas, d'autres mécanismes sont à l'œuvre, comme une incompatibilité morphologique ou des plantes poussant en sympatrie, mais dans des habitats différents (Cornille et al. 2012, Wang et al. 2016, Souto-Vilarós et al. 2018,).

Ici, bien que la comparaison de la variation de la composition des odeurs florales chez *F. hirta* et *F. triloba* ait indiqué que les odeurs de figues de ces espèces étroitement apparentées et sympatriques diffèrent, nos résultats ont néanmoins révélé une similitude interspécifique des odeurs de figues. Les odeurs de figues réceptives varient géographiquement chez *F. hirta*. A Guangdong, Yu et al. (données non publiées) ont observé des pollinisateurs de *F. hirta* se développant dans les figues de *F. triloba*. A Dapu, dans le nord du Guangdong, à environ 100 km de la limite nord (Nanjing, sud du Fujian) de *F. triloba*, il a été trouvé que les pollinisateurs de *F. triloba* visitent les deux hôtes (Yu et al. données non publiées) suggérant une forte similitude des odeurs de figues réceptives, telles que perçues par les pollinisateurs. Il a été documenté que dans la partie nord de son aire de répartition, *F. hirta* est également morphologiquement plus similaire à *F. triloba* présentant des figues plus grandes avec des parois de figues plus épaisses (Yu et al. 2018). D'autres études seront nécessaires pour établir s'il existe une introgression génétique entre les deux espèces et si les odeurs de figues réceptives sont moins différenciées que dans d'autres régions. Dans une situation quelque peu différente chez les espèces de *Yucca* parapatriques, il y avait une différenciation limitée mais significative des odeurs florales entre les deux espèces sœurs *Yucca brevifolia* et *Yucca jaegeriana* (Svensson et al. 2016). Dans une zone de contact entre les deux espèces, leurs pollinisateurs spécifiques respectifs visitent les deux espèces hôtes sans distinction. Chez les individus de plantes introgressés, la corrélation entre les odeurs de fleurs à anthèse et la morphologie florale est rompue (Svensson et al. 2016). Par conséquent, les différences d'odeurs réceptives entre des espèces étroitement apparentées ne sont pas des barrières absolues au flux de pollen interspécifique dans les mutualismes de pollinisation de sites de reproduction.

Seules quelques études évaluent si les pollinisateurs de sites de ponte peuvent être attirés par les odeurs des plantes non-hôtes. Quelques cas de faible préférence olfactive pour un hôte ont été signalés. Par exemple, il a été démontré qu'en dépit de la différence marquée des odeurs réceptives de figues entre deux variétés sympatriques de *Ficus semicordata*, les femelles de leurs pollinisateurs respectifs, les guêpes *Ceratosolen*, étaient attirées par les odeurs des deux variétés d'hôtes dans des tests en tube en Y (Wang et al. 2013). Bien que la spécificité de l'hôte soit renforcée par des composés de contact sur la surface de la figue, certaines guêpes sont entrées dans des figues non hôtes. Les différences fonctionnelles des fleurs peuvent également limiter les flux génétiques interspécifiques, comme cela a été démontré pour des orchidées partageant des pollinisateurs (Gögler et al. 2015). Cependant, aucune divergence morphologique sélectionnée dans l'anatomie florale entre des espèces de *Ficus* apparentées n'a pas encore été mise en évidence. Dans la nature, certaines espèces de *Ficus* sympatriques étroitement liées produisent des odeurs de figues réceptrices similaires et peuvent partager des pollinisateurs (Wang et al. 2016). Ces espèces poussent généralement dans des habitats différents (Wei et al. 2014). Si l'essentiel de la dispersion des pollinisateurs se fait au sein d'un habitat, entre les plantes d'une même espèce, la pollinisation interspécifique peut être marginale même en l'absence de barrières autres.

Perspectives

Notre travail montre que même dans un mutualisme obligatoire très spécifique, où l'odeur émise par la plante est nécessaire et joue le rôle principal dans l'attraction de son pollinisateur spécifique, l'odeur peut être extrêmement diversifiée géographiquement ou bien être constante sur de grandes échelles géographiques. Nous proposons comme hypothèse de travail que cette variation sera corrélée négativement avec le flux génétique chez la plante. Nous prédisons que les odeurs réceptives des figues présentent une variation limitée chez les espèces de *Ficus* monoïques, car leurs pollinisateurs se dispersent en dérivant dans le vent au-dessus de la canopée (Harrison et

Rasplus 2006). Parmi les espèces monoïques, celles pollinisées par des guêpes qui dérivent le plus haut au-dessus de la canopée devraient être les moins structurées. Nous prévoyons également que les espèces monoïques étroitement apparentées seront moins susceptibles que les espèces de *Ficus* dioïques de présenter des odeurs de figues réceptives similaires, en raison de la forte dispersion des pollinisateurs. Réciproquement, nous prévoyons une plus forte structuration spatiale chez les espèces de *Ficus* dioïques et surtout chez les espèces de petite taille. En effet, les petits individus produisent de petites récoltes de figues, qui nécessitent probablement que les guêpes effectuent des recherches plus fines de figues réceptives, car la signalisation olfactive par les figues réceptives individuelles sera limitée. Ceci est plus particulièrement vrai pour les pollinisateurs des petits *Ficus* de sous-bois, qui ne peuvent pas se laisser porter par le vent au-dessus de la canopée comme le font les pollinisateurs des grands *Ficus* monoïques. Il y aura également peu de sélection pour la divergence des odeurs de figues réceptives entre des espèces de *Ficus* dioïques étroitement apparentées si elles occupent des habitats différents. En effet, la dispersion limitée des guêpes limitera automatiquement le transfert de pollen entre les habitats. Ainsi, le mutualisme *Ficus*-guêpe pollinisatrice fournit un ensemble étendu de prédictions qui peuvent être testées dans des travaux futurs.

Une autre perspective est liée à la variation de la réponse à l'odeur de figue réceptive et de la perception des COV par les guêpes. La chromatographie en phase gazeuse couplée à la détection électro-antennographique (GC-EAD) permettra de déterminer s'il existe des variations au sein des espèces d'insectes quant aux COV qu'ils perçoivent. Des expériences sur le comportement des insectes, notamment en utilisant des composés synthétiques, permettront de déterminer quels mélanges de COV attirent les guêpes. Ainsi, il sera possible d'établir si les COV qui contribuent principalement à la différenciation géographique des odeurs de figues sont des composés qui contribuent au signal olfactif que les pollinisateurs détectent et auxquels ils répondent, ou si une telle variation est possible en raison de la plasticité de la réponse olfactive du pollinisateur. Les guêpes pollinisatrices ont un répertoire réduit de gènes olfactifs. Si les tests GC-EAD détectent une variation au sein de l'espèce dans la détection des COV, l'analyse

transcriptomique pourrait permettre d'établir précisément quels gènes sont impliqués dans la détection de COVs particuliers.

General introduction

A major challenge in evolutionary biology is to understand how complex traits of multiple functions have diversified and co-diversified across interacting lineages and geographic ranges (Friberg et al. 2019). There are more than 300,000 species of vascular plants in the world of which 88.7% are angiosperms (Christenhusz and Byng 2016). Interactions between plants and plant-feeding insects are among the most diverse on Earth (Thompson et al. 2005, Thompson et al. 2013, Thompson et al. 2017). Plant-insect pollinator interaction are of particular importance as 90% of angiosperms are pollinated by insects, such as bees, beetles, moths, and flies (Ollerton et al. 2011). Although many complex traits shape these interactions, floral scent usually plays a key role in shaping gene flow in plants and in the specialization of many insects (Raguso 2008, 2009, Schiestl 2015).

Floral signalling in general is essential in any plant-pollinator interaction, and often consist of a combination of visual cues (e.g., colour and shape) and olfactory stimuli (scents, derived for example from petals or pollen) that elicit responses in the pollinators (Lunau 1995, Dobson and Bergström 2000, Chittka and Raine 2006, Raguso 2008). Flowers pollinated by insects emit significantly higher amounts of volatile organic compounds (VOCs) and present a higher diversity of these compounds than wind pollinated flowers (Farré-Armengol et al. 2015).

Although visual cues are important in attracting pollinators, they often are not as specific as olfactory cues (Kunze and Gumbert 2001, Wright et al. 2009). For instance, field tests have shown that olfactory signals emitted by figs, in the absence of any visual cues, are sufficient to attract their specific pollinators (Hossaert-McKey et al. 1994). Plant volatiles are the invisible players in the plant-insect co-evolutionary arms race (Binyameen et al 2021). Single volatiles mediate some interactions between plants and pollinators (Schiest et al. 2003, Chen et al. 2009), but complex floral scent bouquets are more common (Knudsen et al. 2006).

Floral scents may be involved in attracting pollinators, but also in eliciting landing, feeding,

and in some cases mating and oviposition behaviour on the flower depending on flower-insect association (Dobson 2006, Bouwmeester et al. 2019). Despite their importance, the role of floral scent in plant speciation and evolution remains poorly understood (Whitehead and Peakall 2009).

Generalist mutualistic pollination systems

Typical examples of families with species that fall under a generalist pollination syndrome include Rosaceae, Apiaceae, Arecaceae, and Ranunculaceae, where the flowers are pollinated by a diversity of insects (beetles, flies, bees, butterflies) that feed on the exposed nectar and pollen, presented in open and typically bowl-shaped flowers, umbels, cylindrical or globular inflorescences (Dobson 2006). Although various insects are thought to be pollinators, some may be more important and therefore drive selection in scent chemistry more strongly than other ones. Detailed pollination studies are necessary to uncover the relative contribution of the different pollinators (Pelletier et al. 2001, Kandori 2002, Zych 2002, Jürgens 2004). In general, floral scents do not present any particular consolidated pattern of composition other than that they consist of three major chemical classes, namely fatty acid derivatives, terpenoids, and benzenoids, with one class often predominating over the other ones in a given floral odour (Borg-Karlson et al. 1993, Robertson et al. 1993, Tollsten and Bvstedal 1994, Tollsten et al. 1994, Robertson et al. 1995, Knudsen et al. 2001). Causality is difficult to infer in plants with generalist pollination. Reports of variation in floral scents among populations suggest possible adaptation to local suites of pollinators (Dobson 2006, Breitkopf et al. 2013).

Brood pollination mutualisms

Brood site pollination mutualisms (also referred to as nursery pollination mutualisms) are interactions in which specialized insects pollinate as adults and feed as larvae on floral structure of their host plants-have. They have been influential study systems for

coevolutionary biology although authors often restrict the definition to the subset of interactions in which species in which larvae feed on seeds (Hembry and Althoff 2016). Brood site pollination mutualisms are widespread. They occur in multiple species, for instance in genus *Macaranga* (20, probably 40 species (Yamasaki et al. 2015)), in genus *Yucca* (about 50 species; (Pellmyr 2003)), in genus *Ficus* (about 820species), in Cyclanthaceae (230 species; (Franz 2007), in Cycadales (many of the 353 species; (Suinyuy et al. 2010, Toon et al. 2020), in Araceae pollinated by Drosophilidae (about 540 species; (Gibernau 2016, Kawakita et al. 2019), in Phyllantheae pollinated by *Epicephala* moths (maybe some 750 species, including old world and new world species; (Kawakita 2010, Kawakita et al. 2019), in some Arecaceae (palm trees), but probably also in numerous Moraceae, including most species of Castillae (59 species; (Sakai 2001), Dorsteniae (138 species; (Araújo et al. 2017) and Artocarpeae (70 species; (Sakai et al. 2000). Evidence has shown that odours emitted at flower anthesis play a central role in attracting pollinators in most of the brood pollination mutualisms (Miyake and Yafuso 2005, Dotterl et al. 2006, Ibanez et al. 2010, Svensson et al. 2010, Svensson et al. 2011, Proffit et al. 2020). These odours may be produced by flowers but also by diverse organs such as inflorescence bracts and bracteoles (Souza et al. 2015, Yamasaki et al. 2015, Hu et al. 2020) or even by the leaves (Dufaÿ et al. 2003).

What do we know about within-species variation in receptive inflorescence odour in such systems? What can it tell us about the evolutionary processes involved? In Yuccas and yucca moth system, genetic differentiation among *Tegeticula yuccasella* populations on alternative hosts is slight (Leebens-Mack 2004), and no differentiation in floral odour was observed (Svensson et al. 2005). The system suggests high moth-mediated gene flow among sites and conservation of odour composition across populations with different pollinators. In cycad, there is strong interpopulation variation in the cone scent of *Encephalartos villosus*, but pollinator assemblages did not differ across the different populations, which suggest that these

patterns were produced by co-evolution or drift, rather than by pollinator shifts (Suinyuy et al. 2012). Additionally, in a context of strong variation of floral volatiles among populations within species of *Lithophragm*a (Saxifragaceae), there was a strong negative relationship between the pairwise population similarity (1−Bray-Curtis distance) and their geographic distance in the widely distributed *L. parviflorum* (Thompson and Cunningham 2002, Friberg et al. 2019). Hence, obligate nursery pollination mutualisms provide highly constrained model systems, in which we can investigate how plants, their insect associates and floral scent have diversified or codiversified. In this work, we explore these questions taking advantage of the well-known fig-fig wasp system as a study system.

Fig and fig wasp mutualism

There are some 820 species of *Ficus* distributed throughout the world's tropical and subtropical regions. They have a reciprocally obligate pollination relationship with tiny (1–2 mm), short-lived (1–2 days adult lifespan), fig wasps (Chalcidoidea, Agaonidae). Generally, the wasps are associated with a single *Ficus* species, while often a species complex of Agaonid wasps pollinates a *Ficus* species (Darwell et al. 2014, Bain et al. 2016, Rodriguez et al. 2017, Yu et al. 2019, Cook and Rasplus 2003, Cornille et al. 2012, Wang et al. 2016).

The inflorescence of *Ficus* trees, the fig, is a closed urnshaped receptacle the inside of which is lined by uniovulate female flowers and male flowers. Wasps oviposit into female flowers, laying one egg per flower and pollinate. The wasp larva develops within the galled flower feeding on endosperm whose development has been induced by pollination or by the wasp. Male wasps emerge first from their galls and mate with the females. Then the females emerge into the fig cavity, become pollen loaded and leave in search of a tree bearing receptive figs. In monoecious *Ficus* species, *i.e.* in about half of the species, wasp and seeds develop side by side in the figs. In functionally dioecious species, the wasps that enter figs on female trees pollinate, but fail to oviposit (Fig 1.1). This is because the long styles through which the

wasps introduce their ovipositor do not allow it to reach the limit between the inner integument and the nucellus where the egg is laid. Hence, figs on female trees produce seeds but no wasps. When a wasp enters a fig of a functionally male tree, it oviposits into the flowers as the styles are much shorter. In actively pollinating wasp species, pollen is deposited precisely in the stigmas in which the wasps oviposit while in passively pollinated species, but pollen fails to germinate in functionally male trees. As a result, male trees produce wasps, pollen but no seeds.

Generally, monoecious *Ficus* species are hemiepiphytic trees (they germinate as epiphytes, and their roots develop until they reach the soil) or large freestanding trees. Conversely, dioecious *Ficus* species are mainly lianas, shrubs and small trees.

Fig development is usually synchronous over the entire crown of a tree, requiring that wasps emerging from a tree fly to a new tree located by olfaction (Grison et al. 1999). Phenological data and the diversity of pollen donors show that pollinators of monoecious *Ficus* species routinely disperse at 5–14 km (Bronstein et al. 1990, Nason et al. 1998, Harrison 2003). Non- pollinating fig wasps that also develop in fig ovules but without providing pollination services are also attracted olfactively by figs (Bronstein et al. 1990, Proffit et al. 2007). They include galling insects and parasitoids.

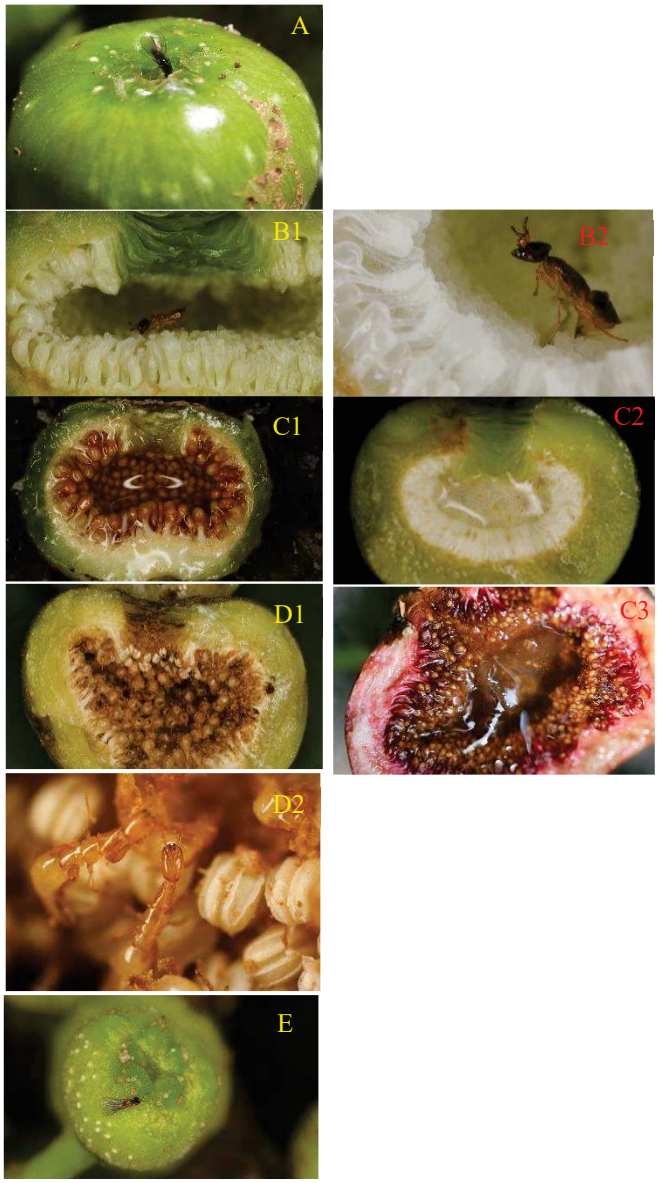


Figure 1.1 Life cycle of wasps in a functionally dioecious *Ficus* species. (A) A foundresses entering a receptive fig through the ostiole. (B1) Upon entering, foundresses lay eggs in the male fig. (B2) foundresses try to lay eggs in the female fig. The stigma forms a tube, the outside of which is covered with papillae allowing easy pollen germination (C1) In male fig, wasp larvae develop in female flowers transformed into galls and the other flowers remain sterile. (C2) Seeds develop in female fig. (D1) Wasps are emerging from their galls. (D2) Males emerge first and mate with the females that are still in their galls. (D3) female fig, which do not have wasps, do not have a male phase and ripen directly. The ripe figs become soft and fleshy. E. A mated pollen bearing female wasp that has dispersed from its natal tree, on the point of entering a receptive fig on another tree.

Fig and fig odors

The volatile organic compounds (VOCs) emitted by receptive figs have been studied in *Ficus* species belonging mainly to four subgenera out of six, namely *Urostigma*, *Sycomorus*, *Sycidium* and *Ficus* (Gibernau et al. 1997, Grison et al. 1999, Hossaert-McKey et al. 2016, Zhang et al. 2017). A role of VOCs in pollinator attraction has been demonstrated in some species (Chen et al. 2009, Proffit et al. 2009, Chen and Wu 2010, Wang et al. 2013, Wang et al. 2016, Zhang et al. 2016, Souto-Vilarós et al. 2018). Studies on the composition of receptive fig odors have now been conducted in more than 30 *Ficus* species (Gibernau et al. 1997, Grison et al. 1999, Delle-Vedove et al. 2011, Cornille et al. 2012, Soler et al. 2012, Hossaert-McKey et al. 2016, Zhang et al. 2017, Souto-Vilarós et al. 2018). In these studies, generally only a few compounds were detected in each species (1 to 50 common compounds for each species), and there were usually 1 to 3 dominant compounds that were far more abundant than other VOCs. The VOCs consist mainly of terpenes (monoterpenes and sesquiterpenes), followed by benzenoids and fatty acid derivatives. The dominant compounds, however, are mostly one or more of the following: (E)-β-ocimene, β- caryophyllene, 1,8-cineole, linalool, benzyl alcohol, α-copaene and limonene. To this list, we can add some compounds that have been detected as main compounds in the odours of a single species, such as oxygenated derivatives of linalool, 6-methyl-5-hepten-2-ol, and 4-methylanisole (Gibernau et al. 1997, Gu et al. 2012, Hossaert-McKey et al. 2016).

Previous studies demonstrate that chemical mediation plays a key role in partner encounters, allowing wasps to locate host plants and find floral rewards, *i.e.* breeding sites (Grison-Pigé et al. 2002, Proffit et al. 2007, Proffit et al. 2009, Hossaert-McKey et al. 2010). A complementary perspective is that specialization in the fig-fig wasp association may result from the integration of several filters, including species-specific odours, non-volatile cuticular chemistry, and ostiole size, into a series of sensory-mechanical filters that exclude non- adapted visitors (Noort and Compton 1996, Raguso 2008, Gibernau et al. 1998). Some data shows that the odors emitted by closely related fig species can be very similar (Grison et al. 1999, Wang et al. 2016). In addition, a case of convergent evolution in floral scent has been reported in the fig-fig wasp system. In South Africa, two sister lineages of wasps, *Elisabethiella*

stuckenbergi and *E. socotrensis*, pollinate *Ficus natalensis* but *E. stuckenbergi* also regularly pollinates *Ficus burkei*. The two wasp species co-occur in individual trees of *F. natalensis* throughout KwaZulu-Natal. The floral volatile blends emitted by *F. natalensis* in KwaZulu-Natal were similar to those emitted by *F. burkei* and different from those produced by other sympatric African *Ficus* species. The fig odour similarity suggests evolutionary convergence to attract particular wasp species (Cornille et al. 2012).

Fig odours and plant-insect coevolution

In the highly specialized fig and fig wasp system, evidence shows that species specific communication is ensured either through a private channel of uncommon or unique compounds (Chen et al. 2009) or, more frequently, through a mixture of a few compounds in which their relative proportions are determinant (Gibernau 2016, Proffit et al. 2020). The specific odour signature is specifically targeted by the pollinators. Hence, reproduction and, as a consequence species survival, of the wasp and fig tree are intimately linked. If floral scents and insect responses are locally coadapted, these chemical cues could function as important isolating traits among populations, and thus function as drivers of diversification (Suinyuy et al. 2015).

Geographic variation of floral scent has been documented in the fig-fig wasp system (Soler et al. 2011, Rodriguez et al. 2017) as well as other brood site pollination mutualisms (Suinyuy et al. 2012, Suinyuy et al. 2015, Cruaud et al. 2017). For instance, *Ficus racemosa*, an extremely wide-ranging *Ficus* species, and its pollinating wasps were structured into four large, vicariant populations which may correspond to distinct (sub)species, located in India, China-Thailand, Borneo, and Australia (Bain et al. 2016). Chemical analysis revealed that it produces a same odour from China to Thailand but a different one in India (Soler et al. 2011). This geographic structuring corresponds to pollinator species and plant genetics co-structuring, but also to climatic and geographic barriers. Conversely, in *F. septica*, a small freestanding tree distributed throughout insular South East Asia, the receptive fig odour varied among islands (Rodriguez et al. 2017). The pattern is similar to the co-structuring of plant gene pools with pollinator

species. However, receptive fig odours in the Negros differed from those from Luzon, while the plants belonged to the same gene pool and were pollinated by the same pollinating wasp species. Hence, do we have local or island-level evolution of plant-insect communication? We have no clue as to the evolutionary mechanisms responsible for odour diversification. How should we understand the forces governing geographic variation in plant- insect communication and their biological consequences?

In the two cases outlined above*, F. racemosa* and its pollinators are high dispersal species, limiting differentiation (Bain et al. 2016), while *F. septica* and its pollinators present reduced gene flow between islands (Rodriguez et al. 2017).

Hence, our hypotheses are:

When gene flow is high, there is no response to local selection, floral scents remain homogeneous over long distances (in *Yucca*, *F. racemosa*).

When plant species present reduced gene flow and share pollinators, their floral scents converge with those of other plants with which they share pollinators (*Encephalartos* cycads, in the *Ficus* species *Ficus natalensis-F. burkei*, *F. auriculata-F. oligodon*).

When the pollinators are specialized on a single host, and gene dispersal is limited in both plants and insects, receptive inflorescence evolution may involve:

local plant-insect coevolution,

or plant odour evolution with pollinators adjusting to this variation,

or the evolution of insect sensory bias with plant odours evolving in response to this bias.

To try to separate between options 1, 2, and 3, we investigate, in a continental setting, three widespread Asian dioecious *Ficus* species presenting low to intermediate gene flow: *Ficus hispida*, *Ficus hirta* and *Ficus triloba*. *Ficus hispida* belong to subgenus *Sycomorus*, while *F. hirta* and *F. triloba* are sister species, but very different in size, belonging to subgenus, section *Eriosycea*. The study species are presented in Figure 1.2, 1.3 and 1.4.

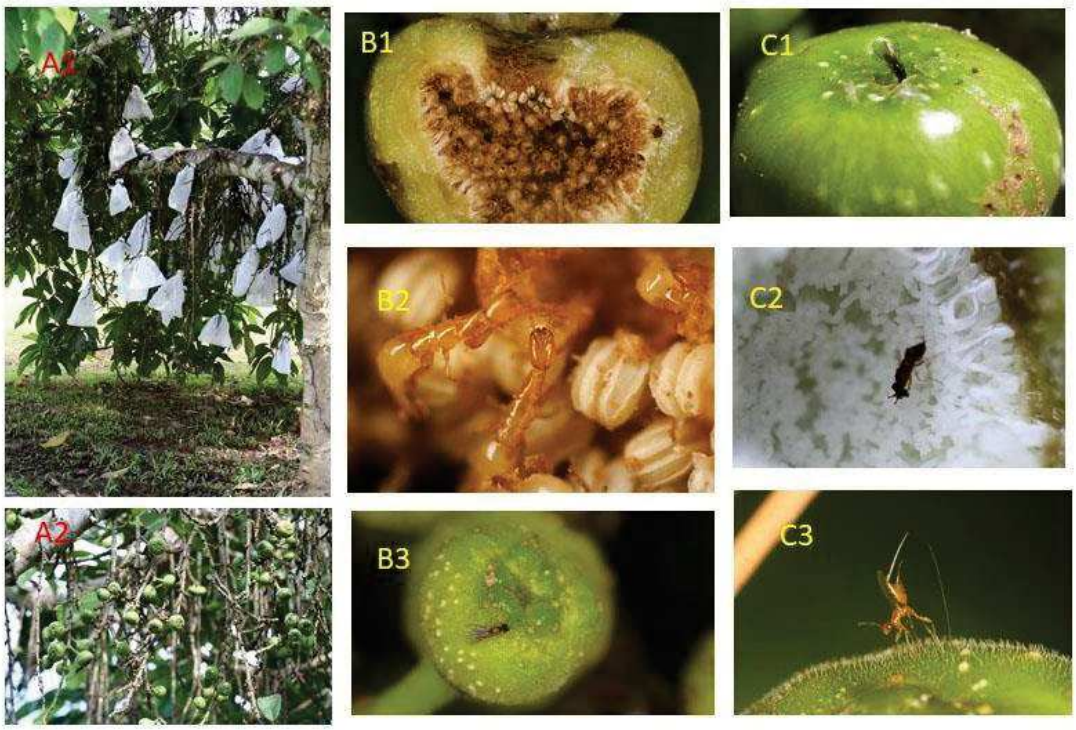


Figure 1.2 *Ficus hispida* and its fig wasps in China (A1) A *Ficus hispida* tree (A2) Branches laden with inflorescence. (B1) Wasps are emerging from their galls. (B2) Male wasps. (B3) Female wasp. (C1) A female wasp entering a receptive fig through the ostiole. (C2) A female wasp pollinating while trying to lay eggs in a female fig (C3) A non-pollinating female wasp laying eggs.

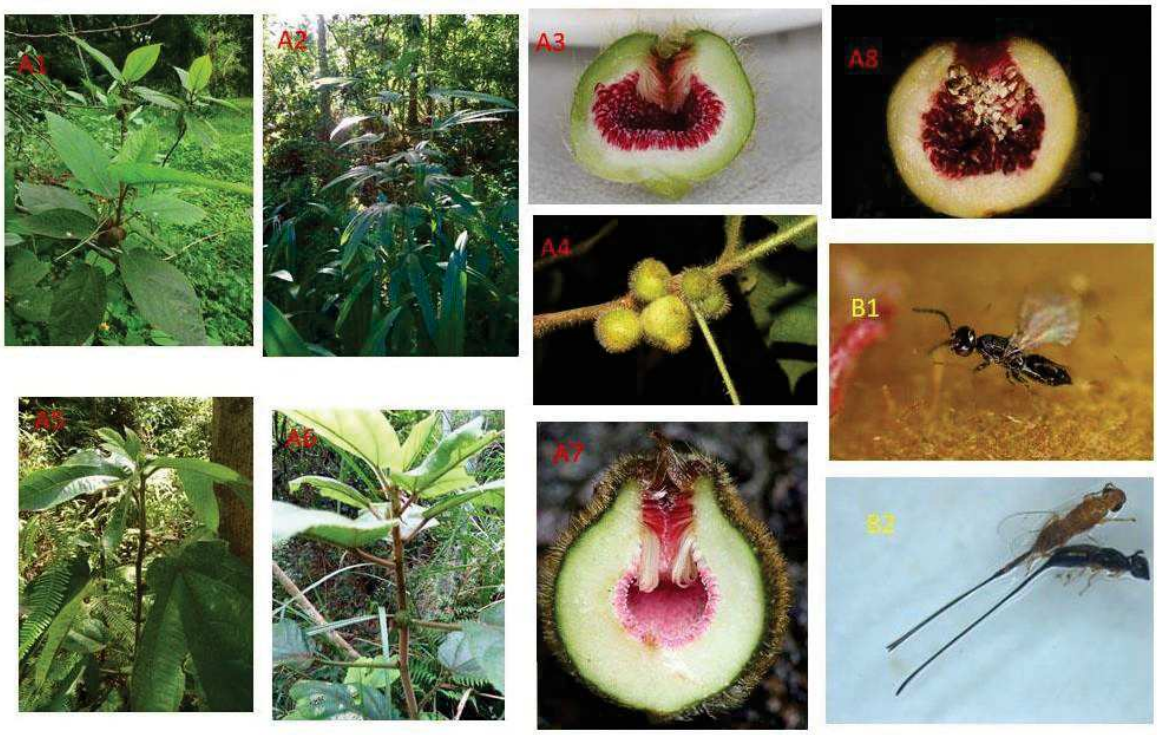


Figure 1.3 *Ficus hirta* and fig wasps in China. (A1) *Ficus hirta* growing in Guangzhou. (A2) *Ficus hirta* growing in Hainan. (A5) *Ficus hirta* growing in Jiangxi province. (A6) *Ficus hirta* growing in Fujian province. (A3) Receptive fig in the south of *F. hirta*'s distribution. (A7) Receptive fig in the north of its distribution. (A4) A branch bearing figs. (A8) Wasps are emerging from their galls. (B1) A female pollinating wasp. (B2) Female non-pollinating wasps.

Figure 1.4 *Ficus triloba* and its pollinating wasp (A1) A *Ficus triloba* tree (A2) A branch laden with figs (B1) Stigma in a receptive female fig (B2) A female pollinating wasp

II. Concise presentation of the results

Detailed results, references and specific discussions are provided in the paper manuscripts. The aim of this chapter is to present a general overview of how the different results obtained and/or analyzed during my PhD form a coherent set. They provide answers as to how receptive fig odors vary geographically within species and between closely related species, on the evolutionary origin of this variation and on its biological consequences.

In order to understand the forces governing geographic variation in plant-insect communication and their biological consequences we need to establish the spatial genetic structure of both the host plant and its pollinating insects. We did this for *Ficus hirta* and its pollinating wasps in *Hui Yu, Enwei Tian, Linna Zheng, **Xiaoxia Deng**, Yufen Cheng, Lianfu Chen, Wei Wu et al. "Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation." Molecular Ecology 28, no. 9 (2019): 2391-2405.*

Previous data have shown that *Ficus hirta* Vahl, a widely distributed species, presents a pattern of spatial genetic structure suggesting genetic isolation by distance across continental South- East Asia. However, a more rapid rate of speciation among fig wasps, compared with their hosts, is to be expected given their much shorter generation times. Two morphologically distinguishable pollinator fig wasp morphotypes, one in Java and one in Hong Kong, have been described from the figs of *F. hirta*, suggesting more than one pollinating wasp species. Hence, we tested the hypothesis that *F. hirta* supports several pollinator species across its range. Our results confirm that across its range *F. hirta* displays clinal genetic variation. It is pollinated by nine parapatric wasp species, with one accidental pollinator in Singapore and eight species belonging to the *Valisia javana* species complex. The parapatric distribution of pollinators suggests competitive exclusion between pollinator species: a local population of trees does not seem to be capable of sustaining more than one local species of pollinator. Local pollinator diversity is constrained by the habitat and not by speciation. The

geographic distribution of the pollinator species does not correlate with host plant genetic structure. Therefore, it will be possible to establish whether plant spatial structure or insect spatial structure drives spatial variation in plant signaling, *i.e.* in receptive fig odors.

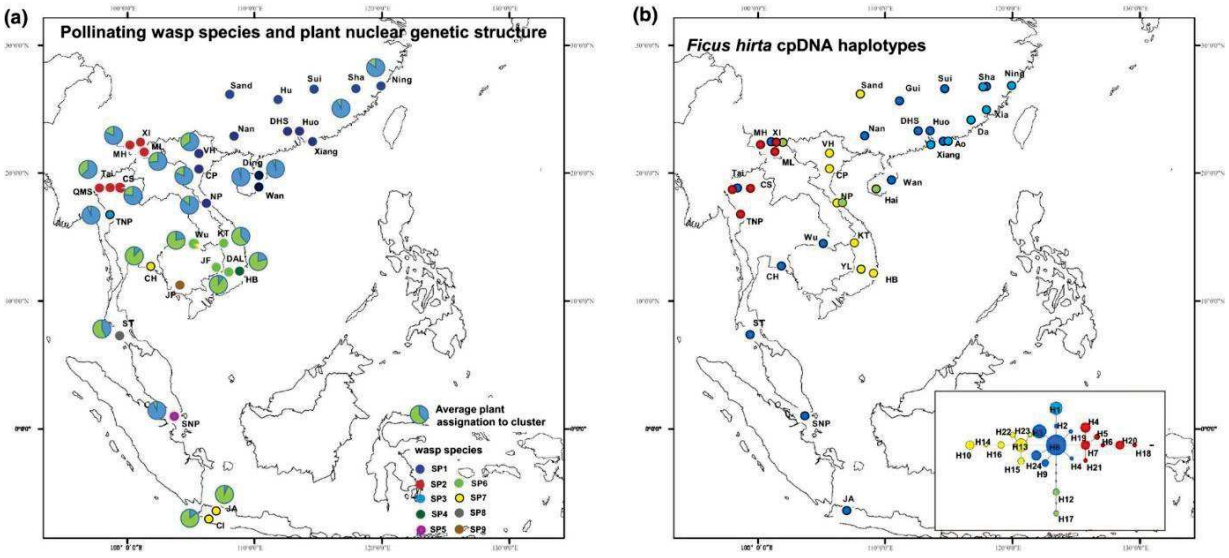


Figure 2.1. Spatial structure of *Ficus hirta* and its pollinating wasps. Panel (a) the distributions of the pollinator fig wasp species associated with *Ficus hirta* and genetic variation in *F. hirta*. Panel (b) the distributions of cpDNA haplotypes of *F. hirta*. Note the disjoint distribution of sp7, the allopatric distribution of the closely related (according to COI data) sp2 and sp9 and the parapatric distribution of the closely related (according to COI data) sp4, sp6 and sp7. For cpDNA, colours are used to show the localized distribution of haplotype H1 and of the different branches of the haplotype network.

To confirm that pollinator species turnover is controlled by competition between pollinator species and not by some plant trait that we did not detect, we investigated the spatial structure of non-pollinating fig wasps associated with *F. hirta* in ***Xiaoxia Deng****, Lianfu Chen, Enwei Tian, Dayong Zhang, Tanming Wattana, Hui Yu, Finn Kjellberg, and Simon T. Segar. "Low host specificity and broad geographic ranges in a community of parasitic non-pollinating fig wasps (Sycoryctinae; Chalcidoidea)." Journal of Animal Ecology (2021).*

Non-pollinating fig wasps (NPFW, belonging to several families of Chalcidoidea) include flower gallers, cleptoparasites and parasitoids. A single wasp larva develops per flower ovule.

NPFWs are generally associated with a single *Ficus* species. Some biological data suggest they disperse more efficiently than pollinators (Sutton etal. 2016), while other data (including our data) suggest they are less dispersive (Kjellberg and Proffit 2016). The local composition of the community of wasps associated with *Ficus hirta* figs is constituted throughout its range by one *Philotrypesis* (a cleptoparasite of the pollinator) and one *Sycoscapter* (a parasitoid of the pollinator) and both oviposit more than a week after the pollinators. NPFWs are also attracted by fig odor, but only those ovipositing at fig receptivity are attracted by receptive fig odors. We document 3 parapatric species of *Sycoscapter* and 4 parapatric species of *Philotrypesis*. On Java, *Sycoscapter* is replaced by a species of *Sycoryctes* and *Philotrypesis* is absent. The distribution limits of the *Valisia*, *Philotrypesis* and *Sycoscapter* species differ. Hence, local wasp community structure is constant throughout most of the range of *Ficus hirta*, while the local species composition vary independently for the different members of the community, and independently from plant genetic structure. There is no hidden structure in *Ficus hirta* that would impose the distribution of its vicariant pollinator species.

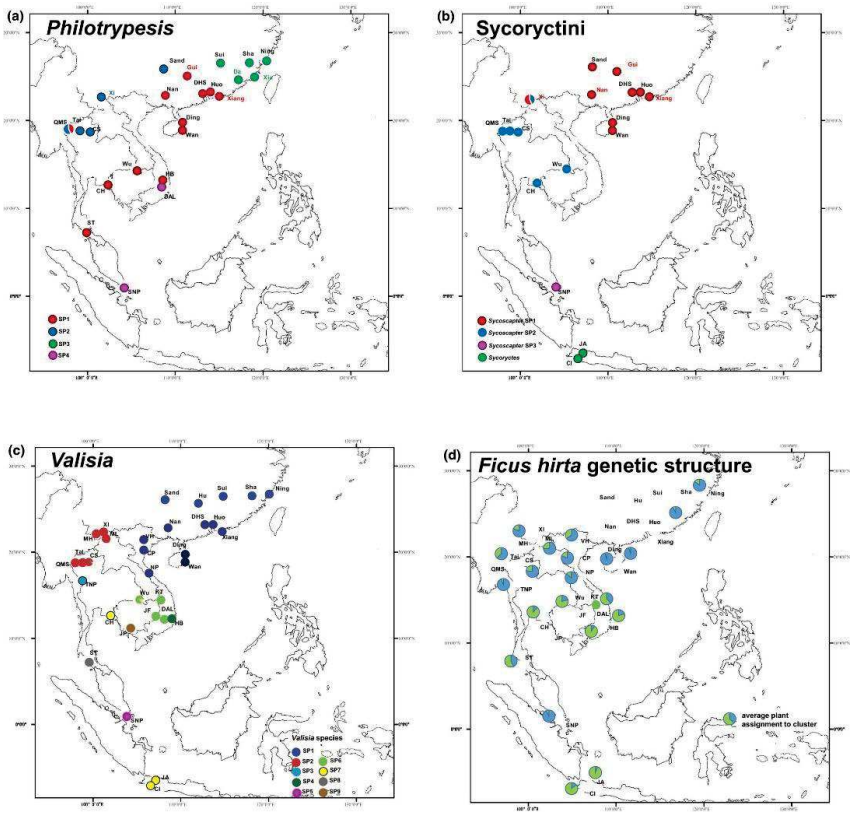


Figure 2.2. Geographic distribution of the three sets of fig wasps associated with *Ficus hirta* and genetic variation of the plant. Panel (a) cleptoparasitic wasp-species distribution, genus *Philotrypesis*; panel (b) parasitoid wasp-species distribution, tribe Sycoryctini; panel (c) pollinating wasp-species distribution, genus *Valisia*; panel (d) host plant spatial genetic structure. Locations indicated with colored lettering are those for which species assignment was based on SSR genotypes. For the plant, genetic variation is clinal and is illustrated here by percentage of assignment to two extreme gene pools.

In China, *Ficus hirta* is pollinated by one population of *Valisia javana hilli* (sp1) in continental South China extending to Fujian province, it is pollinated by another population of *V. javana hilli* in Hainan Island and it is pollinated by *Valisia* sp2 in Yunnan while *F. hirta* presents clinal genetic variation across its range. This allows determining whether receptive fig odors vary geographically in accordance to plant genetic variation or in accordance to pollinating wasp structuring into species or population. This is the topic of ***Xiaoxia Deng***, *Bruno Buatois, Yan- Qiong Peng, Hui Yu, Yufen Cheng, Finn Kjellberg, and Magali Proffit. "Plants are the drivers of geographic variation of floral scents in a highly specialized pollination mutualism: a study of* Ficus hirta *in China." (2021).*

We show that receptive fig odors do not vary according to pollinator species or population. Receptive fig odors follow a pattern of differentiation by distance, similar to the spatial genetic structure of *Ficus hirta*. We conclude that the plant is the driver of receptive fig odor variation and the wasps have to adjust locally.

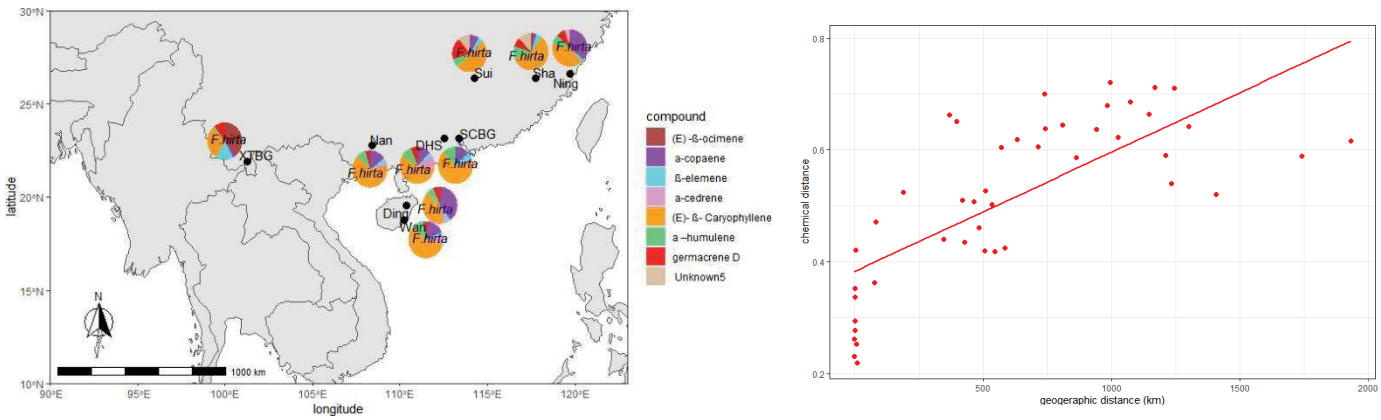


Figure 2.3. Geographical variation of volatile compounds of *Ficus hirta* in the different study sites. Panel a) pie charts depict the percentage in local receptive fig odors of the compounds that represent more than 5% in the average bouquet of scents in at least one site. Panel b) correlation between chemical distance and geographic distance including all the samples at all locations (Mantel statistic r=0.4897, p<0.001).

The pollinating wasps locate receptive figs of their host species, despite receptive fig odor variation (Deng et al. 2021). This variation affected mainly the relative proportions of the constituent volatile organic compounds (VOCs) rather than the presence absence of some compounds. Is pollinating fig wasp attraction to receptive figs fine-tuned to a particular blends of VOCs or do they respond to a broad range of variation in relative concentrations? We investigate this question, using a model system located close to the laboratory, the association between the dioecious *Ficus carica* and *Blastophaga psenes* in *Magali Proffit, Magali, Benoit Lapeyre, Bruno Buatois,* ***Xiaoxia Deng****, Pierre Arnal, Flora Gouzerh, David Carrasco, and Martine Hossaert-McKey. "Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction." Scientific Reports 10, no. 1 (2020): 1-11.*

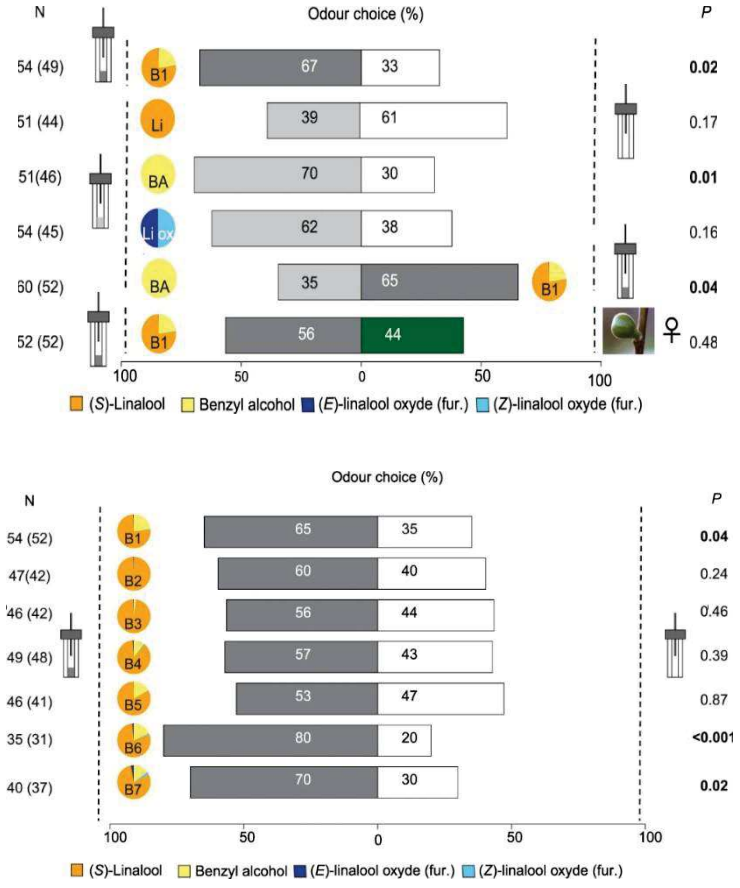


Figure 2.4. Attraction of *Blastophaga psenes* females by blends presenting different proportions of four VOCs. Tests were conducted in Y-tube olfactometers in which female wasps were allowed to choose between synthetic combinations of the four VOCs in different proportions, in one branch, and control odor in the other. For the four VOCs detected by the pollinating females, proportions in each odor source are indicated in the pie chart. The highest attraction rats were achieved for mix B6 and B7, those presenting the closest match of proportions with receptive fig odors. Number of wasps tested (N), number of individuals that made a choice (in parentheses), and P-values (binomial probability under the hypothesis of equal chances of choosing the two branches of the olfactometer) are indicated for each comparison.

We show that *Blastophaga psenes* detects 5 VOCs in receptive fig odors, and these VOCs constitute the bulk of the receptive fig odor. The wasps were only attracted by a mix of VOCs mimicking the proportions found in receptive figs. The geographic variation in the receptive fig odors to which a pollinating wasp species is exposed, that we demonstrate here in *Ficus hirta*, and that has also been demonstrate previously in *Ficus septica*, probably entails that the wasps are adapted to the local mix of VOCs. It suggests that they are not attracted by non-local receptive fig odors. We tested this hypothesis using *Ficus hispida* as a model system in ***Xiaoxia Deng****, Gang Wang, Yan-Qiong Peng, Hui Yu, Aroonrat Kidyoo, Ting Zhang, Finn Kjellberg, Magali Proffit. "Geographic variation in plant-insect signalling: the Achilles heel of the obligate fig-fig wasp mutualism when exposed to climatic accidents?"(submitted).*

We show that receptive fig odors vary geographically *in Ficus hispida*. In particular, receptive fig odors differed between two Chinese locations, the South China Botanical Garden in Guangdong province and the Xishuangbanna Tropical Botanical Garden in Yunnan province. *Ficus hispida* is pollinated by the same wasp species, *Ceratosolen marchali* in those two locations.

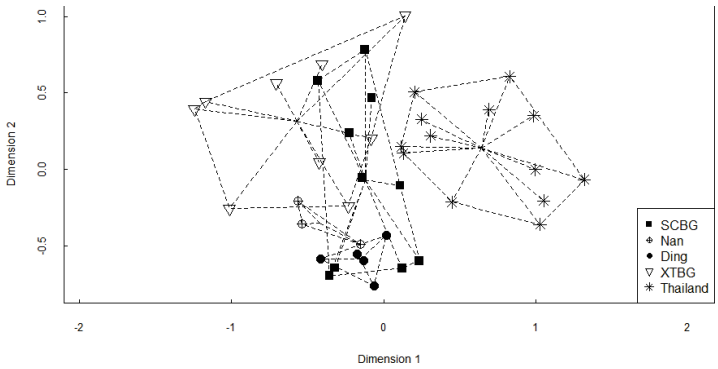


Fig. 2.5. Non-metric multi- dimensional scaling of the relative proportions of VOCs in the global bouquets emitted by receptive figs of *F. hispida* in the different localities based on Bray-Curtis dissimilarity Index (stress=0.190). Samples are grouped (dashed lines) by locality of figs and the centroid of each group is indicated.

We then tested in a Y tube olfactometer the attraction of *Ceratosolen marchali* wasps from these two locations by local and non-local receptive figs. We show that the wasps belonging to a same species were only attracted by the local receptive fig odors from their place of origin.

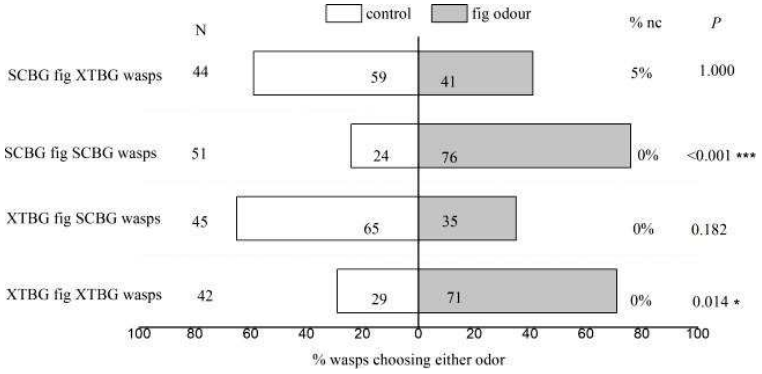


Figure 2.6. Behavioural responses of female *Ceratosolen marchali* fig wasps when confronted with odors of local and non-local receptive figs in Y-tube experiments. Fresh receptive figs were used as odor sources. Behaviour choice to scent combinations in Y- tube test are as follows: nonlocal host versus air and local host versus versus air, (binomial test). control: purified air; N: Numbers of tested fig wasps; NC: percentage of unresponding fig wasps (*P<0.05; ***P<0.001)

Hence, *Ficus* represent an extreme case of pollination specialisation with pollinators only recognising receptive figs of their local or regional host population. Nevertheless, there are reports of wasps locally visiting receptive figs, or of wasps attracted by receptive figs in Y tube olfactometer tests, of close relatives of their host species. Throughout its distribution, *Ficus hirta* co-occurs with a close relative, *Ficus triloba*. In Guangdong province, the local pollinator of *Ficus hirta*, *Valisia javana hilli* was observed to visit figs of *F. triloba* while it has as yet to be detected in figs of *F. triloba* in other locations. We investigate this situation in ***Xiaoxia Deng**, Yufen Cheng, Hui Yu, Magali Proffit, Finn Kjellberg. "Limited interspecific divergence in olfactive signalling coupled with geographic variation may result in localised pollinator sharing between closely related* Ficus *species". (in preparation).*

Closely related fig species may have similar odours because of phylogenetic inertia. To test this assumption, we collected the odour of *F. triloba* throughout its 1200 km range in south China. We show that receptive fig odors of *Ficus triloba* and *F. hirta*, while different, contain the same main VOCs: every compounds representing over 5% of receptive fig odor in one species in one locality was also detected in at least one extract of the other species. Conversely the receptive fig odors of *F. triloba* and *F. hirta* were strikingly different from those of *F. hispida*, as distantly related *Ficus* species.

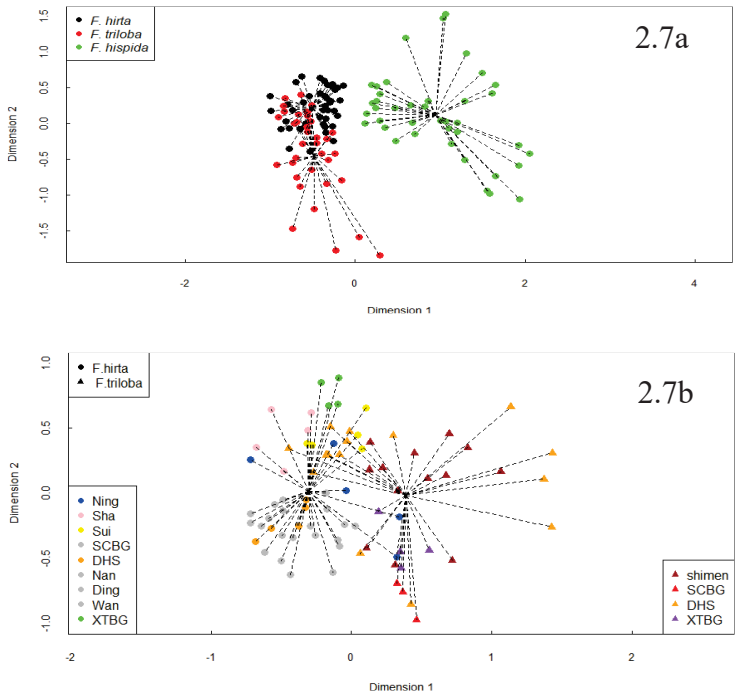


Fig. 2.7. Receptive fig odor variation within and among species. Panel a) comparison between *Ficus triloba*, *F. hirta* and *F. hispida*. Panel b) comparison between *F. triloba* and *F. hirta* from different locations. Non-metric multi-dimentional scaling representation of the relative proportions of VOCs in the odors emitted by individual plants based on Bray-Curtis dissimilarity Index (stress=0.18 for 2a and stress= 0.20 for 2b).

In Dinghu mountain, *V. javana hilli* was found to frequently develop in figs of *F. triloba* while *V. esquirolianae* was not observed to develop in figs of *F. hirta*. We tested the attraction of *Valisia javana hilli* and *V. esquirolianae* from Dinghu mountain, to local receptive figs of the two species. When confronted with a choice between clean air and receptive fog odors, both species were only attracted by figs of their usual host species. However, when given a choice between receptive fig odor of the two species, *V. javana hilli* was equally attracted by figs of the two species. In places where *V. javana hilli* is stimulated by *F. hirta* receptive fig odours, it may also visit *F. triloba*.

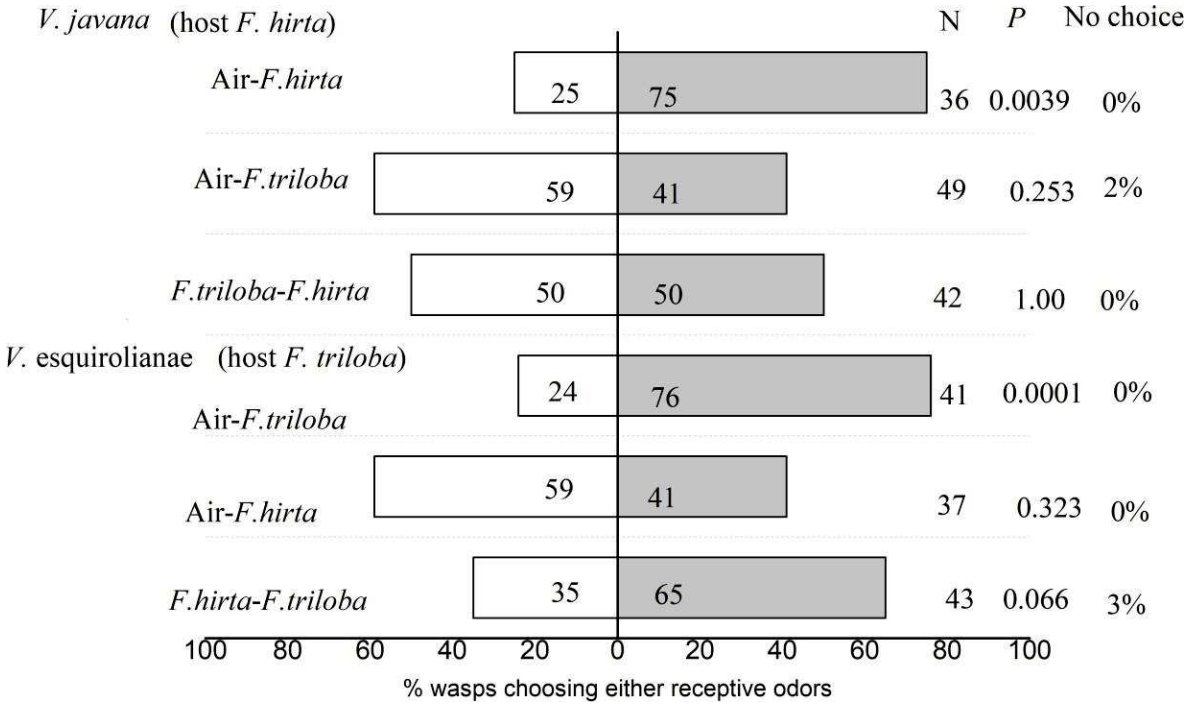


Fig. 2.8. Choices made by *Valisia javana hilli* and *V. esquirolianae* when confronted with different odour sources (receptive figs) in a Y-tube olfactometer. We used binomial tests for statistical comparisons between the number of choices for odour versus clean air or choice between odours of the two *Ficus* species. N: number of tested wasps. P: probability, two tailed. No choice: percentage of insects that did not make a choice.

We conclude that geographic variation in receptive fig odors, as illustrated below, and local co- occurrence of the two host species may result in overlap in the odours produced by the two *Ficus* species up to a point where one or the other species of pollinator may locally visit both *Ficus hirta* and *F. triloba*. This may occur despite the sensitivity of the wasps to the relative proportion of the different VOCs it percieves. It will only occur between *Ficus species* sharing a sufficient portion of their olfactive signalling, *i.e.* between closely related species.

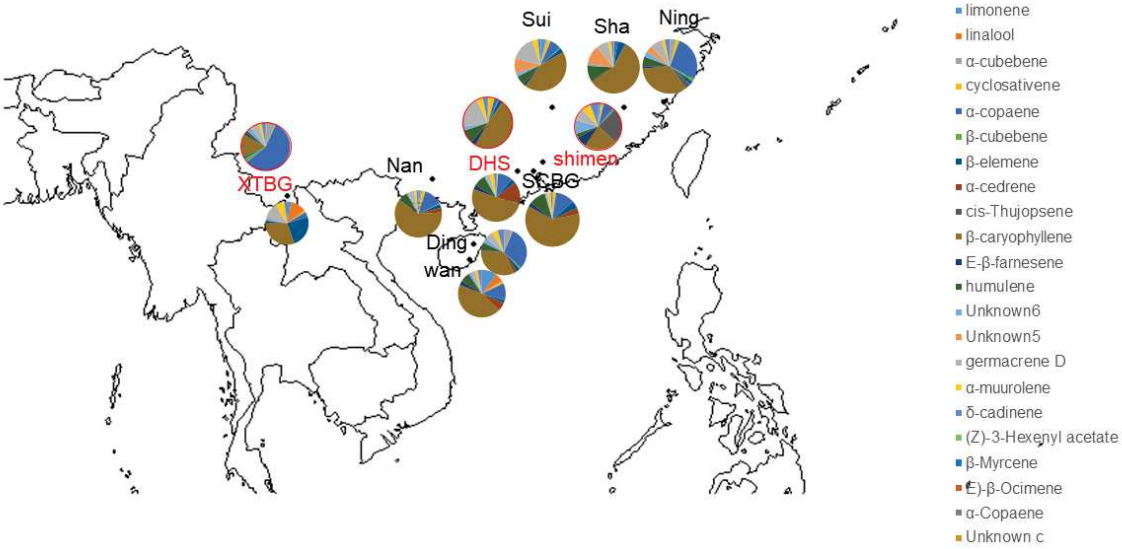


Fig. 2.9. Geographic variation in receptive fig odour composition for *Ficus hirta* and *Ficus triloba*. The pie charts depict the local representation in receptive fig odors of the compounds that represent more than 5% in the average bouquet of odors of at least one species in at least one site.

In summary, we have shown that geographic variation in receptive fig odors corresponds to plant genetic structure and not to pollinator genetic structure. Geographic variation of receptive fig odors affects the insect's capacity to locate non-local receptive figs as the insects are adapted to the local odors. Nevertheless, the olfactive signaling of related *Ficus* species may overlap so that in some locations, pollinators may be attracted by non-host receptive figs.

General Discussion, Conclusions, and Perspectives

This work explores the forces governing geographic variation in plant-insect communication and their biological consequences, focusing on the relationship between receptive fig odour and host plant and pollinating fig wasp genetic structure and wasp behaviour. We focus on the current ongoing diversification processes in three widely distributed dioecious *Ficus* species, namely *Ficus hispida*, *Ficus hirta*, and *Ficus triloba* in a continental setting. Figs and their pollinating wasps (Agaonidae) constitute one of the most tightly integrated pollination mutualism known, originating some 70 million years ago (Cruaud et al. 2012). Empirical studies have pointed out the decisive role of odourodours emitted at anthesis and composed of a mixture of volatile organic compounds (VOCs) in the olfactive attraction of pollinators in brood site pollination mutualisms (Dotterl et al. 2006, Chen et al. 2009, Hossaert-McKey et al. 2010, Svensson et al. 2010, Song et al. 2014, Proffit et al. 2020, Terry et al. 2021). Integrating inflorescence odourodours, pollination ecology and population genetics have opened up new possibilities for understanding the functioning and evolution of these specialized systems. After the investigations on the above three *Ficus* species, several conclusions and generalizations can be proposed.

Several lines of evidence obtained through interspecific comparisons of brood site pollination mutualism indicate a strong role for pollinator-driven floral scent evolution. For instance, when two plant species interact with the same group of pollinators, the composition of the odourodour emitted at anthesis converges (Svensson et al. 2011, Cornille et al. 2012, Huang et al. 2015, Wang et al. 2016). Clearly, pollinators mediate selective pressures on the evolution of floral odourodours in the sense that they are attracted by these odours and their behaviour determines plant reproductive success. It has been suggested that any variation in their occurrence and/or their impact on plant reproduction should also influence the force and the direction of selection

on plant signalling (Delle-Vedove et al. 2017). However, empirical studies, including our results, show that pollinator-driven selection does not explain geographical variation in olfactive signalling in obligate brood site pollination mutualisms (Knudsen 2002, Svensson et al. 2005, Suinyuy et al. 2012, Rodriguez et al. 2017, Friberg et al. 2019, Deng et al. 2021). For instance, recent data in *Lithophragma* shows high floral odour similarity between closely located populations within species but not among closely located populations of plant species belonging to different lineages but sharing pollinators (Friberg et al. 2019). These results do not support pollinator-driven selection of floral odours in *Lithophragma*. Evidence also shows that different plant chemotypes producing extremely different cone volatiles are associated with a same pollinating insect assemblage in the African cycad *Encephalartos villosus* (Suinyuy et al. 2012). Indeed, *E. villosus* is pollinated by different pollinator assemblages according to location but mainly by a single species, *Porthetes sp*., in all locations (Donaldson 1997, Suinyuy et al. 2012). The intraspecific variation in cone volatile composition among populations suggests local convergence of receptive inflorescence odours between cycad species sharing pollinators and pollinators evolving response to the local receptive inflorescence odours (Suinyuy et al. 2012). However, we cannot confirm whether variation in cone volatile composition matches cycad plant spatial genetic structure. Indeed, limited molecular data is available for *Encephalartos* due to notoriously low genetic variation within the genus (Treutlein et al. 2005), and among cycads in general (Ellstrand et al. 1990, González and Vovides 2002). Similarly, in *Encephalartos ghellinckii Lem*, a species present in habitats presenting wide temperature variation from freezing cold with snow in winter in the Drakensberg to extreme heat and drying winds in summer in the populations closer to the coast (Suinyuy and Johnson 2018), the observed pattern of cone odour variation suggests the occurrence of two different chemotypes, a mountain chemotype and a lowland chemotype (Suinyuy and Johnson 2018, Suinyuy and

Johnson 2020), which corresponded with differences in insect assemblages, but with the presence of shared pollinator species (Suinyuy and Johnson 2018).

In our study, *Ficus hirta* is pollinated by one population of *Valisia javana* hilli (sp1) in continental South China extending to Fujian province, it is pollinated by another population of *V. javana hilli* in Hainan Island and it is pollinated by sp2 in Yunnan while *F. hirta* presents clinal genetic variation across its range. We evidenced progressive geographic divergence of receptive fig odours in *F. hirta*, which corresponds with plant genetic structure. Hence, our results show that geographic variation of receptive fig odour corresponds to plant genetics and not to insect genetic structure. This suggests that plants evolve first and then the wasps have to adapt to local odours. Plants are the drivers of geographic variation of floral odours and insects the followers (Deng et al. 2021). Although the genetic structure of *F. hispida* is not yet available, unpublished molecular data evidence clinal genetic variation and genetic and morphological data on the insects have confirmed that in SCBG and XTBG *F. hispida* is pollinated by the same pollinating wasp, but in Thailand by a different species. Our results show that *F. hispida* emitted a similar odour in south China mainland and Hainan island pollinated by a different assemblage of fig wasps while floral odours varied between SCBG and XTBG pollinated by the same fig wasp species. Hence, our results show that odour variation does not correlate with pollination by different pollinator species.

We hypothesize that in the larger, probably more dispersive, *F. triloba*, we will observe less geographic variation in receptive fig odours. Further work on the species is required to test the hypothesis. Indeed, geographic variation in receptive fig odour seems to correlate negatively with plant gene flow. In flowering plants, gene flow occurs through the movement of pollen and seeds, with pollen flow often contributing most (Campbell 1991). Long-distance pollen flow between fig trees (*Ficus* spp., Moraceae) has been documented (Harrison and Rasplus 2006, Ahmed et al. 2009), while routine dispersal of wasp pollinators in a tropical rainforest

was estimated at 6–14 km (Nason et al. 1998). Accumulating data on brood site mutualisms show that more dispersive species present less spatial structuring of receptive odours (Svensson et al. 2005, Soler et al. 2011, Bain et al. 2016, Friberg et al. 2019), comparatively to limited dispersal species (Suinyuy et al. 2012, Rodriguez et al. 2017, Friberg et al. 2019). In *Encephalartos villosus*, pollinators lost a substantial amount of pollen within a few hours after they left the pollen shedding cones and plants situated over 5 km from source populations were not visited by insect pollinators (Donaldson 1997, Suinyuy et al. 2012). Long-range pollen dispersal between plant patches (> 5 km) appears to be uncommon in *E. villosus*, facilitating the evolution of strong interpopulation variation in cone odours (Suinyuy et al. 2012). Extreme diversification of floral odours within species is likely to be associated with limited pollen and seed dispersal in *Lithophragma* (Furches et al. 2008, Friberg et al. 2019). In *Yucca*, the homogeneity of floral odours among populations is consistent with the prediction of extensive pollen-mediated gene flow among host populations by dispersing female moths (Massey and Hamrick 1998, Svensson et al. 2005). Nevertheless, analyses have shown that most pollen was transferred to flowers of the same plant or neighboring plants, and the quantity of pollen transferred declined rapidly with distance from the source (Marr et al. 2000). Although the study showed restricted dispersal in moths, the method used could not detect long-range dispersal, and therefore the frequency of such events remains unknown. The important question is what is the contribution of long-range dispersal events to the genetic structure and do these events limit the drift that could lead to local differentiation?

The floral odour often differs between sympatric closely related species, suggesting that pollinator behavior mediated by sensory traits can be a key factor for the establishment of reproductive isolation between co-occurring closely related plant species (Okamoto et al. 2007, Svensson et al. 2016, Okamoto and Su 2021). Reciprocally, allopatrically distributed closely related species, such as *Glochidion obovatum* and *G. rubrum* (Kawakita and Kato 2004), may

share a same floral odour (Okamoto et al. 2007). The same is true for the *Yucca*-*Yucca* moth mutualism, where yucca species that have non-overlapping ranges may share the same floral odour while sympatrically occurring species with overlapping flowering phonologies from other sections of the genus *Yucca* presented differences in their floral odours (Svensson et al. 2011, Svensson et al. 2016). These studies support the idea that pollinators often discriminate among sympatric hosts based on chemical cues. Nevertheless, in a number of cases, other mechanisms are at work, such as morphological incompatibility or the plants growing in different habitats (Cornille et al. 2012, Wang et al. 2016, Souto-Vilarós et al. 2018).

Here, although floral odour composition differed between *F. hirta* and *F. triloba* our results nevertheless revealed interspecific similitude in fig odours despite widespread co-occurrence of these two closely related species. Receptive fig odours vary geographically in *F. hirta*. In Guangdong, Yu et al. (unpublished data) observed pollinators of *F. hirta* developing in the figs of *F. triloba*. In Dapu, in north-east Guangdong, about 100 km away from the northern limit (Nanjing, south Fujian) of *F. triloba*, its pollinators were attracted to both hosts (Yu et al. unpublished data). From Dapu, northwards, *F. hirta* is also morphologically more similar to *F. triloba* presenting larger figs with thicker fig walls (Yu et al. 2018). Further studies will be required to establish whether there is genetic introgression between the two species and whether receptive fig odours are less differentiated than in other locations. In a somewhat different situation in *Yucca*, there was limited but significant floral odour differentiation between two parapatric sister species, *Yucca brevifolia* and *Yucca jaegeriana* (Svensson et al. 2016). In the contact zone between the two species, their respective specific pollinators visit both *Yucca* species indiscriminately. In introgressed plant individuals the correlation between receptive floral odours and floral morphology was broken (Svensson et al. 2016). Hence, receptive odours differences between

closely related species are not absolute barriers to interspecific pollen flow in brood site pollination mutualisms.

Only a few studies evaluate whether nursery pollinators may be attracted by the odours of non- host plants. Some cases of weak olfactory-based host preference have been reported. For example, despite the marked difference in receptive fig odours between two sympatric varieties of *Ficus semicordata*, females of their respective *Ceratosolen* fig wasp pollinators were attracted to the odours of both host varieties in Y-tube tests (Wang et al. 2013). Although host specificity was reinforced by contact cues on the fig surface, some wasps entered non-host figs. Flower functional differences may also limit interspecific gene flow as has been shown for orchids sharing pollinators (Gögler et al. 2015). However, a selected morphological divergence in floral anatomy between related *Ficus* species has yet to be evidenced. In nature, some closely related sympatric *Ficus* species produce similar receptive fig odours, and may share pollinators (Wang et al. 2016). Such species usually grow in different habitats (Wei et al. 2014). If most pollinator dispersal is within habitat, between plants of a same species, there may be only limited interspecific pollination despite pollinator sharing.

Perspectives

Our work shows that even in a highly specific obligate mutualism, where the odour emitted by the plant is necessary and central in its specific partner attraction, the scent can be extremely diversified geographically or can be constant over large geographical scales. We propose as a working hypothesis that this variation will be correlated negatively with the plant's gene flow. We predict that receptive fig odours present limited variation in monoecious *Ficus* species as their pollinators disperse by drifting in the wind above the canopy (Harrison and Rasplus 2006). Among monoecious species, those pollinated by wasps drifting highest above the canopy should be the least structured. We also predict that closely related monoecious species will be less likely than dioecious *Ficus* species to present similar receptive fig odours, because of strong

pollinator dispersal. Reciprocally we predict more spatial structure in dioecious *Ficus* species and especially in small sized species. Indeed, small individuals produce small fig crops, that probably require the wasps to do more fine grained searches for receptive figs as signalling by individual receptive figs will be limited. This is particularly true for the pollinators of small understory *Ficus* species that cannot disperse by drifting in the wind as the pollinators of large monoecious *Ficus* species. There will also be little selection for the divergence of receptive fig odours between closely related dioecious *Ficus* species if they occupy different habitats. Indeed, limited wasp dispersal will automatically limit pollen transfer between habitats. Hence, the *Ficus*-pollinating wasp mutualism provides an extensive set of predictions that can be tested in future work.

Another perspective has to do with variation in receptive fig odour response and VOC perception by the wasps. Gas chromatography coupled with electroantennographic detection (GC-EAD) will allow determining if there is within insect species variation in which VOCs they perceive. Insect behavioural experiments, especially using synthetic compounds will allow determining which mixes of VOCs elicit wasp attraction. Hence, it will be possible to establish whether the VOCs principally contributing to geographical differentiation of receptive odours are compounds that contribute to the olfactory signal that pollinators detect and respond to, or whether such variation is possible due to plasticity in the olfactory response of the pollinator. Fig pollinating wasps have a reduced repertoire of olfactory genes. If the GC-EAD tests detect within species variation in which VOCs are detected, the transcriptomic analysis may enable to establish precisely which genes are involved in detecting particular VOCs.

Bibliography

Ahmed, S., S. G. Compton, R. K. Butlin, and P. M. Gilmartin. 2009. Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. Proceedings of the National Academy of Sciences 106:20342-20347.

Araújo, L. M., A. Valentin-Silva, G. W. Fernandes, and M. F. Vieira. 2017. From anthesis to diaspore dispersal: reproductive mechanisms of rare herbaceous Moraceae species endemic to Brazil. Darwiniana, nueva serie **5**:83-92.

Bain, A., R. M. Borges, M. H. Chevallier, H. Vignes, N. Kobmoo, Y. Q. Peng, A. Cruaud, J. Y. Rasplus, F. Kjellberg, and M. Hossaert-Mckey. 2016. Geographic structuring into vicariant species-pairs in a wide-ranging, high-dispersal plant–insect mutualism: the case of *Ficus racemosa* and its pollinating wasps. Evolutionary Ecology 30:663-684.

Binyameen, M., Ali, Q., Roy, A., & Schlyter, F. 2021. Plant volatiles and their role in insect olfaction. In Plant-Pest Interactions: From Molecular Mechanisms to Chemical Ecology (pp. 127-156). Springer, Singapore.

Borg-Karlson, A.-K., I. Valterová, and L. A. Nilsson. 1993. Volatile compounds from flowers of six species in the family Apiaceae: bouquets for different pollinators? Phytochemistry 35:111-119.

Bouwmeester, H., R. C. Schuurink, P. M. Bleeker, and F. Schiestl. 2019. The role of volatiles in plant communication. The Plant Journal 100:892-907.

Breitkopf, H., P. M. Schluter, S. Xu, F. P. Schiestl, S. Cozzolino, and G. Scopece. 2013. Pollinator shifts between *Ophrys sphegodes* populations: might adaptation to different pollinators drive population divergence? Journal of Evolutionary Biology 26:2197-2208.

Bronstein, J. L., P. H. Gouyon, C. Gliddon, F. Kjellberg, and G. Michaloud. 1990. The ecological consequences of flowering asynchrony in monoecious figs: a simulation study. Ecology 71:2145-2156.

Campbell, D. R. 1991. Comparing pollen dispersal and gene flow in a natural population. Evolution:1965-1968.

Chen, C., Q. Song, M. Proffit, J.-M. Bessière, Z. Li, and M. Hossaert-McKey. 2009. Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*. Functional Ecology 23:941-950.

Chen, Y., and W. Wu. 2010. Volatile compounds from the syconia of *Ficus awkeotsang* Makino and their attractiveness to pollinator wasps. Acta Ecologica Sinica 30: 2212-2219.

Chittka, L., and N. E. Raine. 2006. Recognition of flowers by pollinators. Current opinion in plant biology 9:428-435.

Christenhusz, M. J. M., and J. W. Byng. 2016. The number of known plants species in the world and its annual increase. Phytotaxa 261:201.

Cook, J. M., and J.-Y. Rasplus. 2003. Mutualists with attitude: coevolving fig wasps and figs. Trends in Ecology & Evolution 18:241-248.

Cornille, A., J. G. Underhill, A. Cruaud, M. Hossaert-McKey, S. D. Johnson, K. A. Tolley, F. Kjellberg, S. van Noort, and M. Proffit. 2012. Floral volatiles, pollinator sharing and diversification in the fig-wasp mutualism: insights from *Ficus natalensis*, and its two wasp pollinators (South Africa). Proceedings of the Royal Society B: Biological Sciences 279:1731-1739.

Cruaud, A., N. Rønsted, B. Chantarasuwan, L. S. Chou, W. L. Clement, A. Couloux, B. Cousins, G. Genson, R. D. Harrison, P. E. Hanson, M. Hossaert-mckey, R. Jabbour-zahab, E. Jousselin, C. Kerdelhué, F. Kjellberg, C. Lopez-vaamonde, J. Peebles, Y.-q. Peng, R. A. S. Pereira, T. Schramm, R. Ubaidillah, S. V. Noort, G. D. Weiblen, D.-r. Yang, A. Yodpinyanee, R. Libeskind-hadas, J. M. Cook, J.-y. Rasplus, and V. Savolainen. 2012. An extreme case of plant-insect codiversification: figs and fig-pollinating wasps. Systematic Biology 61:1029-1047.

Cruaud, P., J. Y. Rasplus, L. J. Rodriguez, and A. Cruaud. 2017. High-throughput sequencing of multiple amplicons for barcoding and integrative taxonomy. Scientific reports 7:41948.

Darwell, C. T., S. Al-Beidh, and J. M. Cook. 2014. Molecular species delimitation of a symbiotic fig-pollinating wasp species complex reveals extreme deviation from reciprocal partner specificity. BMC Evolutionary Biology 14:1-10.

Delle-Vedove, R., B. Schatz, and M. Dufay. 2017. Understanding intraspecific variation of floral scent in light of evolutionary ecology. Annals of Botany 120:1-20.

Delle-Vedove, R., N. Juillet, J. M. Bessiere, C. Grison, N. Barthes, T. Pailler, L. Dormont, and B. Schatz. 2011. Colour-scent associations in a tropical orchid: three colours but two odours. Phytochemistry 72:735-742.

Deng, X., B. Bruno, P. Yan-Qiong, H. Yu, C. Yufen, K. Finn, and P. Magali. 2021. Plants are the drivers of geographic variation of floral scents in a highly specialized pollination mutualism: a study of *Ficus hirta* in China.

Dobson, H. E. 2006. Relationship between floral fragrance composition and type of pollinator. Biology of floral scent:147-198.

Dobson, H. E. M., and G. Bergstr6m. 2000. The ecology and evolution of pollen odors. Plant Systematics and Evolution 222:63-87.

Donaldson, J. S. 1997. Is there a floral parasite mutualism in cycad pollination? The pollination biology of *Encephalartos villosus* (Zamiaceae). American Journal of Botany 84:1398-1406.

Dotterl, S., A. Jurgens, K. Seifert, T. Laube, and B. Weissbecker. 2006. Nursery pollination by a moth in *Silene latifolia*: the role of odours in eliciting antennal and behavioural responses. New Phytologist 169:707-718.

Dufaÿ, M., M. Hossaert-McKey, and M. C. Anstett. 2003. When leaves act like flowers: how dwarf palms attract their pollinators. Ecology Letters 6:28-34.

Ellstrand, N. C., R. Ornduff, and J. M. Clegg. 1990. Genetic structure of the Australian cycad, Macrozamia communis (Zamiaceae). American Journal of Botany 77:677-681.

Farré-Armengol, G., I. Filella, J. Llusià, and J. Peñuelas. 2015. Pollination mode determines floral scent. Biochemical Systematics and Ecology 61:44-53.

Franz, N. M. 2007. Reproductive trade-offs in a specialized plant/pollinator system involving *Asplundia uncinata* Harling (Cyclanthaceae) and a derelomine flower weevil (Coleoptera: Curculionidae). Plant Systematics and Evolution 269:183-201.

Friberg, M., C. Schwind, P. R. G. Jr., R. A. Raguso, and J. N. Thompson. 2019. Extreme diversification of floral volatiles within and among species of *Lithophragma* (Saxifragaceae). Proceedings of the National Academy of Sciences 116: 4406-4415.

Furches, M. S., L. E. Wallace, and K. Helenurm. 2008. High genetic divergence characterizes populations of the endemic plant *Lithophragma maximum* (Saxifragaceae) on San Clemente Island. Conservation Genetics 10:115-126.

Gibernau, M. 1997. Odeurs et spécificité dans les mutualismes figuier-pollinisateur: Le cas de *Ficus carica* L. et *Blastophaga psenes* L. PhD Thesis. Université Montpellier II, France.

Gibernau, M. 2016. Pollinators and visitors of aroid inflorescences III-phylogenetic & chemical insights. Aroideana 39:4-22.

Gibernau, M., H. R. Buser, J. E. Frey, and M. Hossaert-McKey. 1997. Volatile compounds from extracts of figs of *Ficus carica*. Phytochemistry 46:241-244.

Gibernau, M., M. HossaertMcKey, J. Frey, and F. Kjellberg. 1998. Are olfactory signals sufficient to attract fig pollinators? Ecoscience 5:306-311.

Gögler, J., J. Stökl, P. Cortis, H. Beyrle, M. R. Barone Lumaga, S. Cozzolino, and M. Ayasse. 2015. Increased divergence in floral morphology strongly reduces gene flow in sympatric sexually deceptive orchids with the same pollinator. Evolutionary Ecology 29:703-717.

González, D., and A. P. Vovides. 2002. Low intralineage divergence in *Ceratozamia* (Zamiaceae) detected with nuclear ribosomal DNA ITS and chloroplast DNA trnL-F non-coding region. Systematic Botany 27:654-661.

Grison, L., A. A. Edwards, and M. Hossaert-McKey. 1999. Interspecies variation in floral fragrances emitted by tropical *Ficus* species. Phytochemistry 52:1293-1299.

Grison-Pigé, L., J.-M. Bessière, and M. Hossaert-McKey. 2002. Specific attraction of fig-pollinating wasps: role of volatile compounds released by tropical figs. Journal of Chemical Ecology 28:283-295.

Gu, D., S. G. Compton, and D. Yang. 2012. 'Push'and 'pull'responses by fig wasps to volatiles released by their host figs. Chemoecology 22:217-227.

Harrison, R. D. 2003. Fig wasp dispersal and the stability of a keystone plant resource in Borneo. Proceedings of the Royal Society B: Biological Sciences 270 Suppl 1:S76-79.

Harrison, R. D., and J.-Y. Rasplus. 2006. Dispersal of fig pollinators in Asian tropical rain forests. Journal of Tropical Ecology 22:631-639.

Hembry, D. H., and D. M. Althoff. 2016. Diversification and coevolution in brood pollination mutualisms: Windows into the role of biotic interactions in generating biological diversity. American Journal of Botany 103:1783-1792.

Hossaert-McKey, M., C. Soler, B. Schatz, and M. Proffit. 2010. Floral scents: their roles in nursery pollination mutualisms. Chemoecology 20:75-88.

Hossaert-McKey, M., M. Gibernau, and J. E. Frey. 1994. Chemosensory attraction of fig wasps to substances produced by receptive figs. Entomologia Experimentalis et Applicata 70:185-191.

Hossaert-McKey, M., M. Proffit, C. C. Soler, C. Chen, J. M. Bessiere, B. Schatz, and R. M. Borges. 2016. How to be a dioecious fig: Chemical mimicry between sexes matters only when both sexes flower synchronously. Scientific reports 6:1-11.

Hu, R., P. Sun, H. Yu, Y. Cheng, R. Wang, X. Chen, and F. Kjellberg. 2020. Similitudes and differences between two closely related *Ficus* species in the synthesis by the ostiole of odors attracting their host-specific pollinators: A transcriptomic based investigation. Acta Oecologica 105:103554.

Huang, D., F. Shi, M. Chai, R. Li, and H. Li. 2015. Interspecific and intersexual differences in the chemical composition of floral scent in *Glochidion* species (Phyllanthaceae) in South China. Journal of Chemistry 2015:1-14.

Ibanez, S., S. Dötterl, M.-C. Anstett, S. Baudino, J.-C. Caissard, C. Gallet, and L. Després. 2010. The role of volatile organic compounds, morphology and pigments of globeflowers in the attraction of their specific pollinating flies. New Phytologist 188:451-463.

Jürgens, A. 2004. Flower scent composition in diurnal *Silene* species (Caryophyllaceae): phylogenetic constraints or adaption to flower visitors? Biochemical Systematics and Ecology 32:841-859.

Kandori, I. 2002. Diverse visitors with various pollinator importance and temporal change in the important pollinators of *Geranium thunbergii* (Geraniaceae). Ecological Research 17:283-294.

Kawakita, A. 2010. Evolution of obligate pollination mutualism in the tribe Phyllantheae (Phyllanthaceae). Plant Species Biology 25:3-19.

Kawakita, A., A. A. W. Sato, J. R. L. Salazar, and M. Kato. 2019. Leafflower-leafflower moth mutualism in the Neotropics: Successful transoceanic dispersal from the Old World to the New World by actively-pollinating leafflower moths. PLoS One 14:e0210727.

Kawakita, A., and M. Kato. 2004. Obligate pollination mutualism in *Breynia* (Phyllanthaceae): further documentation of pollination mutualism involving *Epicephala* moths (Gracillariidae). American Journal of Botany 91:1319-1325.

Knudsen, J. T. 2002. Variation in floral scent composition within and between populations of *Geonoma macrostachys* (Arecaceae) in the western Amazon. American Journal of Botany 89:1772-1778.

Knudsen, J. T., L. Tollsten, and F. Ervik. 2001. Flower scent and pollination in selected neotropical palms. Plant Biology 3:642-653.

Knudsen, J. T., R. Eriksson, J. Gershenzon, and B. Stahl. 2006. Diversity and distribution of floral scent. The botanical review 72:1-120.

Kunze, J., and A. Gumbert. 2001. The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. Behavioral Ecology and Sociobiology 12:447-456.

Leebens-Mack, J. 2004. Patterns of Genetic Structure among Populations of an Oligophagous Pollinating Yucca Moth (*Tegeticula yuccasella*). Journal of Heredity 95:127-135.

Lunau, K. 1995. Notes on the colour of pollen. Plant Systematics and Evolution 198:235-252.

Marr, D. L., J. Leebens-Mack, L. Elms, and O. Pellmyr. 2000. Pollen dispersal in *Yucca filamentosa* (Agavaceae): the paradox of self-pollination behavior by *Tegeticula yuccasella* (Prodoxidae). American Journal of Botany 87:670-677.

Massey, L. K., and J. L. Hamrick. 1998. Genetic diversity and population structure of *Yucca filamentosa* (Agavaceae). American Journal of Botany 85:340-345.

Miyake, T., and M. Yafuso. 2005. Pollination of *Alocasia cucullata* (Araceae) by two *Colocasiomyia* flies known to be specific pollinators for *Alocasia odora*. Plant Species Biology 20:201-208.

Nason, J. D., E. A. Herre, and J. L. Hamrick. 1998. The breeding structure of a tropical keystone plant resource. Nature 391:685-687.

Noort, S. v., and S. G. Compton. 1996. Convergent evolution of agaonine and sycoecine (Agaonidae, Chalcidoidea) head shape in response to the constraints of host fig morphology. Journal of Biogeography 23:415-424.

Okamoto, T., A. Kawakita, and M. Kato. 2007. Interspecific variation of floral scent composition in *Glochidion* and its association with host-specific pollinating seed parasite (*Epicephala*). Journal of Chemical Ecology 33:1065-1081.

Okamoto, T., and Z.-H. Su. 2021. Chemical analysis of floral scents in sympatric *Ficus* species: highlighting different compositions of floral scents in morphologically and phylogenetically close species. Plant Systematics and Evolution 307.

Ollerton, J., R. Winfree, and S. Tarrant. 2011. How many flowering plants are pollinated by animals? Oikos 120:321-326.

Pelletier, L., A. Brown, B. Otrysko, and J. N. McNeil. 2001. Entomophily of the cloudberry (*Rubus chamaemorus*). Entomologia Experimentalis et Applicata 101:219-224.

Pellmyr, O. 2003. Yuccas, yucca moths, and coevolution: a review. Annals of the Missouri Botanical Garden 35-55.

Proffit, M., B. Lapeyre, B. Buatois, X. Deng, P. Arnal, F. Gouzerh, D. Carrasco, and M. Hossaert-McKey. 2020. Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction. Scientific reports 10:1-11.

Proffit, M., B. Schatz, R. M. Borges, and M. Hossaert-McKey. 2007. Chemical mediation and niche partitioning in non-pollinating fig-wasp communities. Journal of Animal Ecology 76:296-303.

Proffit, M., C. Chen, C. Soler, J.-M. Bessière, B. Schatz, and M. Hossaert-McKey. 2009. Can chemical signals, responsible for mutualistic partner encounter, promote the specific exploitation of nursery pollination mutualisms? – The case of figs and fig wasps. Entomologia Experimentalis et Applicata 131 46–57.

Raguso, R. A. 2008. Wake up and smell the roses: the ecology and evolution of floral scent. Annual Review of Ecology, Evolution, and Systematics 39:549-569.

Raguso, R. A. 2009. Floral scent in a whole-plant context: moving beyond pollinator attraction. Functional Ecology 23:837-840.

Robertson,W. G., D. W. Griffiths, J. A. T. Woodford, A. N. E. Birch, J. A. Picket, and L. J. Wadhams. 1993. A comparison of the flower volatiles from hawthorn and four raspberry cultivars. Phytochemistry 33:1047-1053.

Robertson,W. G., D. W. Griffiths, J. A. T. Woodford, and A. N. E. Birch. 1995. Changes in the chemical composition of volatiles released by the flowers and fruits of the red raspberry (Rubus idaeus) cultivar Glen Prosen. Phytochemistry 38:1175-1179.

Rodriguez, L. J., A. Bain, L.-S. Chou, L. Conchou, A. Cruaud, R. Gonzales, M. Hossaert-McKey, J.-Y. Rasplus, H.-Y. Tzeng, and F. Kjellberg. 2017. Diversification and spatial structuring in the mutualism between *Ficus septica* and its pollinating wasps in insular South East Asia. BMC Evolutionary Biology 17:207.

Sakai, S. 2001. Thrips pollination of androdioecious *Castilla elastica* (Moraceae) in a seasonal tropical forest. American Journal of Botany 88:1527-1534.

Sakai, S., M. Kato, and H. Nagamasu. 2000. *Artocarpus* (Moraceae)-gall midge pollination mutualism mediated by a male-flower parasitic fungus. American Journal of Botany 87:440-445.

Schiest, F. P., R. Peakal, J. G. Mant, F. Ibarra, C. Schulz, S. Franke, and W. Francke. 2003. The chemistry of sexual deception in an orchid-wasp pollination system. Science 302:437-438.

Schiestl, F. P. 2015. Ecology and evolution of floral volatile-mediated information transfer in plants. New Phytologist 206:571-577.

Soler, C. C. L., M. Proffit, J.-M. B. re, M. Hossaert-McKey, and B. Schatz. 2012. Evidence for intersexual chemical mimicry in a dioecious plant. Ecology Letters 15:978-985.

Soler, C., M. Hossaert-McKey, B. Buatois, J. M. Bessiere, B. Schatz, and M. Proffit. 2011. Geographic variation of floral scent in a highly specialized pollination mutualism. Phytochemistry 72:74-81.

Soler, C., M. Hossaert-McKey, B. Buatois, J. M. Bessiere, B. Schatz, and M. Proffit. 2011. Geographic variation of floral scent in a highly specialized pollination mutualism. Phytochemistry 72:74-81.

Song, B., G. Chen, J. Stocklin, D. L. Peng, Y. Niu, Z. M. Li, and H. Sun. 2014. A new pollinating seed-consuming mutualism between *Rheum nobile* and a fly fungus gnat, *Bradysia sp*., involving pollinator attraction by a specific floral compound. New Phytologist 203:1109-1118.

Souto-Vilarós, D., M. Proffit, B. Buatois, M. Rindos, M. Sisol, T. Kuyaiva, B. Isua, J. Michalek, C. T. Darwell, M. Hossaert-McKey, G. D. Weiblen, V. Novotny, and S. T. Segar. 2018. Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. Journal of Ecology 106:2256-2273.

Souza, C. D., R. A. Pereira, C. R. Marinho, F. Kjellberg, and S. P. Teixeira. 2015. Diversity of fig glands is associated with nursery mutualism in fig trees. American Journal of Botany 102:1564-1577.

Suinyuy, T. N., and S. D. Johnson. 2018. Geographic variation in cone volatiles and pollinators in the thermogenic African cycad *Encephalartos ghellinckii* Lem. Plant Biology 20:579-590.

Suinyuy, T. N., and S. D. Johnson. 2020. Evidence for pollination ecotypes in the African cycad *Encephalartos ghellinckii* (Zamiaceae). Botanical Journal of the Linnean Society.

Suinyuy, T. N., J. S. Donaldson, and S. D. Johnson. 2010. Scent chemistry and patterns of thermogenesis in male and female cones of the African cycad *Encephalartos natalensis* (Zamiaceae). South African Journal of Botany 76:717-725.

Suinyuy, T. N., J. S. Donaldson, and S. D. Johnson. 2012. Geographical variation in cone volatile composition among populations of the African cycad *Encephalartos villosus*. Biological Journal of the Linnean Society 106:514-527.

Suinyuy, T. N., J. S. Donaldson, and S. D. Johnson. 2015. Geographical matching of volatile signals and pollinator olfactory responses in a cycad brood-site mutualism. Proceedings of the Royal Society B: Biological Sciences 282:20152053.

Svensson, G. P., J. Michael O. Hickman, S. Bartram, W. Boland, O. Pellmyr, and R. A. Raguso. 2005. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). American Journal of Botany 92:1624-1631.

Svensson, G. P., O. Pellmyr, and R. A. Raguso. 2011. Pollinator attraction to volatiles from virgin and pollinated host flowers in a yucca/moth obligate mutualism. Oikos 120:1577-1583.

Svensson, G. P., R. A. Raguso, R. Flatz, and C. I. Smith. 2016. Floral scent of Joshua trees (*Yucca brevifolia* sensu lato): Divergence in scent profiles between species but breakdown of signal integrity in a narrow hybrid zone. American Journal of Botany 103:1793-1802.

Svensson, G. P., T. Okamoto, A. Kawakita, R. Goto, and M. Kato. 2010. Chemical ecology of obligate pollination mutualisms: testing the 'private channel' hypothesis in the *Breynia-Epicephala* association. New Phytologist 186:995-1004.

Terry, L. I., C. J. Moore, R. B. Roemer, D. R. Brookes, and G. H. Walter. 2021. Unique chemistry associated with diversification in a tightly coupled cycad-thrips obligate pollination mutualism. Phytochemistry 186:112715.

Thompson, A. R., C. E. Thacker, and E. Y. Shaw. 2005. Phylogeography of marine mutualists: parallel patterns of genetic structure between obligate goby and shrimp partners. Molecular Ecology 14:3557-3572.

Thompson, J. N., and B. M. Cunningham. 2002. Geographic structure and dynamics of coevolutionary selection. Nature 417:735-738.

Thompson, J. N., C. Schwind, and M. Friberg. 2017. Diversification of trait combinations in coevolving plant and insect lineages. The American Naturalist 190:171-184.

Thompson, J. N., C. Schwind, P. R. Guimaraes, Jr., and M. Friberg. 2013. Diversification through multitrait evolution in a coevolving interaction. Proceedings of the National Academy of Sciences 110:11487-11492.

Tollsten, L., and D. O. Bvstedal. 1994. Differentiation in floral scent chemistry among populations of Conopodium majus (Apiaceae). Nordic Journal of Botany 14:361-368.

Tollsten, L., J. T. Knudsen, and L. G. Bergström. 1994. Floral scent in generalistic *Angelica* (Apiaceae)-an adaptive character? Biochemical Systematics and Ecology 22:161-169.

Toon, A., L. I. Terry, W. Tang, G. H. Walter, and L. G. Cook. 2020. Insect pollination of cycads. Austral ecology.

Treutlein, J., P. Vorster, and M. Wink. 2005. Molecular relationships in *Encephalartos* (Zamiaceae, Cycadales) based on nucleotide sequences of nuclear ITS 1&2, rbcL, and genomic ISSR fingerprinting. Plant Biology 7:79-90.

Wang, G., C. H. Cannon, and J. Chen. 2016. Pollinator sharing and gene flow among closely related sympatric dioecious fig taxa. Proceedings of the Royal Society B: Biological Sciences 283:20152963.

Wang, G., S. G. Compton, and J. Chen. 2013. The mechanism of pollinator specificity between two sympatric fig varieties: a combination of olfactory signals and contact cues. Annals of Botany 111:173-181.

Wei, Z. D., N. Kobmoo, A. Cruaud, and F. Kjellberg. 2014. Genetic structure and hybridization in the species group of *Ficus auriculata*: can closely related sympatric *Ficus* species retain their genetic identity while sharing pollinators? Molecular Ecology 23:3538-3550.

Whitehead, M. R., and R. Peakall. 2009. Integrating floral scent, pollination ecology and population genetics. Functional Ecology 23:863-874.

Wright, G. A., A. F. Choudhary, and M. A. Bentley. 2009. Reward quality influences the development of learned olfactory biases in honeybees. Proceedings of the Royal Society B: Biological Sciences 276:2597-2604.

Yamasaki, E., A. Kawakita, and S. Sakai. 2015. Diversity and evolution of pollinator rewards and protection by *Macaranga* (Euphorbiaceae) bracteoles. Evolutionary Ecology 29:379-390.

Yu, H., D. Liang, E. Tian, L. Zheng, and F. Kjellberg. 2018. Plant geographic phenotypic variation drives diversification in its associated community of a phytophagous insect and its parasitoids. BMC Evolutionary Biology 18:134.

Yu, H., E. Tian, L. Zheng, X. Deng, Y. Cheng, L. Chen, W. Wu, W. Tanming, D. Zhang, S. G. Compton, and F. Kjellberg. 2019. Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation. Molecular Ecology 28:2391-2405.

Zhang, C., J. Yan, Y. Chen, W. Wu, S. Tu, Y. Xu, X. Chen, and S. Huang. 2016. Responses of two *Eupristina* wasp species to fig volatiles of *Ficus altissima* and infochemicals. Acta Ecologica Sinica 36:5581-5589.

Zhang, T., W. Bo, M. Bai-Ge, and P. Yan-Qiong. 2017. Chemical composition of volatiles from the syconia of *Ficus microcarpa* and host recognition behavior of pollinating fig wasps. Chinese Journal of Plant Ecology 41:549-558.

Zych, M. 2002. Pollination biology of *Heracleum sphondylium* L.(Apiaceae): the advantages of being white and compact. Acta societatis botanicorum Poloniae 71:163-170.

Received: 16 March 2018 | Revised: 31 January 2019 | Accepted: 1 February 2019

DOI: 10.1111/mec.15046

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation

Hui Yu[1,2] | Enwei Tian[1] | Linna Zheng[1] | Xiaoxia Deng[1] | Yufen Cheng[1] | Lianfu Chen[1] | Wei Wu[1] | Wattana Tanming[3] | Dayong Zhang[4] | Stephen G. Compton[5] | Finn Kjellberg[6]

[1]Guangdong Provincial Key Laboratory of Digital Botanical Garden and Key Laboratory of Plant Resource Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou, China

[2]Centre for Plant Ecology, CAS Core Botanical Gardens, Guangzhou, China

[3]Queen Sirikit Botanic Garden, Chiang Mai, Thailand

[4]State Key Laboratory of Earth Surface Processes and Resource Ecology and MOE Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing, China

[5]School of Biology, University of Leeds, Leeds, UK

[6]CEFE, CNR, EPHE, IRD, Université de Montpellier, Université Paul-Valéry Montpellier, Montpellier, France

Correspondence
Hui Yu, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou, China; Centre for Plant Ecology, CAS Core Botanical Gardens, Guangzhou, China.
Email: yuhui@scib.ac.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 31670395, 31630008 and 31370409; Guangzhou Science and Technology Plan Project, Grant/Award Number: 201707010351; External Cooperation Program of Bureau of International Co-operation Chinese Academy of Sciences, Grant/Award Number: GJHZ201321; CAS President's International Fellowship Initiative, Grant/Award Number: 2018VBA0040; Vietnam National Foundation for Science and Technology Development, Grant/Award Number: 106.11-2012.82

Abstract

The ways that plant-feeding insects have diversified are central to our understanding of terrestrial ecosystems. Obligate nursery pollination mutualisms provide highly relevant model systems of how plants and their insect associates have diversified and the over 800 species of fig trees (*Ficus*) allow comparative studies. Fig trees can have one or more pollinating fig wasp species (Agaonidae) that breed within their figs, but factors influencing their number remain to be established. In some widely distributed fig trees, the plants form populations isolated by large swathes of sea, and the different populations are pollinated by different wasp species. Other *Ficus* species with continuous distributions may present genetic signatures of isolation by distance, suggesting more limited pollinator dispersal, which may also facilitate pollinator speciation. We tested the hypothesis that *Ficus hirta*, a species for which preliminary data showed genetic isolation by distance, would support numerous pollinator species across its range. Our results show that across its range *F. hirta* displays clinal genetic variation and is pollinated by nine parapatric species of *Valisia*. This is the highest number of pollinators reported to date for any *Ficus* species, and it is the first demonstration of the occurrence of parapatric pollinator species on a fig host displaying continuous genetic structure. Future comparative studies across *Ficus* species should be able to establish the plant traits that have driven the evolution of pollinator dispersal behaviour, pollinator speciation and host plant spatial genetic structure.

KEYWORDS
co-evolution, dispersal, gene flow, mutualism, plant growth form, speciation

[Correction added on 05 May 2020: the affiliation of the first author has been updated in this version.]

1 | INTRODUCTION

Insects feeding on plants represent one of the most common forms of trophic interaction seen in terrestrial ecosystems, and the relationship between plants and insects has generated the most species-rich animal taxa ever seen on the planet (Price, 1980, 2002). These insects are typically associated with one or a few related host plants, so different plants host different insects (Strong, Lawton, & Southwood, 1984). Processes such as local adaptation, the geography of speciation and the consequences of secondary sympatry are key issues in understanding the dynamics of community diversification (Mittelbach & Schemske, 2015). However, almost no studies have explored how the insect community on a plant changes across its geographic range (Lawton, Lewinsohn, & Compton, 1993; Leather, 1986; Lewinsohn & Roslin, 2008). A study across Papuan rainforests found little variation in herbivorous insect communities over distances of up to 1,000 km (Craft et al., 2010; Novotny et al., 2007), while a study of nonpollinating fig wasps on *Ficus rubiginosa* Desf. ex Ventenat in Australia detected, within a set of seven widespread morphospecies, four pairs of parapatric cryptic species, along a 2,000-km transect (Darwell & Cook, 2017).

In nursery pollination mutualisms, pollinating insects breed in floral structures of the plants they pollinate (Dufaÿ & Anstett, 2003). Insects involved in nursery pollination mutualisms constitute particularly favourable biological models for investigating the geographic variation of diversity on a host plant as the insect's prevalence on a host is high and their presence is easy to detect. Furthermore, some of these mutualisms are species rich allowing comparative studies. Nursery pollination systems, such as those involving *Yucca* and Yucca moths (Pellmyr, 2003), *Glochidion* and *Epicephala* moths (Kawakita, 2010) and fig trees (*Ficus*) and fig wasps (Agaonidae) (Cook & Rasplus, 2003), were initially envisioned as systems in which a single species of pollinating insect breeds in and pollinates a single host plant species. This simple pattern is now largely rejected because of accumulating examples where one insect species pollinates several host species (Cornille et al., 2012; Hembry et al., 2018; Pellmyr, 1999; Wachi, Kusumi, Tzeng, & Su, 2016; Wang, Cannon, & Chen, 2016), or cases where several insect species pollinate a single host (Darwell, al-Beidh, & Cook, 2014; Li, Wang, & Hu, 2015; Yang et al., 2015). A general model of plant–insect diversification in these nursery pollination mutualisms must therefore take into account the factors influencing their relative rates of speciation and explain why some of the plants have a single pollinator when others have two or more.

Fig trees (*Ficus*) represent the most species-rich group of plants offering nursery pollination rewards and the most diversified lineage within the Moraceae (Brunn-Lund, Verstraete, Kjellberg, & Rønsted, 2018). Fig trees are pollinated by female fig wasps that enter *Ficus* inflorescences to lay their eggs. Because fig wasps carry pollen from their natal plants, they only transfer pollen from those plants in which their larvae can develop. This is a more direct link between suitability for offspring development and subsequent pollen transfer than in any other brood pollination mutualism and may favour co-adaptation (Anstett, Hossaert-McKey, & Kjellberg, 1997). However, a more rapid rate of speciation among fig wasps, compared with their hosts, is to be expected given their much shorter generation times (Petit & Hampe, 2006; Thomas, Welch, Lanfear, & Bromham, 2010). From this perspective, the long-held assumption that each fig tree was pollinated by a single species of pollinator was problematic.

More extensive sampling and molecular techniques that have facilitated separation of morphologically close ("cryptic") species have revealed an increasing number of *Ficus* species with several pollinators (Darwell et al., 2014). However, we still know little about how genetic diversity is structured within *Ficus* species and how this is related to the distributions and genetic diversity among the fig wasp pollinators that it supports (Bain et al., 2016; Rodriguez et al., 2017; Wachi et al., 2016). Where several pollinator species share a single *Ficus* host, they are generally sister species (Yang et al., 2015). The recorded exceptions, where nonsister pollinator species occupy the same host species, have almost all been within two *Ficus* clades that have diversified recently, and this appears to have favoured host shifts (Cruaud et al., 2012; Jousselin et al., 2008; Machado, Robbins, Gilbert, & Herre, 2005; Yang et al., 2015). The main modes of fig wasp speciation therefore appear to vary across lineages.

Widely distributed plants grow in areas with a wide range of environmental conditions, which will vary in suitability for their associated insects, including their pollinators. Reflecting this, plant-eating insects usually only occupy a subset of the ranges of their hosts (Strong et al., 1984), but fig trees require an effective and specific pollinator to be present wherever they grow. Widely distributed *Ficus* species would therefore be predicted to support additional species of pollinator compared to species with narrow, more climatically homogeneous, ranges. Only a few widely distributed *Ficus* species have been sampled at multiple sites across their geographic ranges. *Ficus racemosa* L. is pollinated by a single fig wasp across India and by another in China and SE Asia, with additional sister species of pollinators in the island of Borneo and Australia (Bain et al., 2016; Kobmoo, Hossaert-McKey, Rasplus, & Kjellberg, 2010). More localized differentiation is evident among island populations of *Ficus septica* Burm. f., in the Philippines (Conchou, Cabioch, Rodriguez, & Kjellberg, 2014; Lin, Yeung, Fong, Tzeng, & Li, 2011; Rodriguez et al., 2017), and along elevation gradients (Segar et al., 2017; Souto-Vilarós et al., 2018), where several different pollinators are present.

The absence of divergence among the pollinators of *F. racemosa* across large swathes of its continental range is likely to reflect their ability to disperse over large distances. Spatial genetic structuring across the range of a plant species provides an indication of the geographic extent of their gene flow, and in the case of *Ficus*, it has revealed a widely varying extent of dispersal among their pollinators. Paternity analyses have shown that some fig wasps transport pollen between large fig trees growing over 100 km apart (Ahmed, Compton, Butlin, & Gilmartin, 2009), whereas population structuring shows that other, smaller, species have much more localized gene flow (Chen, Zhu, Compton, Liu, & Chen, 2011; Liu, Compton, Peng,

Zhang, & Chen, 2015). These differences in dispersal behaviour among the pollinators of different trees appear highly likely to influence differentiation and speciation of both their own populations as well as those of the host plants they pollinate.

Previous data have shown that *Ficus hirta* Vahl presents a pattern of spatial genetic structure suggesting genetic isolation by distance across continental South-East Asia (Yu & Nason, 2013). Here, based on extensive sampling, we describe the population genetic structure of *F. hirta* and its pollinating fig wasps across most of their continental range. We addressed the following questions: (a) To what extent is the population structure of the plant co-incident with that of its pollinator(s)—have they diversified at the same spatial scales? (b) Even in the absence of major geographic barriers, is this widespread host tree pollinated by a diverse assemblage of fig wasps within its range? And (c) if so, what are the ecological consequences—do individual plants have the chance to receive pollination services from more than one fig wasp species? This is the first study comparing spatial genetic structuring in a widespread fig species and its pollinating wasps that encompasses most of their ranges. It is also the first broad-scale study of joint genetic structuring between pollinator fig wasps and a fig tree species displaying genetic isolation by distance. We discuss the results in terms of the factors that may have resulted in the patterns of diversification we observed and what they suggest about modes of speciation.

2 | MATERIALS AND METHODS

2.1 | Study species

Like all *Ficus* species, *F. hirta* (family Moraceae, subgenus *Ficus*, section *Eriosycea*, Berg, 2003) has unique protogynous inflorescences called figs. These are hollow and lined internally with numerous tiny male and female florets. Pollination can only be achieved when pollen is carried by female fig wasps from their natal fig into receptive figs through a narrow aperture called the ostiole. Like about half of all *Ficus* species, *F. hirta* is functionally dioecious, with figs that differ in floral structure on "male" and "female" trees. Figs of the former support the development of the fig wasp offspring that when mature can transport pollen to receptive figs. In contrast, the figs on female trees develop seeds and no pollinator offspring. *Ficus hirta* is a species of secondary, disturbed habitats and typically grows as a shrub or treelet. It can reach a maximum height of about 5 m, but most mature individuals are much shorter (Berg & Corner, 2005). Its figs are small and produced in the leaf axils. Female figs ripen to a bright red and are mainly eaten by birds (Corlett, 2006). As many as 50 figs can be present on a tree, but their development is asynchronous, which reduces the peak numbers of figs available for pollination or dispersal at any one time and can also allow pollinator cycling between figs on the same male tree (Jia, Dan, Zhang, & Chen, 2007).

The distribution of *F. hirta* extends northwards from the island of Java (Indonesia) in the south to China in the north and westwards into northeast India, Nepal and Sikkim (Berg & Corner, 2005). This extensive geographic range (covering over 30° of latitude and 30° of longitude) encompasses a range of tropical and subtropical biomes. Berg (2007) restricted *F. hirta* to what was previously known as *F. h. subsp. hirta*. In the following, we will use the name *F. hirta* for *F. hirta* sensu Berg (2007). Following that definition, *F. hirta* has a disjunct distribution. South of Thailand, it is absent from Peninsular Malaysia and it is only present in South Sumatra and North Java (Berg & Corner, 2005). Population genetic studies, mainly from China, detected some spatial genetic structuring across *F. hirta* populations and concluded that low nuclear differentiation, combined with high interpopulation differentiation and geographic structuring of chloroplast variation, indicated that gene flow via seed dispersal was more limited than via dispersal of pollen (Yu & Nason, 2013). Genetic differentiation in nuclear genes between populations of *F. hirta* on the Chinese mainland and Hainan Island has also been detected, and corresponding differences were also present between the populations of the tree's pollinators (Tian et al., 2015). Two morphologically distinguishable pollinator fig wasps have been described from the figs of *F. hirta*. *Valisia javana javana* Mayr was reared from *F. hirta* figs collected in Java, and a second subspecies, *Valisia j. hilli* Wiebes, was described from figs collected in Hong Kong (Wiebes, 1993).

2.2 | Sampling

Between 2006 and 2014, we sampled the leaves of *F. hirta* (31 locations) and its pollinating fig wasps (32 locations) across mainland South-East Asia and south to the island of Java (Supporting Information Table S1). The samples were up to 4,100 km apart (north–south) and reached the northern, eastern and southern limits of the range of *F. hirta*.

Within locations, *F. hirta* individuals were typically sampled at intervals of 3–5 m, with no individuals located more than 200 m apart. Their leaves were collected and dried in plastic bags containing silica gel. At each location, 10–30 figs containing mature fig wasp offspring were also removed from the plants and placed individually in fine-mesh bags, where the fig wasps were allowed to emerge. The pollinators were then separated from other species of fig wasps and preserved in 95% ethanol that was stored at −20°C until DNA extraction. A single female fig wasp per fig was used for genetic analyses.

2.3 | Pollinating wasps

2.3.1 | Pollinating wasp DNA extraction, amplification and sequencing

The mitochondrial genetic marker mtCOI was sequenced from an average of 9.9 individuals per location (range 1–28, total 330), and microsatellite loci were genotyped from an average of 18.9 other individuals from the same locations (range 2–32, total 568). To complement the data, the nuclear ITS2 nuclear gene was amplified for 201 individuals.

Genomic DNA was extracted from the whole body of each fig wasp using the EasyPure Genomic DNA Extraction Kit (TransGen, Beijing, China). A 681 bp fragment of the mtCOI gene was then sequenced following the protocol used in previous studies (Tian et al., 2015). A 689 bp fragment of the ITS2 gene was amplified in 201 individuals using the universal primer pair (ITSR: 5′-CGCCTGATCTGAGGTCGTGA-3′, ITSF: 5′-ATTCCCGCACCACGCCTGGCTGA-3′; Lopez-Vaamonde, Rasplus, Weiblen, & Cook, 2001) and the same PCR amplification reaction volume as for the COI gene. The reaction was optimized and programmed on a MJ Thermal Cycler (PTC 200) as one cycle of denaturation at 94°C for 5 min, 35 cycles of 30-s denaturation at 94°C, 30 s at a 55°C annealing temperature and 30-s extension at 72°C, followed by 8-min extension at 72°C. All amplified PCR products were purified using QIAquick spin columns (Qiagen) and were sequenced in an ABI 3730xl capillary sequencer using BIGDYE TERMINATOR V 3.1 chemistry (Applied Biosystems).

Previously published protocols were also used to genotype individuals at nine unlinked microsatellite loci (1–78, 1–141, A34, A80, A99, B30, C25, F17 and H33) that had been previously developed for *Valisia j. hilli* (Tian, Yu, Zhang, & Nason, 2011). The alleles used in the present study were the same as those used previously to analyse southeast China and Hainan populations (Tian et al., 2015).

2.3.2 | Pollinating fig wasp sequence analysis

We did not detect any indications of pseudogenes, such as multiple peaks in chromatograms, stop codons or frameshift mutations (Song, Buhay, Whiting, & Crandall, 2008). Sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) with manual corrections.

Previous work has shown that the fig wasp genus *Valisia*, including samples collected from *F. hirta*, is monophyletic (Cruaud et al., 2010). We downloaded from GenBank the COI sequences of the eight *Valisia* species available including one sequence (FJ619191) of *V. javana*. Published phylogenies have shown that the genera *Ceratosolen* and *Kradibia* constitute an outgroup relative to all other pollinating fig wasps (Cruaud et al., 2010, 2012). We included representatives of these two genera (two species of *Ceratosolen* and 11 species of *Kradibia*) in the phylogenetic analysis.

Dated phylogenetic trees that included the downloaded sequences and all our sequenced haplotypes were estimated using Bayesian methods. The best-fit model, GTR+I+G, was selected by hierarchical likelihood ratio tests in the program MODELTEST 3.7 (Posada & Crandall, 1998), where GTR+I+G was favoured. We ran BEAST 2.3.1 (Bouckaert et al., 2014) to explore the best combinations of substitution, clock and population models, with 10 million generations. Twice the difference in Ln harmonic mean of the likelihood of each model combination (LnBF; Nicholls et al., 2010) was calculated with TRACER 1.6 to assess the preferred models, based on the LnBF table (Kass & Raftery, 1995). Using the GTR+I+G model, we ran BEAST under a strict clock, an uncorrelated exponential relaxed clock or lognormal relaxed clock with each population model (constant size, exponential growth, yule process, birth–death process). Bayes factors indicated that the combination of an uncorrelated exponential clock and the exponential population growth model was the best (with LnBFs from 0.47 to 198.19 and most >15).

Two independent runs of 30 million iterations were performed, with genealogies and model parameters sampled every 1,000 iterations. The chain convergence was checked based on ESSs (effective sample sizes) viewed in TRACER 1.6. All ESSs for each parameter had to be larger than 200. The phylogenetic tree was summarized by TREEANNOTATOR 1.8.1 and then viewed by FIGTREE 1.3.1 (Rambaut, 2006). The dates of the most recent common ancestor were scaled by a mutation rate of 1 because no suitable fossil record was available to calibrate node ages. We used the published 34.8 (46.5–24.2) Ma age for the crown group of the genus *Valisia* that had been estimated previously for a very similar set of *Valisia* species that used numerous genetic markers and a comprehensive set of species within Agaonidae (Cruaud et al., 2012). We used the phylogenetic tree to detect candidate species characterized by low within-clade genetic distances and much larger between-clade genetic distances.

We calculated Kimura 2-parameter (K2P) distances within and between clades for COI haplotypes using MEGA 6.06. Tajima's *D*, Fu and Li's *D*, and Fu and Li's *F* (Fu & Li, 1993) were used to detect signatures of population expansion or selection using DNASP 5.0 (Librado & Rozas, 2009). We then explored the relationships of COI haplotypes within each clade using phylogenetic networks built separately for each clade with TCS 1.21 (Clement, Posada, & Crandall, 2000) using the 95% statistical parsimony criterion as a connection limit, with loops in the network resolved following Crandall and Templeton's methods (1993). Variation in COI sequences among populations within clades and within populations was partitioned using hierarchical analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.0 (Excoffier, Laval, & Schneider, 2005). Significance tests were based on 10,000 permutations.

We used jMOTU as a complementary method to detect molecular operational taxonomic units (MOTUs) using a range of threshold differences. All the COI sequences used in the phylogenetic analysis were analysed using jMOTU at cut-offs from 0 to 21 bases (Jones, Ghoorah, & Blaxter, 2011). The aggregation parameter was 97%, and the input minimum sequence percentage was set to 95%.

As ITS2 evolves more slowly than COI, we applied a simplified analysis of the data. We calculated K2P distances within and between clades as for COI haplotypes using MEGA 6.06, and we used a maximum-likelihood tree to reconstruct the phylogenetic relationships based on all ITS haplotypes. The ML tree was reconstructed using MEGA 6.06 (Tamura et al., 2013), and node supports were assessed based on 2,000 bootstrap replicates.

2.3.3 | Pollinating fig wasp microsatellite data analysis

Classical indices of genetic diversity were estimated using GENALEX 6.1 (Peakall & Smouse, 2006). In order to represent the global data, we performed a factorial correspondence analysis following

Nenadić and Greenacre (2007) as implemented in Genetix (Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme, 1996–2004). Because multiple locus population genetics data should not be analysed using phylogenetic trees, we constructed an unweighted neighbour-joining tree of multilocus microsatellite genotypes using DARWIN v6 (Perrier & Jacquemoud-Collet, 2006), thus grouping genotypes according to the numbers of shared alleles, without taking into account gene evolution. Bootstrap number was set at 2,000. Finally, we used Bayesian clustering to assign multilocus microsatellite genotypes to clusters using STRUCTURE 2.2 (Pritchard, Stephens, & Donnelly, 2000). The admixture ancestry and correlated allele frequencies model were used with five independent runs each of 500,000 MCMC iterations and 500,000 burn-in steps. We ran STRUCTURE with varying *K* values (the number of clusters) from 2 to 30 (the maximum number of populations available for microsatellite analysis) to explore how different values of *K* resulted in the assignment of species and populations to different clusters. The resulting assignment patterns were explored to detect co-occurrence of different clades within a sampling location. Results obtained from the factorial correspondence analysis, neighbour-joining tree and Bayesian clustering were compared.

The presence of a relationship between (log) geographic distance and genetic differentiation $F_{ST}/(1 - F_{ST})$ values (isolation by distance) was evaluated with GENALEX 6.1 within the two clades detected in the above analysis and for which data from more than three locations were available, using a Mantel test with 10,000 permutations. When structuring was detected within clades, the distribution of pairwise F_{ST} values was explored to detect potential structuring into subclades.

2.4 | Ficus hirta

2.4.1 | *Ficus hirta* DNA extraction and amplification

We used previously published methodological details for cpDNA and nuclear microsatellite sequencing and genotyping (Yu & Nason, 2013), and re-used all the previously published cytoplasmic data. The set of analysed microsatellite loci was reduced to avoid some amplification/reading problems, leaving seven microsatellite loci available: FS4-11, Frub38, Frub398 and Frub436 (Yu & Nason, 2013), and FH3, FH10 and FH47 (Zheng, Nason, Liang, Ge, & Yu, 2015). Using these, we analysed plant microsatellite data from two locations in southeast China and two locations in Hainan, and cytoplasmic data from 14 locations in southeast China and Hainan. This was in addition to the microsatellite data and cytoplasmic data from 17 locations elsewhere within the range of the plant, so we had a total of 21 locations with microsatellite data and 31 locations with cytoplasmic data.

2.4.2 | *Ficus hirta* cytoplasmic DNA analysis

The sequences of the two cpDNA regions were concatenated (1,367 bp) and then aligned using MUSCLE in MEGA 6.06 with manual corrections. A matrix of combined sequences for trnL-trnF and trnS-trnG was constructed, and haplotypes were distinguished on the basis of nucleotide and insertion/deletion differences. The same procedures as for wasp COI data were used to determine polymorphism indices, detect signatures of population expansion and establish haplotype networks.

We used AMOVA to test the significance of cpDNA differentiation among populations (999 permutations) and tested for genetic isolation by distance by conducting a Mantel test of the correlation between $F_{ST}/(1 - F_{ST})$ and log-transformed geographic distance for all population pairs (999 permutations).

2.4.3 | *Ficus hirta* microsatellite analyses

Parameters of genetic diversity were calculated as for pollinator microsatellite data. As with the pollinating wasp microsatellite data, we performed a factorial correspondence analysis, we build a neighbour-joining tree, and we used Bayesian clustering to assign multilocus microsatellite genotypes to clusters. The number of clusters was set to vary from 1 to 21.

The relationship between F_{ST} and geographic distance was plotted to visualize genetic differentiation. The significance of the correlation between $F_{ST}/(1 - F_{ST})$ and the log geographic distance was evaluated as with the insects.

3 | RESULTS

3.1 | Pollinating fig wasps

3.1.1 | Pollinating fig wasp gene sequences

After alignment and exclusion of sites with gaps, 502 bp long COI sequences were obtained for phylogenetic analysis. A total of 162 haplotypes were obtained and 267 COI polymorphic sites were identified from the 330 sequenced fig wasps (Supporting Information Table S2). Haplotype sequences have been deposited in GenBank under Accession nos. KR873011–KR873047 and MF472722–MF472846. A 689 bp fragment of the ITS2 gene was amplified in 201 individuals. A total of 18 haplotypes were obtained. The sequences have been deposited in GenBank under Accession nos. MF467418–MF467426 and MF467428–MF467436.

The Bayesian COI tree separated the *F. hirta* pollinating fig wasps into nine clades (Figure 1). Gene sequence differences were weak within clades (Kimura-2-parameter = 0.001–0.014) and high between clades (0.064–0.272) (Figure 1, Supporting Information Figure S1, Supporting Information Table S3). The between-clade distances are similar to those between currently recognized Agaonidae species (Chen, Compton, Liu, & Chen, 2012; Yang et al., 2015).

The ITS2 maximum-likelihood tree separated the *F. hirta* pollinating wasps into seven clades (Supporting Information Figure S2, Supporting Information Table S4). ITS2 data were obtained for several individuals from each of 30 of the 31 locations for which COI data were obtained (Supporting Information Table S1). The ITS2 sequences grouped the genotypes according to sampling location (Supporting Information Table S5) in the same way as the

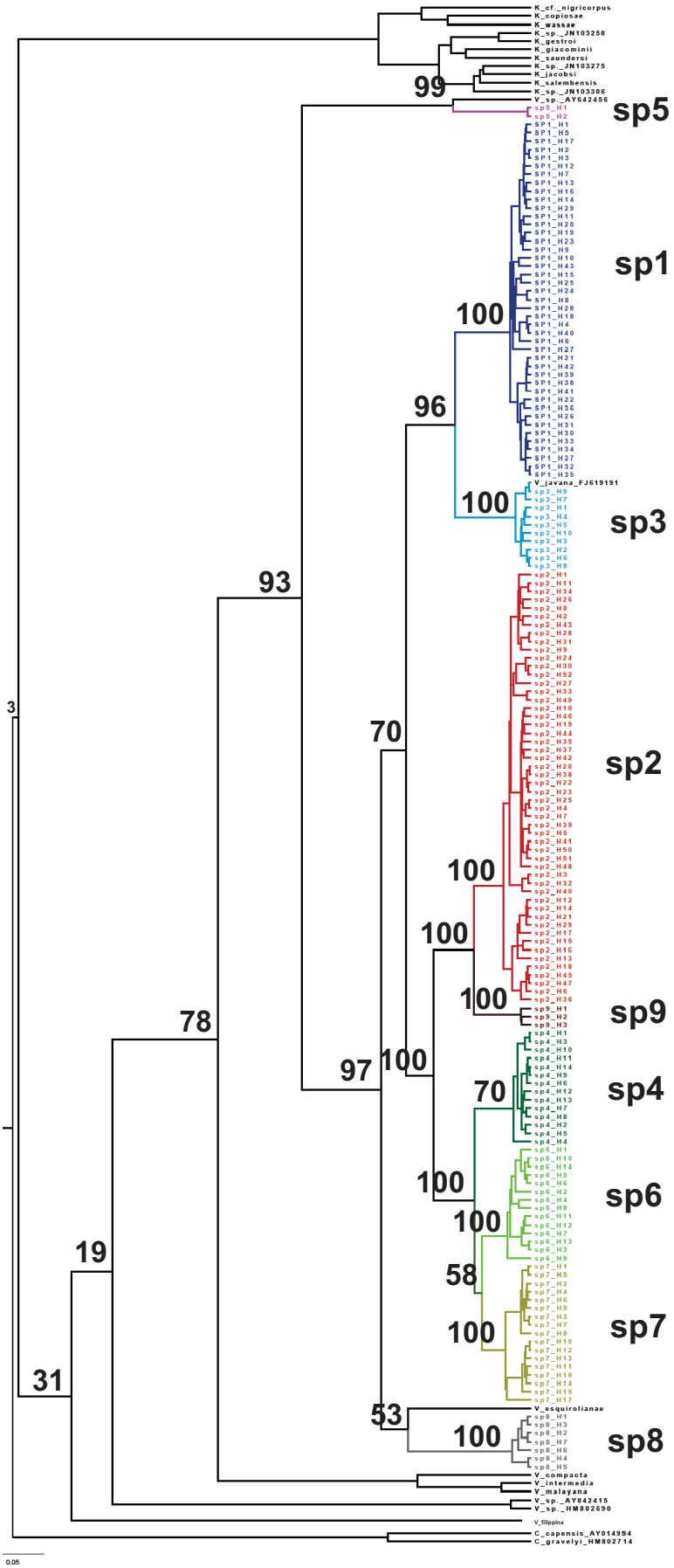


FIGURE 1 COI Bayesian phylogenetic tree of the *Valisia* fig wasp pollinators associated with *Ficus hirta*, including all GenBank sequences of pollinating wasps reared from *Ficus* subg. *Ficus* sect. *Eriosycea* Miq. and some sequences of *Ceratosolen* and *Kradibia* used as outgroups. Posterior probabilities of the nodes are indicated as percentages [Colour figure can be viewed at wileyonlinelibrary.com]

COI sequences (Supporting Information Table S2), except that the ITS2 sequences provided less resolution and pooled the locations that gave COI clade 2 and COI clade 9 and pooled the locations that gave COI clade 6 and COI clade 7. Hence, cytoplasmic and nuclear sequence data provided consistent location-level assignment to clades, although the ITS2 sequences provided less resolution.

The program jMOTU groups sequences into clades (molecular taxonomic units) so that the minimum difference, expressed in numbers of bases, between two different MOTUs is larger than the chosen cut-off value. With increasing cut-off values, the number of inferred MOTUs drops rapidly and then stabilizes when within-species variation is included within a single MOTU, before dropping again when closely related species are pooled. The number of clades inferred from the COI sequences dropped to 33 MOTUs for a cut-off value of 9 and remained constant up to a cut-off value of 11 (Supporting Information Figure S3). For these cut-off values, all the clades recognized in the COI tree were separated into single MOTUs except for clade 7, which was separated into three MOTUs and clade 6 that was also separated into 3 MOTUs. Starting at the cut-off value of 17 bases (which is the 2.5% cut-off threshold value proposed as a general rule-of-thumb for species discrimination with COI; Jones et al., 2011) and up to a cut-off value of 21 bases, jMOTU gave 29 MOTUs. Each of the nine clades recognized from the phylogenetic tree (Figure 1) was recognized as a single MOTU, and the two sequences of fig wasps (AY842415 and HM802690) collected from *Ficus langkokensis* grouped into a single MOTU. Hence, the jMOTU analysis supports the proposition that each clade recognized in the COI tree is a separate operational taxonomic unit, that is a species.

Overall, the different analyses of sequence data support the conclusion that clades 1–9 can be considered as distinct species, and this conclusion is applied from here on (as sp1–sp9).

The pollinators collected in Singapore (sp5) formed part of a distinct clade that had the largest gene sequence differences relative to the other species pollinating *F. hirta* (for cytoplasmic COI, Figure 1, Supporting Information Table S3; for nuclear ITS2, Supporting Information Figure S2, Supporting Information Table S4). This clade included a COI GenBank sequence (AY642456) of a fig wasp collected from *F. androchaete* Corner, a *Ficus* species endemic to Borneo, where *F. hirta* is absent (Berg & Corner, 2005). The other major clade comprised sp1–4 and sp6–9 reared from *F. hirta*, plus the GenBank sequence for *Valisia esquiroliana* Chen & Chou. The dated tree obtained with BEAST (Supporting Information Figure S1) suggests that separation of sp5 from the remaining eight species associated with *F. hirta* began about 16 (11–22) Ma and the two tightly knit subgroups of species (sp2 + sp9) and (sp4 + sp6 + sp7) diverged from each other about 8 Ma (Supporting Information Figure S1). These two species groups were also recovered in the ITS2 phylogeny (Supporting Information Figure S2). The estimated dates of divergence among the taxa within these subgroups that we are recognizing as distinct species varied between 4.6 (sp2 + sp9 and sp4 + sp6 + sp7) and 6.0 (sp1 + sp3) Ma.

A striking feature was the limited overlap in the distribution of species. We only observed examples of a pair of species occurring together in two locations, location CS (with sp2 and a low frequency of sp3) in north Thailand and Wu in northeast Thailand (with sp4 together with small numbers of sp7; Figure 2a). In addition, GenBank Accession no. FJ619191 belonged to sp3 and was recorded from XI in SW China, an area where we only collected sp2. Where species were collected from several sites, their distributions were generally geographically coherent, but sp7 has a disjoint distribution. It is present in east Thailand and in Java, but absent from peninsular Malaysia

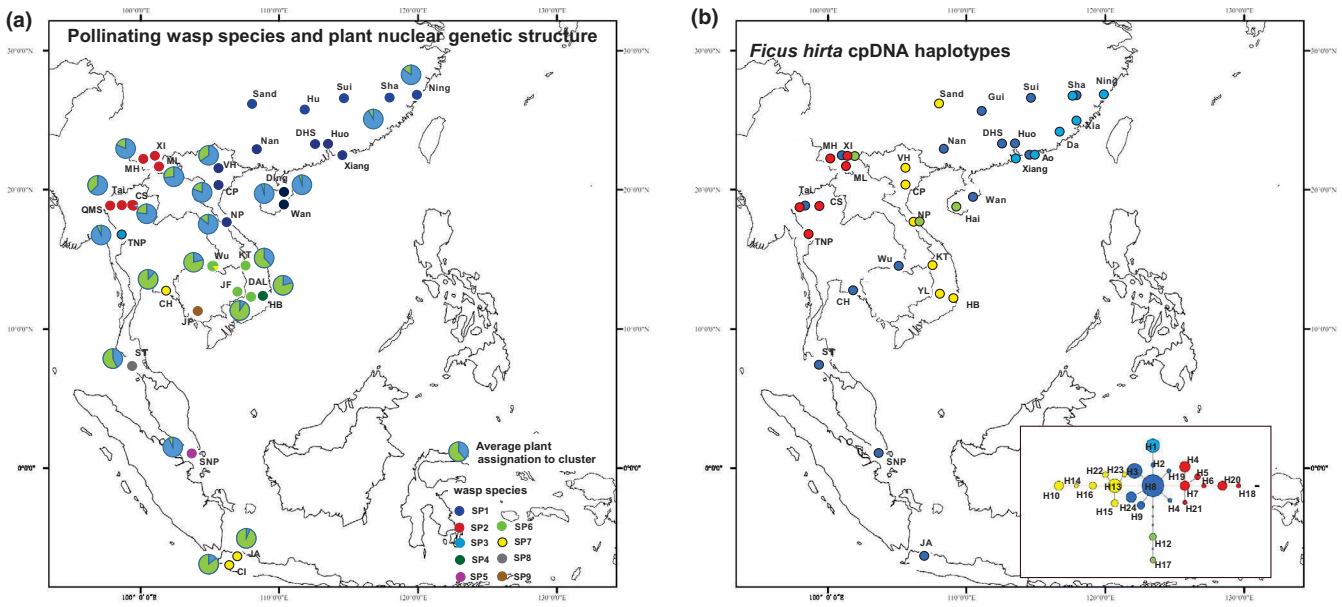


FIGURE 2 (a) The distributions of the pollinator fig wasp species associated with *Ficus hirta*. (b) The distributions of cpDNA haplotypes of *F. hirta*. Note the disjoint distribution of sp7, the allopatric distribution of the closely related (according to COI data) sp2 and sp9 and the parapatric distribution of the closely related (according to COI data) sp4, sp6 and sp7. For cpDNA, colours are used to show the localized distribution of haplotype H1 and of the different branches of the haplotype network

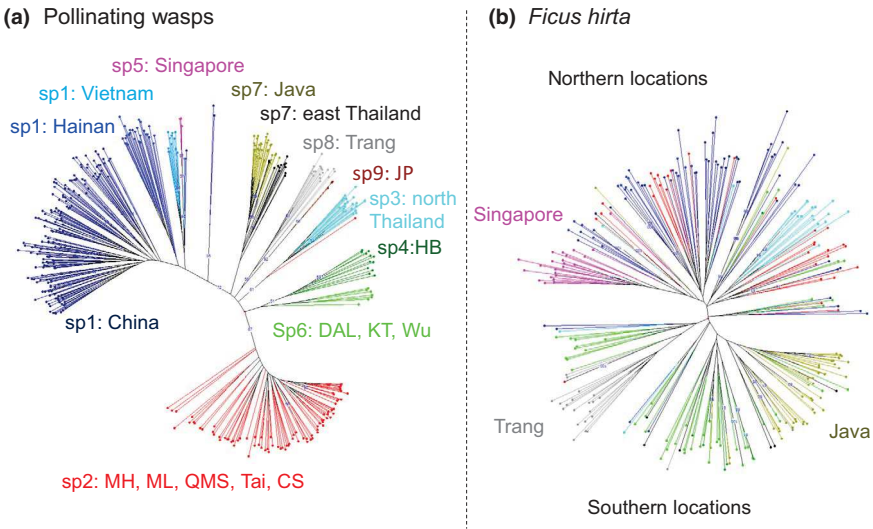


FIGURE 3 Neighbour-joining microsatellite genotype networks for (a) the pollinating wasps and (b) *Ficus hirta*. The colours indicate the most common pollinator species recorded at the location where a plant genotype was collected

where its host plant is also absent and is apparently replaced in peninsular Thailand by sp8 (Figure 2a).

AMOVA revealed considerable haplotype variation within species, as well as within and among locations (Supporting Information Table S6). The exception was sp5, where haplotype diversity was strikingly low, with only two almost identical haplotypes represented in the 17 individuals (Supporting Information Figure S4). In the two more extensively sampled species, we observed negative deviations from neutral assumptions in both sp1 (Tajima's D value = −1.51, p = 0.055; Fu and Li's D = −2.75, p < 0.05; and Fu and Li's F = −2.69, p < 0.05) and sp2 (Tajima's D = −1.88, p < 0.05; Fu and Li's D = −3.49, p < 0.01; and Fu and Li's F = −3.40, p < 0.01). Deviations were not significant in the other species. The COI haplotype networks (Supporting Information Figure S4) revealed a subdivision in sp1 into a southeast China group of haplotypes, a Hainan Island group of haplotypes and a Vietnamese group of haplotypes, with some haplotype exchange between southeast China and Hainan Island.

3.1.2 | Pollinating fig wasps microsatellite data

Diversity indices for microsatellite markers are given in Supporting Information Table S7. Pairwise genetic differentiation between location values (F_{ST}) is given in Supporting Information Table S8.

The first eight components of the discriminant analysis separated the nine species (Supporting Information Figure S5). The analysis also shows differentiation within sp1 according to geographic origin (China, Hainan and North Vietnam), as suggested by the COI analysis (Supporting Information Figure S4). While ITS2 sequences did not allow separation of sp2 from sp9 and sp6 from sp7, the discriminant analysis of microsatellite data separates them without ambiguity on component 2. Hence, the discriminant analysis confirms the presence of nine nuclear genetic clusters corresponding to the species detected using the cytoplasmic COI sequences.

In the neighbour-joining tree (Figure 3a), the different species are again separated, except for sp5, which seems to be close to sp1 from Vietnam, a result that is not compatible with the nuclear and cytoplasmic sequence data. The subdivision of sp1 into three geographic entities is supported. The close proximity between sp4 and sp6 is also supported, as is the slight separation within sp7 depending on geographic origin.

The bar plots of assignments of individuals to clusters using STRUCTURE with variable numbers of clusters are presented in Supporting Information Figure S6a. Irrespective of the different values of K, assignments to clusters are generally strong, except for the south China locations for sp1 for which mixed assignments suggest the presence of some variation among locations. From K = 6 to 22, the populations of sp1 from Hainan island and Vietnam are each assigned to their own cluster. For K = 11, all the species are separated into different clusters except for sp4 and sp6 (Figure 4a). For K = 20 and above, sp4 and sp6 are separated into distinct clusters. For K = 22 and above, the individuals of sp7 collected in Thailand were perfectly separated from those collected in Java. Consistently, the separation of sp3 to sp9 is somewhat unstable across the different values of K. As with the COI data, and despite different individuals being used for COI and microsatellite analyses, the microsatellite data suggested assignment of one individual to sp3 in location CS and another individual to sp7 in location Wu.

Genetic isolation by distance could only be explored for sp1 and sp2 as the other species were only sampled in 1–3 locations. There was significant isolation by distance, as shown by the relationship of $F_{ST}/(1 - F_{ST})$ with distance (Mantel test) in sp1 (R^2 = 0.13, p = 0.008), but not in sp2 (R^2 = 0.023, p = 0.459). However, the signature of isolation by distance in sp1 was generated by the differentiation of sp1 into the three genetically differentiated geographic entities already detected in the COI, in the discriminant and in the STRUCTURE analyses (located in southeast China, Hainan and Vietnam, Figure 5), despite obtaining a single ITS2 haplotype for sp1 (with 33, 11 and 9 individuals genotyped, respectively). There was no genetic isolation by distance within any of these three geographic entities.

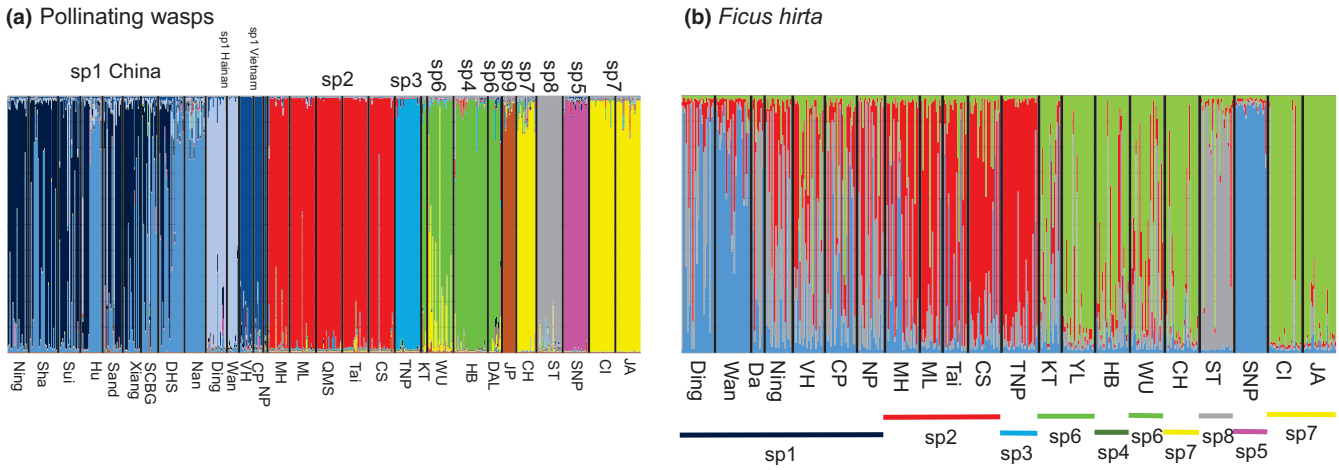


FIGURE 4 Bar plots of membership probabilities of individuals to the different clusters (as vertical bars) from the STRUCTURE analysis. (a) Genetic structure of the pollinating wasps obtained at $K = 11$; (b) genetic structure of the plant host, *Ficus hirta*, obtained at $K = 4$. For the pollinator, sp1 genotypes from continental China present mixed assignments to two clusters suggesting genetic isolation by distance, while genotypes from Hainan and genotypes from Vietnam are each assigned to a particular cluster. For *F. hirta*, there is no clear global pattern of clustering, suggesting clinal variation in gene frequencies. The Singapore genotypes (SNP) cluster with Hainan Island (south China) genotypes Ding and Wan

3.2 | Ficus hirta

3.2.1 | *Ficus hirta* cpDNA analysis

We detected 24 haplotypes (H1–H24). Sequences of the trnL-trnF and trnS-trnG regions have been deposited in GenBank under Accession nos. GQ452019–GQ452032 and MF467405–MF467416. We did not detect any signature of selection or of population expansion on cpDNA sequences. The cpDNA network and the distribution of these haplotypes among populations are presented in Figure 2b and Supporting Information Table S9. The haplotype network is centred on haplotype H8, and no haplotype differed from H8 by more than six mutations (Figure 2b). Haplotype H8 was observed throughout the range of *F. hirta*. Haplotype diversity was high in the north-central part of our sampling zone, where sampling density was highest. AMOVA confirmed significant differentiation among populations ($F_{ST} = 0.799$; $p < 0.001$). The colours correspond to different branches of the network, and their geographic distribution suggests spatial structuring. The regression of population pairwise F_{ST} on the natural logarithm of geographic distance was also significant ($R^2 = 0.0472$, $p = 0.003$, Mantel test), confirming that there was spatial genetic structure among populations of the plant.

3.2.2 | *Ficus hirta* microsatellite analysis

Genetic diversity parameters are given in Supporting Information Table S10. Pairwise genetic differentiation between location values (F_{ST}) is given in Supporting Information Table S11.

The first four components of the discriminant analysis organized the data along a north–south axis, except for the genotypes from Singapore, which were placed close to the northern genotypes (Supporting Information Figure S7). The discriminant analysis suggests much more continuous genetic variation in *F. hirta* than in its pollinators (compare Supporting Information Figures S5 and S7).

As with the discriminant analysis, the neighbour-joining tree separates the data along a north–south axis (Figure 3b). The Singapore samples and the Trang samples each form highly homogeneous clusters. As in the discriminants analysis, the Trang samples are placed with southern locations while the Singapore samples are placed with northern locations.

The bar plots of assignments of individuals to clusters of varying total number using STRUCTURE are presented in Supporting Information Figure S6b. For $K = 2$, genotypes from all northern locations plus Singapore were mainly assigned to cluster one and genotypes from southern locations were mainly assigned to cluster two (Supporting Information Figure S6b). The geographic distribution of assignments (Figure 2a) showed: (a) progressive geographic genetic variation of *F. hirta* along a north–south axis; (b) some geographic east–west geographic genetic structure; and (c) a placement of the Singapore samples that does not correspond to its geographic location. For $K = 4$, genotypes from the northern locations had mixed assignments to two clusters, supporting an east–west spatial genetic structure, genotypes from Singapore still clustered with genotypes from the north, and genotypes from Trang formed a distinct group (Figure 4b).

We also explored spatial genetic structure by plotting genetic differentiation between locations against geographic distance (Figure 6). Comparisons between locations showed a general pattern of genetic isolation by distance ($R^2 = 0.204$, permutation test, $p = <10^{-3}$). Singapore was an exception, as it was most similar to the geographically distant northern locations.

Taken together, the factorial analysis, the STRUCTURE assignments to clusters and the neighbour-joining tree all suggest that the genetic diversity of *F. hirta* is organized according to a pattern of genetic

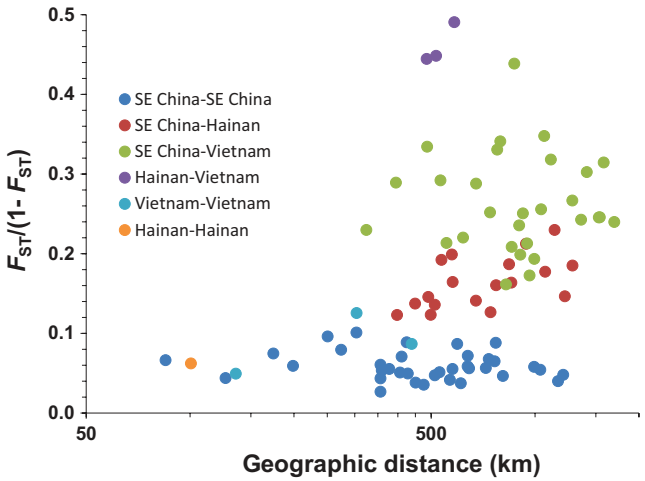


FIGURE 5 Spatial genetic structuring of the pollinator fig wasp sp1 based on nuclear microsatellite data. Genetic differentiation between locations is plotted according to geographic distance. The colours indicate the geographic origin of the locations being compared. Dark blue: comparison between two locations from southeast China; red: comparison between a location in southeast China and one in Hainan Island; green: comparison between a location from southeast China and one from Vietnam; violet: comparison between a location from Hainan Island and one from Vietnam; light blue, comparison between two locations from Vietnam; orange: comparison between the two Hainan Island locations. The species is structured into three genetic groups: southeast China, Hainan and Vietnam, the same structure as revealed by the cytoplasmic data (Supporting Information Figure S2)

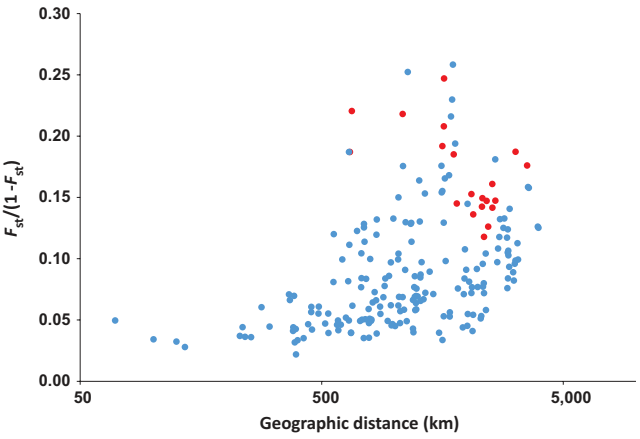


FIGURE 6 Pairwise genetic differentiation between locations in *Ficus hirta*, as a function of distance, based on microsatellite data. Red points: comparisons involving the Singapore location [Colour figure can be viewed at wileyonlinelibrary.com]

isolation by distance. Despite their southern location, the Singapore genotypes belong with the northern genotypes. Furthermore, the local pollinator (sp5) is only distantly related to the other pollinator species.

4 | DISCUSSION

4.1 | Species status of the pollinators

Although different pollinating wasp individuals were genotyped using COI, ITS2 and microsatellites, the parapatric distribution of the wasp clades allows direct comparison of results obtained with the different markers. Cytoplasmic and nuclear markers subdivide the pollinating wasps into the same nine clades. The COI divergence between clades (ranging from 5.4% to 28%, with most values above 10%) is larger than the divergence previously reported between sister species of fig pollinating wasps that share the same host (2.4%–7.4%; Yang et al., 2015). Furthermore, the nine clades can be separated morphologically (Wiebes, 1993; J.Y. Rasplus, com. pers.) and we therefore conclude that *F. hirta* is pollinated by nine species of fig wasps in our study zone, which covered most but not all of the range of the plant.

Sp5 is not closely related to the other pollinators of *F. hirta*. It was recorded from Singapore, where our data confirm an earlier proposition that *F. hirta* is not native (Berg & Corner, 2005). We suggest that sp5 is the regular pollinator of another *Ficus* species that has colonized *F. hirta* in Singapore. Fig trees planted outside their native range are known to similarly be capable of supporting successful development by fig wasps that usually breed in the figs of another *Ficus* species (Cook & Segar, 2010; Wang et al., 2016). Sequences from GenBank suggest that *Valisia esquiroliana* Chen & Chou is nested within the group of *Valisia* species associated with *F. hirta*. This fig wasp was described from *Ficus triloba* Buch.-Ham. ex Voigt, a species closely related to *F. hirta* (Berg, 2007; Berg & Corner, 2005). Genetic analyses incorporating these two *Ficus* species will be required to clarify their evolutionary relationships.

Genetic differentiation was detected within the distribution of pollinator sp1. Its populations are divided into three genetic groups located in continental China, Hainan Island and Vietnam. Such differentiation could be a first step towards speciation. Experimental data are needed to determine whether these genetic groups are interfertile. Reproductive isolation could arise rapidly in fig pollinating wasps as they display a high prevalence of *Wolbachia* that could cause cytoplasmic incompatibilities (Haine & Cook, 2005) and they display systematic assortative mating due to mating in their natal figs before dispersal (Anstett et al., 1997).

4.2 | The biogeography of *Ficus* and their pollinators

Ficus hirta displays a signature of genetic isolation by distance with both nuclear microsatellites and the cytoplasmic haplotypes, with no marked subdivision into distinct gene pools. It has been proposed that speciation in nursery pollinators and their host plants may be decoupled (Hembry & Althoff, 2016) and our results provide an example of this phenomenon.

The disjunct distribution of sp7, with a 2,000-km gap and with three species of pollinating wasps (sp5, sp8 and sp9) located in between, suggests that pollinators can successfully colonize distant host plants and establish populations there. A similar example is

known from Australia, where one pollinator of *F. rubiginosa* is present in the cool south and at a higher altitude in the warmer north, resulting in a 1,500-km distribution gap (Sutton, Riegler, & Cook, 2016). This suggests that pollinating fig wasp distributions are plastic and do not necessarily reflect a classical biogeographic history. Indeed, the biogeographic history of *F. racemosa* populations differs from that of its pollinator species, despite their identical separation into large gene pools (Bain et al., 2016). Similarly, in *F. septica*, an ecologically divergent pollinating fig wasp has colonized a 2,000-km range previously only occupied by three parapatric pollinating wasp species (Rodriguez et al., 2017). Thus, the distributions of fig pollinating wasp species seem to be dynamic and reveal cases of successful establishment of long-distance migrants. The long-range dispersal abilities of at least some fig wasps should facilitate this (Ahmed et al., 2009).

The mismatch distributions of COI sequences in the two best-sampled species (sp1 and sp2) provide signatures of either selective sweeps or population expansion. This is similar to what has been found in the pollinators of *F. pumila* (Chen et al., 2012) and in a pollinator of *F. septica* (Lin, Yeung, & Li, 2008). It has been proposed that these signatures are due to recurrent regional extinctions of pollinating wasps during extreme climatic events, but not of their host plants, followed by population expansions (Tian et al., 2015). Contemporary examples of this phenomenon have been documented in Florida (hurricane), southern France (frost) and the north of Borneo Island (El Nino-associated draught; Tian et al., 2015). Climatic episodes such as these make fig wasp species ranges intrinsically dynamic.

The establishment of new disjunct populations by long-distance migrants depends on a combination of suitable environmental and biological conditions in their new locations. Founder populations will also usually be small, which can lead to Allee effects. These result from (a) genetic inbreeding and loss of heterozygosity; (b) demographic stochasticity (including sex-ratio fluctuations); and (c) a reduction in cooperative interactions when individuals are at low density (Courchamp, Clutton-Brock, & Grenfell, 1999). As a fig is often colonized by a single fig wasp and given that their offspring mate in their natal fig before dispersal, inbreeding among fig wasps is routine (Molbo, Machado, Herre, & Keller, 2004), and consequently, we expect founder populations of fig wasps to be little affected by inbreeding. Furthermore, although offspring sex ratios are heavily female-biased, foundress females typically begin by laying male eggs in a clutch, so female offspring rarely have difficulty finding mates (Raja, Suleman, Compton, & Moore, 2008). Finally, foundress females compete within figs for oviposition sites, so no cooperation between them is expected (Herre, 1989). Consequently, pollinating fig wasps are probably not sensitive to Allee effects and they should be much less dependent on progressive geographic range expansion than many other organisms. Within this context, the parapatric distributions of many pollinating fig wasps suggest an important role for interspecific competition in shaping their distributions, as has been proposed for nonpollinating fig wasps (Darwell & Cook, 2017).

4.3 | Diversification in plant–insect mutualisms

The total of nine pollinator species found on *F. hirta* represents the highest ever-reported number of pollinator species for a *Ficus* species. Almost as high pollinator diversity has been documented for some other *Ficus* species (five for *F. rubiginosa*, Darwell et al., 2014; five for *F. racemosa*, Bain et al., 2016; four for *F. septica*, Rodriguez et al., 2017). This is strikingly different from the comprehensively known *Yucca* moth–*Yucca* association, where from a total of 24 pollinating moth species (Pellmyr, Balcázar-Lara, Segraves, Althoff, & Littlefield, 2008) there are only five instances of two copollinators sharing on a host (Smith, Drummond, Godsoe, Yoder, & Pellmyr., 2009). Available data on the less comprehensively investigated *Epicephala*–Phyllantheae association suggest an intermediate situation with up to four pollinator species associated with one host (Li et al., 2015). The comparatively low pollinator diversity observed in Yucca moths could be related to several factors. First, Yuccas have much more restricted distribution ranges than *Ficus* species, with only the distribution ranges of the two most northern Yucca species reaching 2,000 km (Althoff, Segraves, Smith, Leebens-Mack, & Pellmyr, 2012). Second, Yucca moths can exhibit prolonged diapause, a factor that will slow down the dynamics of population divergence (Powel, 1992). Third, assortative mating is not systematic, unlike in fig wasps (Powel, 1992).

In addition to their contrasting rates of differentiation, the population genetics of *F. hirta* and its pollinators are strikingly different. Whereas *F. hirta* exhibits clinal genetic variation with a signature of genetic isolation by distance, its pollinator species sp1 and sp2 lack any such signature. The same pattern has been observed for *F. pumila* L. and one of its pollinating wasps in southeast China (Liu et al., 2015). Hence, although pollinating fig wasps diversify faster than their host figs, they also display signatures of stronger gene flow. This apparent contradiction suggests that factors which facilitate speciation, such as a short generation time, infection by *Wolbachia* and assortative mating, can compensate for extensive gene flow and be decisive factors facilitating pollinating fig wasp speciation. Some nonpollinating fig wasps (NPFW, belonging to several families of Chalcidoidea) may disperse as widely as pollinator fig wasps (Kjellberg & Proffit, 2016; Sutton et al., 2016). NPFW have a largely similar biology to the pollinators, but exhibit varying intensities of local mate competition due to varying patterns of oviposition and mating sites. Obligatory assortative mating among NPFW ranges from minimal (among species that mate outside natal figs), through intermediate (NPFW with offspring dispersed across numerous figs that mate in the cavity of their natal figs) to being highly similar to that of pollinating wasps (with offspring aggregated in a single or small number of figs and mating inside these natal figs; Cook & Rasplus, 2003). If assortative mating indeed facilitates speciation, then we would expect a correlation between NPFW reproductive behaviour and speciation rates in different NPFW clades.

The mismatch between genetic structuring of host plant and fig wasps shows that, as in *Yucca* moths (Godsoe, Yoder, Smith, Drummond, & Pellmyr, 2010) and *Epicephala* moths (Hembry et al.,

2018), fine population-level phenotypic matching between mutualists is not required to allow population persistence. Nevertheless, on Hainan Island, the populations of *F. hirta* and its pollinator present the same signature of marked genetic differentiation from continental populations. Hence, the mismatch in population genetic structure is not consistent over the whole range of the association. Such differences in genetic costructuring are also encountered among associations in other species-specific plant–insect interactions. For instance, in a specialized association between two ants and an ant plant, with populations arranged as stepping stones, the ants and the plant presented very similar genetic signatures of population geographic expansion (Léotard et al., 2009). In a similar association showing a history of restriction to refugia followed by expansion leading to more continuous populations, the plants and insects had the same pattern of genetic geographic structuring, but different histories (Blatrix et al., 2017). The latter study also showed that the trees had evolved stronger reproductive isolation than their ant symbionts. Hence, available case studies suggest that patterns of diversification in plant–insect mutualistic interactions are not uniform among representatives of the same type of interaction. As more case studies become available, exploring how variation is explained by the biology of individual species is the next challenge.

4.4 | Relationships between tree characteristics and diversity of pollinators

Pollinator diversity has now been explored throughout large parts of the distribution ranges of four widely distributed Asian and Australasian *Ficus* species (*F. hirta*, *F. septica*, *F. racemosa* and *F. rubiginosa*; Bain et al., 2016; Darwell et al., 2014; Rodriguez et al., 2017). Their pollinator fig wasps each form old monophyletic species complexes (dating back >10 Ma) associated with a single host (Bain et al., 2016; Cruaud et al., 2012), but the species groups display varying geographic scales of pollinator species diversification. The host figs also display varying scales of genetic differentiation. Further studies are required to investigate whether plant traits such as crop size, crop synchrony and growth form may combine to select for different pollinator dispersal behaviour and may result in pollinator and fig genetic diversity being expressed at different geographic scales.

ACKNOWLEDGEMENTS

We thank Prof. Xia Nianhe, Peng Yanqiong, Vu Quang Nam (Vietnam National Foundation for Science and Technology Development under grant numbers 106.11-2012.82), Tien Tran, Nong Van Duy, Zhang Xianchun (collecting permission number in Indonesia: No. 1799IPH.1.02/KS.01/XI/2014), Harry Wiriadinata, Arief Hidayat, Wei Ran, Chen Wenli, Mashhor Mansor, Nora Sikin, Serena Lee (provided Singapore samples), Khin Me Aung, Chuck Cannon, Thitima Tharawoot and Liang Dan for sample collection and help in the field. We thank Mei Qiming, Liu Qing, Kong Hanghui and Tu Tieyao for their help with cluster computers. We also thank Liu Yunxiao for her help with figures. We thank Simon Segar, Tatiana Giraud and the anonymous reviewers for helpful comments on the manuscript. This work was supported by the National Natural Science Foundation of China (31670395; 31630008; 31370409), the Guangzhou Science and Technology Plan Project (201707010351), the External Cooperation Program of Bureau of International Co-operation Chinese Academy of Sciences (No. GJHZ201321) and the CAS President's International Fellowship Initiative (2017VBA0039).

CONFLICT OF INTEREST

The authors declared that they have no competing interests.

AUTHOR CONTRIBUTIONS

Y.H. designed research, collected samples, analysed data and cowrote the manuscript. S.G.C. cowrote the manuscript. F.K. contributed to data analyses and cowrote the manuscript. E.W.T. and Z.L.N. collected samples, performed laboratory work and analysed data. D.X.X. and Y.F.C. performed laboratory work. D.Y.Z. designed research. C.L.F. and W.W. analysed data. W.T. collected samples in Thailand. All authors contributed substantially to revisions.

DATA ACCESSIBILITY

Microsatellite genotype data, final assembly mtDNA sequences for all individuals, ITS2 nrDNA sequences for each species and each haplotypes of *Valasia javana*, and microsatellite genotype data and final assembly cpDNA sequences for all individuals of *Ficus hirta* are archived in the Dryad Digital Repository at http://datadryad.org, https://doi.org/10.5061/dryad.k7t33tg.

ORCID

Hui Yu https://orcid.org/0000-0003-0074-9153

REFERENCES

Ahmed, S., Compton, S. G., Butlin, R. K., & Gilmartin, P. M. (2009). Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 20342–20347. https://doi.org/10.1073/pnas.0902213106

Althoff, D. M., Segraves, K. A., Smith, C. I., Leebens-Mack, J., & Pellmyr, O. (2012). Geographic isolation trumps coevolution as a driver of yucca and yucca moth diversification. *Molecular Phylogenetics and Evolution*, *62*, 898–906. https://doi.org/10.1016/j.ympev.2011.11.024

Anstett, M. C., Hossaert-McKey, M., & Kjellberg, F. (1997). Figs and fig pollinators: Evolutionary conflicts in a coevolved mutualism. *Trends in Ecology and Evolution*, *12*, 94–99.

Bain, A., Borges, R. M., Chevallier, M. H., Vignes, H., Kobmoo, N., Peng, Y. Q., … Hossaert-Mckey, M. (2016). Geographic structuring into vicariant species-pairs in a wide-ranging, high-dispersal plant-insect mutualism: The case of *Ficus racemosa* and its pollinating wasps. *Evolutionary Ecology*, *30*, 663–684. https://doi.org/10.1007/s10682-016-9836-5

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., & Bonhomme, F. (1996–2004). *GENETIX 4.05*, *logiciel sous Windows TM pour la génétique des populations*. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France). http://kimura.univ-montp2.fr/genetix/

Berg, C. C. (2003). Flora Malesiana precursor for the treatment of Moraceae 3: *Ficus* subgenus *Ficus*. *Blumea*, *48*, 529–550. https://doi.org/10.3767/000651903X489537

Berg, C. C. (2007). Precursory taxonomic studies on *Ficus* (Moraceae) for the Flora of Thailand. *Thai Forest Bulletin (Botany)*, *35*, 4–28.

Berg, C. C., & Corner, E. J. H. (2005). Moraceae (*Ficus*). In H. P. Nooteboom (Ed.), *Flora Malesiana* (pp. 1–730). Leiden, the Netherlands: National Herbarium Nederland.

Blatrix, R., Peccoud, J., Born, C., Piatscheck, F., Benoit, L., Sauve, M., … McKey, D. (2017). Comparative analysis of spatial genetic structure in an ant–plant symbiosis reveals a tension zone and highlights speciation processes in tropical Africa. *Journal of Biogeography*, *44*, 1856–1868. https://doi.org/10.1111/jbi.12972

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., … Drummond, A. J. (2014). BEAST 2: A software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, *10*, e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Bruun-Lund, S., Verstraete, B., Kjellberg, F., & Rønsted, N. (2018). Rush hour at the Museum – Diversification patterns provide new clues for the success of figs (*Ficus* L., Moraceae). *Acta Oecologica*, *90*, 4–11. https://doi.org/10.1016/j.actao.2017.11.001

Chen, Y., Compton, S. G., Liu, M., & Chen, X. Y. (2012). Fig trees at the northern limit of their range: The distributions of cryptic pollinators indicate multiple glacial refugia. *Molecular Ecology*, *21*, 1687–1701. https://doi.org/10.1111/j.1365-294X.2012.05491.x

Chen, Y., Zhu, X. J., Compton, S. G., Liu, M., & Chen, X. Y. (2011). Genetic diversity and differentiation of the extremely dwarf *Ficus tikoua* in Southwestern China. *Biochemical Systematics and Ecology*, *39*, 441–444. https://doi.org/10.1016/j.bse.2011.06.006

Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, *9*, 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Conchou, L., Cabioch, L., Rodriguez, L. J. V., & Kjellberg, F. (2014). Daily rhythm of mutualistic pollinator activity and scent emission in *Ficus septica*: Ecological differentiation between co-occurring pollinators and potential consequences for chemical communication and facilitation of host speciation. *PLoS ONE*, *9*, e103581. https://doi.org/10.1371/journal.pone.0103581

Cook, J. M., & Rasplus, J. Y. (2003). Mutualists with attitude: Coevolving fig wasps and figs. *Trends in Ecology and Evolution*, *18*, 241–248. https://doi.org/10.1016/S0169-5347(03)00062-4

Cook, J. M., & Segar, S. T. (2010). Speciation in fig wasps. *Ecological Entomology*, *35*(Suppl. 1), 54–66. https://doi.org/10.1111/j.1365-2311.2009.01148.x

Corlett, R. T. (2006). Figs (*Ficus*, Moraceae) in Urban Hong Kong, South China. *Biotropica*, *38*, 116–121.

Cornille, A., Underhill, J. G., Cruaud, A., Hossaert-McKey, M., Johnson, S. D., Tolley, K. A., … Proffit, M. (2012). Floral volatiles, pollinator sharing and diversification in the fig-wasp mutualism: insights from *Ficus natalensis*, and its two wasp pollinators (South Africa). *Proceedings of the Royal Society of London Series B-Biological Sciences*, *279*, 1731–1739. https://doi.org/10.1098/rspb.2011.1972

Courchamp, F., Clutton-Brock, T., & Grenfell, B. (1999). Inverse density dependence and the Allee effect. *Trends in Ecology and Evolution*, *14*, 405–410. https://doi.org/10.1016/S0169-5347(99)01683-3

Craft, K. J., Pauls, S. U., Darrow, K., Miller, S. E., Hebert, P. D. N., Helgen, L. E., … Weiblen, G. D. (2010). Population genetics of ecological communities with DNA barcodes: An example from New Guinea Lepidoptera. *Proceedings of the National Academy of Sciences*, *107*, 5041–5046. https://doi.org/10.1073/pnas.0913084107

Crandall, K. A., & Templeton, A. R. (1993). Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics*, *134*, 959–969.

Cruaud, A., Jabbour-Zahab, R., Genson, G., Cruaud, C., Couloux, A., Kjellberg, F., & Rasplus, J. Y. (2010). Laying the foundations for a new classification of Agaonidae (Hymenoptera: Chalcidoidea), a multilocus phylogenetic approach. *Cladistics*, *26*, 359–387.

Cruaud, A., Rønsted, N., Chantarasuwan, B., Chou, L. S., Clement, W. L., Couloux, A., … Savolainen, V. (2012). An extreme case of plant–insect codiversification: Figs and fig-pollinating wasps. *Systematic Biology*, *61*, 1029–1047. https://doi.org/10.1093/sysbio/sys068

Darwell, C. T., al-Beidh, S., & Cook, J. M. (2014). Molecular species delimitation of a symbiotic fig-pollinating wasp species complex reveals extreme deviation from reciprocal partner specificity. *BMC Evolutionary Biology*, *14*, 189. https://doi.org/10.1186/s12862-014-0189-9

Darwell, C. T., & Cook, J. M. (2017). Cryptic diversity in a fig wasp community—Morphologically differentiated species are sympatric but cryptic species exhibit competitive exclusion. *Molecular Ecology*, *26*, 937–950.

Dufaÿ, M., & Anstett, M. C. (2003). Conflicts between plants and pollinators that reproduce within inflorescences: Evolutionary variations on a theme. *Oikos*, *100*, 3–14. https://doi.org/10.1034/j.1600-0706.2003.12053.x

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797. https://doi.org/10.1093/nar/gkh340

Excoffier, L., Laval, G., & Schneider, S. (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics*, *1*, 47–50.

Fu, Y. X., & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics*, *133*, 693–709.

Godsoe, W., Yoder, J. B., Smith, C. I., Drummond, C. S., & Pellmyr, O. (2010). Absence of population-level phenotype matching in an obligate pollination mutualism. *Journal of Evolutionary Biology*, *23*, 2739–2746. https://doi.org/10.1111/j.1420-9101.2010.02120.x

Haine, E. R., & Cook, J. M. (2005). Convergent incidences of *Wolbachia* infection in fig wasp communities from two continents. *Proceedings of the Royal Society of London Series B-Biological Sciences*, *272*, 421–429.

Hembry, D. H., & Althoff, D. M. (2016). Diversification and coevolution in brood pollination mutualisms: Windows into the role of biotic interactions in generating biological diversity. *American Journal of Botany*, *103*, 1783–1792. https://doi.org/10.3732/ajb.1600056

Hembry, D. H., Raimundo, R. L. G., Newman, E. A., Atkinson, L., Guo, C., Guimarães, P. R. Jr, & Gillespie, R. G. (2018). Does biological intimacy shape ecological network structure? A test using a brood pollination mutualism on continental and oceanic islands. *Journal of Animal Ecology*, *87*, 1160–1171. https://doi.org/10.1111/1365-2656.12841

Herre, E. A. (1989). Coevolution and reproductive characteristics in 12 species of New World figs and their pollinator wasps. *Experientia*, *45*, 637–647.

Jia, X. C., Dan, Y., Zhang, Y., & Chen, Y. Z. (2007). Direct evidence for the cycling of fig wasps within one male fig tree. *Nordic Journal of Botany*, *25*, 119–124. https://doi.org/10.1111/j.0107-055X.2007.00021_23.x

Jones, M., Ghoorah, A., & Blaxter, M. (2011). jMOTU and taxonerator: Turning DNA barcode sequences into annotated operational taxonomic units. *PLoS ONE*, *6*, e19259. https://doi.org/10.1371/journal.pone.0019259

Jousselin, E., van Noort, S., Berry, V., Rasplus, J. Y., Rønsted, N., Erasmus, J. C., & Greeff, J. M. (2008). One fig to bind them all: Host conservatism in a fig wasp community unravelled by cospeciation analyses among pollinating and nonpollinating fig wasps. *Evolution*, *62*, 1777–1797.

Kass, R. E., & Raftery, A. E. (1995). Bayes factors. *Journal of American Statistical Association*, *90*, 773–795. https://doi.org/10.1080/01621459.1995.10476572

Kawakita, A. (2010). Evolution of obligate pollination mutualism in the tribe Phyllantheae (Phyllanthaceae). *Plant Species Biology*, *25*, 3–19. https://doi.org/10.1111/j.1442-1984.2009.00266.x

Kjellberg, F., & Proffit, M. (2016). Tracking the elusive history of diversification in plant–herbivorous insect–parasitoid food webs: Insights from figs and fig wasps. *Molecular Ecology*, *25*, 843–845. https://doi.org/10.1111/mec.13533

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. *Molecular Ecology*, *19*, 2700–2712. https://doi.org/10.1111/j.1365-294X.2010.04654.x

Lawton, J., Lewinsohn, T., & Compton, S. (1993). Patterns of diversity for the insect herbivores on bracken. In R. E. Ricklefs, & D. Schluter (Eds.), *Species diversity in ecological communities: Historical and geographical perspectives* (pp. 178–184). Chicago, IL: University of Chicago Press.

Leather, S. R. (1986). Insect species richness of the British Rosaceae: The importance of host range, plant architecture, age of establishment, taxonomic isolation and species-area relationships. *Journal of Animal Ecology*, *55*, 841–860. https://doi.org/10.2307/4420

Léotard, G., Debout, G., Dalecky, A., Guillot, S., Gaume, L., McKey, D., & Kjellberg, F. (2009). Range expansion drives dispersal evolution in an Equatorial three-species symbiosis. *PLoS ONE*, *4*, e5377.

Lewinsohn, T. M., & Roslin, T. (2008). Four ways towards tropical herbivore megadiversity. *Ecology Letters*, *11*, 398–416. https://doi.org/10.1111/j.1461-0248.2008.01155.x

Li, H., Wang, Z., & Hu, B. (2015). Four new species of *Epicephala* Meyrick, 1880 (Lepidoptera, Gracillariidae) associated with two species of *Glochidion* (Phyllanthaceae) from Hainan Island in China. *ZooKeys*, *508*, 53–67. https://doi.org/10.3897/zookeys.508.9479

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*, 451–1452. https://doi.org/10.1093/bioinformatics/btp187

Lin, R. C., Yeung, C. K. L., Fong, J. J., Tzeng, H. Y., & Li, S. H. (2011). The lack of pollinator specificity in a dioecious fig tree: Sympatric fig-pollinating wasps of *Ficus septica* in southern Taiwan. *Biotropica*, *43*, 200–207. https://doi.org/10.1111/j.1744-7429.2010.00686.x

Lin, R. C., Yeung, C. K., & Li, S. H. (2008). Fig trees at the northern limit of their range: The distributions of cryptic pollinators indicate multiple glacial refugia. *Molecular Ecology*, *21*, 1687–1701.

Liu, M., Compton, S. G., Peng, F. E., Zhang, J., & Chen, X. Y. (2015). Movements of genes between populations: Are pollinators more effective at transferring their own or plant genetic markers? *Proceedings of the Royal Society of London Series B-Biological Sciences*, *282*, 20150290.

Lopez-Vaamonde, C., Rasplus, J. Y., Weiblen, G. D., & Cook, J. M. (2001). Molecular phylogenies of fig wasps: Partial cocladogenesis of pollinators and parasites. *Molecular Phylogenetics and Evolution*, *21*, 55–71. https://doi.org/10.1006/mpev.2001.0993

Machado, C. A., Robbins, N., Gilbert, M. T. P., & Herre, E. A. (2005). Critical review of host specificity and its coevolutionary implications in the fig fig-wasp mutualism. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 6558–6565. https://doi.org/10.1073/pnas.0501840102

Mittelbach, G. C., & Schemske, D. W. (2015). Ecological and evolutionary perspectives on community assembly. *Trends in Ecology and Evolution*, *30*, 241–247. https://doi.org/10.1016/j.tree.2015.02.008

Molbo, D., Machado, C. A., Herre, E. A., & Keller, L. (2004). Inbreeding and population structure in two pairs of cryptic fig wasp species. *Molecular Ecology*, *13*, 1613–1623. https://doi.org/10.1111/j.1365-294X.2004.02158.x

Nenadić, O., & Greenacre, M. (2007). Correspondence analysis in R, with two- and three-dimensional graphics: The ca package. *Journal of Statistical Software*, *20*, 1–13.

Nicholls, J. A., Preuss, S., Hayward, A., Melika, G., Csóka, G., Nieves-Aldrey, J.-L., … Stone, G. N. (2010). Concordant phylogeography and cryptic speciation in two Western Palaearctic oak gall parasitoid species complexes. *Molecular Ecology*, *19*, 592–609. https://doi.org/10.1111/j.1365-294X.2009.04499.x

Novotny, V., Miller, S. E., Hulcr, J., Drew, R. A. I., Basset, Y., Janda, M., … Weiblen, G. D. (2007). Low beta diversity of herbivorous insects in tropical forests. *Nature*, *448*, 692–695. https://doi.org/10.1038/nature06021

Peakall, R., & Smouse, P. E. (2006). GENALEX *6*: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*, 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Pellmyr, O. (1999). Systematic revision of the yucca moths in the *Tegeticula yuccasella* complex (Lepidoptera: Prodoxidae) north of Mexico. *Systematic Entomology*, *24*, 243–271. https://doi.org/10.1046/j.1365-3113.1999.00079.x

Pellmyr, O. (2003). Yuccas, yucca moths, and coevolution: A review. *Annals of the Missouri Botanical Garden*, *90*, 35–55. https://doi.org/10.2307/3298524

Pellmyr, O., Balcázar-Lara, M., Segraves, K. A., Althoff, D. M., & Littlefield, R. J. (2008). Phylogeny of the pollinating yucca moths, with revision of Mexican species (*Tegeticula* and *Parategeticula*; Lepidoptera, Prodoxidae). *Zoological Journal of the Linnean Society*, *152*, 297–314. https://doi.org/10.1111/j.1096-3642.2007.00361.x

Perrier, X., & Jacquemoud-Collet, J. P. (2006). *DARwin software*. Retrieved from http://darwin.cirad.fr/

Petit, R. J., & Hampe, A. (2006). Some evolutionary consequences of being a tree. *Annual Review of Ecology Evolution and Systematics*, *37*, 187–214. https://doi.org/10.1146/annurev.ecolsys.37.091305.110215

Posada, D., & Crandall, K. A. (1998). MODELTEST: Testing the model of DNA substitution. *Bioinformatics*, *14*, 817–818. https://doi.org/10.1093/bioinformatics/14.9.817

Powel, J. A. (1992). Interrelationships of yuccas and yucca moths. *Trends in Ecology and Evolution*, *7*, 10–15. https://doi.org/10.1016/0169-5347(92)90191-D

Price, P. W. (1980). *Evolutionary biology of parasites. Princeton Monographs in Population Biology No. 15*. Princeton, NJ: Princeton University Press.

Price, P. W. (2002). Species interactions and the evolution of biodiversity. In O. Pellmyr & C. Herrera (Eds.), *Plant-animal interactions* (pp. 3–26). Oxford, UK: Blackwell Science.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multi-locus genotype data. *Genetics*, *155*, 945–959.

Raja, S., Suleman, N., Compton, S. G., & Moore, J. C. (2008). The mechanism of sex ratio adjustment in a pollinating fig wasp. *Proceedings of the Royal Society of London Series B-Biological Sciences*, *275*, 1603–1610.

Rambaut, A. (2006). *FigTree v1.3.1*. Retrieved from http://tree.bio.ed.ac.uk/software/figtree

Rodriguez, L. J., Bain, A., Chou, L.-S., Conchou, L., Cruaud, A., Gonzales, R., … Kjellberg, F. (2017). Diversification and spatial structuring in the mutualism between *Ficus septica* and its pollinating wasps in insular South East Asia. *BMC Evolutionary Biology*, *17*, 207. https://doi.org/10.1186/s12862-017-1034-8

Segar, S. T., Volf, M., Zima Jnr, J., Isua, B., Sisol, M., Sam, L., … Novotny, V. (2017). Speciation in a keystone plant genus is driven by elevation: A case study in New Guinean *Ficus*. *Journal of Evolutionary Biology*, *30*, 512–523.

Smith, C. I., Drummond, C. S., Godsoe, W., Yoder, J. B., & Pellmyr, O. (2009). Host specificity and reproductive success of yucca moths (*Tegeticula* spp. Lepidoptera: Prodoxidae) mirror patterns of gene flow between host plant varieties of the Joshua tree (*Yucca brevifolia*: Agavaceae). *Molecular Ecology*, *18*, 5218–5229. https://doi.org/10.1111/j.1365-294X.2009.04428.x

Song, H., Buhay, J. E., Whiting, M. F., & Crandall, K. A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 13486–13491. https://doi.org/10.1073/pnas.0803076105

Souto-Vilarós, D., Proffit, M., Buatois, B., Rindos, M., Sisol, M., Kuyaiva, T., … Segar, S. T. (2018). Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. *Journal of Ecology*, *106*, 2256–2273. https://doi.org/10.1111/1365-2745.12995

Strong, D. R., Lawton, J. H., & Southwood, R. (1984). *Insects on plants: Community patterns and mechanisms*. New York, NY: Harvard University Press.

Sutton, T. L., Riegler, M., & Cook, J. M. (2016). One step ahead: A parasitoid disperses farther and forms a wider geographic population than its fig wasp host. *Molecular Ecology*, *25*, 882–894. https://doi.org/10.1111/mec.13445

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729. https://doi.org/10.1093/molbev/mst197

Thomas, J. A., Welch, J. J., Lanfear, R., & Bromham, L. (2010). A generation time effect on the rate of molecular evolution in invertebrates. *Molecular Biology and Evolution*, *27*, 1173–1180. https://doi.org/10.1093/molbev/msq009

Tian, E. W., Nason, J. D., Machado, C. A., Zheng, L., Yu, H., & Kjellberg, F. (2015). Lack of genetic isolation by distance, similar genetic structuring but different demographic histories in a fig-pollinating wasp mutualism. *Molecular Ecology*, *24*, 5976–5991. https://doi.org/10.1111/mec.13438

Tian, E. W., Yu, H., Zhang, D. Y., & Nason, J. D. (2011). Development of microsatellite loci for *Blastophaga javana* (Agaonidae), the pollinating wasp of *Ficus hirta* (Moraceae). *American Journal of Botany*, *98*, e41–e43.

Wachi, N., Kusumi, J., Tzeng, H. Y., & Su, Z. H. (2016). Genome-wide sequence data suggest the possibility of pollinator sharing by host shift in dioecious figs (Moraceae, *Ficus*). *Molecular Ecology*, *25*, 5732–5746.

Wang, G., Cannon, C. H., & Chen, J. (2016). Pollinator sharing and gene flow among closely related sympatric dioecious fig taxa. *Proceedings of the Royal Society of London Series B-Biological Sciences*, *283*, 20152963.

Wiebes, J. T. (1993). Agaonidae (Hymenoptera Chalcidoidea) and *Ficus* (Moraceae): Fig wasps and their figs, xi (*Blastophaga*) *s.l. Proceedings of the Koninklijke Nederlandse Akademie Van Wetenschappen Series C*, *96*, 347–367.

Yang, L. Y., Machado, C. A., Dang, X. D., Peng, Y. Q., Yang, D. R., Zhang, D. Y., & Liao, W. J. (2015). The incidence and pattern of copollinator diversification in dioecious and monoecious figs. *Evolution*, *69*, 294–304. https://doi.org/10.1111/evo.12584

Yu, H., & Nason, J. D. (2013). Nuclear and chloroplast DNA phylogeography of *Ficus hirta*: Obligate pollination mutualism and constraints on range expansion in response to climate change. *New Phytologist*, *197*, 276–289.

Zheng, L. N., Nason, J. D., Liang, D., Ge, X. J., & Yu, H. (2015). Development and characterization of microsatellite loci for *Ficus hirta* (Moraceae). *Applications in Plant Sciences*, *3*, 1500034.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Yu H, Tian E, Zheng L, et al. Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation. *Mol Ecol*. 2019;28:2391–2405. https://doi.org/10.1111/mec.15046

Table S1. Sampling locations and sample sizes. * collected close to DAL at YL (lat 12.415, long 108.176). In bold, abbreviation used throughout the text.

Location	Name	Latitude	longitude	Wasp mtCOI	Wasp nSSR	Wasp ITS2	Fig cpDNA	Fig nSSR
S.E. China	**Gui**lin	25.077	110.306				11	
	Aomen	22.199	113.640				10	
	Dapu	24.258	116.806				10	8
	Xiamen	24.742	118.072				10	
	Ningde	26.664	119.549	10	20	3	4	19
	Shaxian	26.419	117.818	7	27		9	
	Suichuan	26.476	114.239	11	20	3	9	
	Xianggang	22.424	114.306	8	16	5	6	
	Huolu Mountain	23.170	113.373	20	15	4	9	
	Dinghu mountain	23.166	112.543	14	24	9	10	
	Hunan	25.571	111.946	7	20	3		
	Sandu	25.984	107.874	6	18	2	10	
	Nanning	22.787	108.389	9	19	4	9	
Hainan	**Hai**	18.642	109.701				2	
	Dingan	19.697	110.328	8	19	6		24
	Wan	18.795	110.391	10	10	5	12	25
Yunnan	**Xi**shuangbanna	21.913	101.264	3	0	3	9	
	Meng**H**ai	21.979	100.450	14	20	6	4	24
	Meng**L**un	21.447	101.568	22	24	13	3	15
Thailand	Temple Hill **Tai**	18.894	98.858	15	24	12	18	17
	Chao **S**on	18.84	99.47	17	24	9	10	23
	Other side of TempleHill **QMS**	18.809	98.914	16	24	8		
	Taksinmaharat **TNP**	16.782	98.928	13	24	8	14	26
	Wuwen	14.443	105.273	7	24	6	7	24
	CHantaburi	12.774	102.096	12	18	10	9	24
	Trang **ST**	7.467	99.639	11	24	11	22	24
Vietnam	Vinh Yen **VH**	21.467	105.581	8	12	7	10	22
	Cuc **P**huong	20.253	105.712	2	8	1	12	22
	Mengxi **NP**	17.579	106.308	2	2	2	12	19
	Hon Ba **HB**	12.248	108.796	28	32	14	10	24
	Dalat/Lac Thien (**DAL**/**YL**)	12.157	108.137	4	12	4	12*	23*
	Kon Tum (**KT**)	14.516	107.639	3	4	3	9	15
Singapore	**Si**N**ga**P**ore**	-1.312	103.816	17	24	18	12	23
Indonesia	**CI**nangneng	-6.566	106.706	7	24	8		24
	JAkarta	-6.368	106.830	11	24	7	12	24
Cambodia	Pnom-penh **JP**	11.353	104.153	7	12	9		
	North **JF**	12.499	107.318	1				

Table S2. Pollinating wasps, COI, haplotype data. Sample size (N), number of haplotypes (K), haplotype diversity (Hd), nucleotide diversity (π), and COI haplotypes with number of individuals.

		Pop.	N	K	Hd	π	COI haplotype (number of individuals)
sp1		Ning	10	7	0.911	0.00346	2 (2), 3 (1), 5 (1), 6 (3), 12 (1), 13 (1), 14 (1)
		Sha	7	5	0.905	0.00406	1 (1), 2 (2), 3 (1), 4 (2), 5 (1)
		Sui	11	8	0.945	0.00358	1 (2), 4 (1), 5 (2), 6 (1), 10 (1), 11 (2), 13 (1), 15 (1)
		Xiang	8	7	0.964	0.00393	5 (1), 6 (2), 7 (1), 8 (1), 9 (1), 10 (1), 11 (1)
		Huo	20	9	0.789	0.00738	1 (1), 5 (9), 6 (2), 12 (1), 14 (1), 23 (3), 27 (1), 28 (1), 29 (1)
		DHS	14	9	0.912	0.00332	4 (2), 5 (4), 6 (2), 14 (1), 22 (1), 23 (1), 24 (1), 25 (1), 26 (1)
		Hu	7	2	0.571	0.00168	5 (4), 6 (3)
		Sand	6	4	0.800	0.00274	5 (1), 6 (3), 12 (1), 16 (1)
		Nan	9	7	0.917	0.00653	5 (1), 6 (1), 17 (3), 18 (1), 19 (1), 20 (1), 21 (1)
		Ding	8	5	0.786	0.00184	30, 31, 32, 33, 34 (4)
		Wan	10	6	0.867	0.00723	30 (3), 31, 34, 35 (3), 36, 37
		VH	8	5	0.786	0.00603	29(4), 38, 39, 40, 41
		CP	2	2	1	0.00294	42, 29
		NP	2	1	0	0	43 (2)
		TOTAL	122	43	**0.933**	**0.00018**	
sp2		XI	3	3	1.000	0.00685	1, 2,3
		MH	14	6	0.824	0.00494	1, 2(5), 4, 5(3), 6(3), 7
		ML	22	16	0.961	0.00835	5(3), 8, 9(3), 10,11,12,13(3),14,15,16,17,18,19,20, 21, 22
		Tai	15	14	0.990	0.00951	13,23,24,25,26,27,28,29(2),30, 31, 32, 33, 34, 35
		CS	16	11	0.908	0.00664	13(5), 28, 36,37(2), 38, 39, 40, 41, 42, 43, 44
		QMS	16	14	0.983	0.00901	13(2),26,28,31,37,39,45,46, 47, 48, 49(2), 50, 51, 52
		TOTAL	86	52	**0.972**	**0.00008**	
sp3		TNP	13	9	0.910	0.00401	1, 2 , 3(4), 4, 5(2), 6, 7, 8, 9
		CS-1.12	1	1			10
		TOTAL	14	10	**0.923**	**0.00365**	
sp4		HB	28	14	**0.881**	**0.00422**	1 (2),2 ,3,4,5,6,7 (8),8 ,9 ,10,11(6), 12,13(2),14
sp5		SNP	17	2	**0.309**	**0.00045**	1 (3),2(14)
sp6		DAL	4	4	1	0.1371	1,2,3,4
		KT	3	3	1	0.01077	5,6,7
		Wu	6	6	1	0.00861	8,9,10,11,12,13
		JF	1	1			14
		TOTAL	14	14	**1.000**	**0.00073**	
sp7		CI	7	5	0.857	0.0028	1,2(3),3,4,5
		JA	11	5	0.855	0.00315	2(3),6,7(2),8(3),9(2)
		CH	12	7	0.909	0.00258	10(2),11 (3),12(2),13,14,15(2),16
		Wu-11	1	1	-	-	17
		TOTAL	31	17	**0.944**	**0.00057**	
sp8		ST	11	7	**0.909**	**0.00833**	1,2(3),3,4(2),5,6,7
sp9		JP	7	3	**0.714**	**0.00420**	1(3),2, 3(3)

Figure S1. Dated Bayesian tree for wasp COI gene sequences. Dates in million years. Age of the different nodes with 95% lower and upper highest posterior distribution inferred by BEAST reported between parentheses: sp 1-3 crown 6.0 (2.0-10.7); sp2-9 crown 4.6 (1.5, 8.3); sp 4-6-7 crown 4.6 (1.5, 8.1); clade (2-9)-(4-6-7) crown 7.8 (3.0, 13.5); clade (sp1-2-3-4-6-7-9) crown 9.5 (3.8, 15.9); clade (sp1-2-3-4-6-7-8-9) crown 11.5 (4.3, 19.2); clade (1-2-3-4-5-6-7-8-9) crown 15.9 (6.7, 26.3)

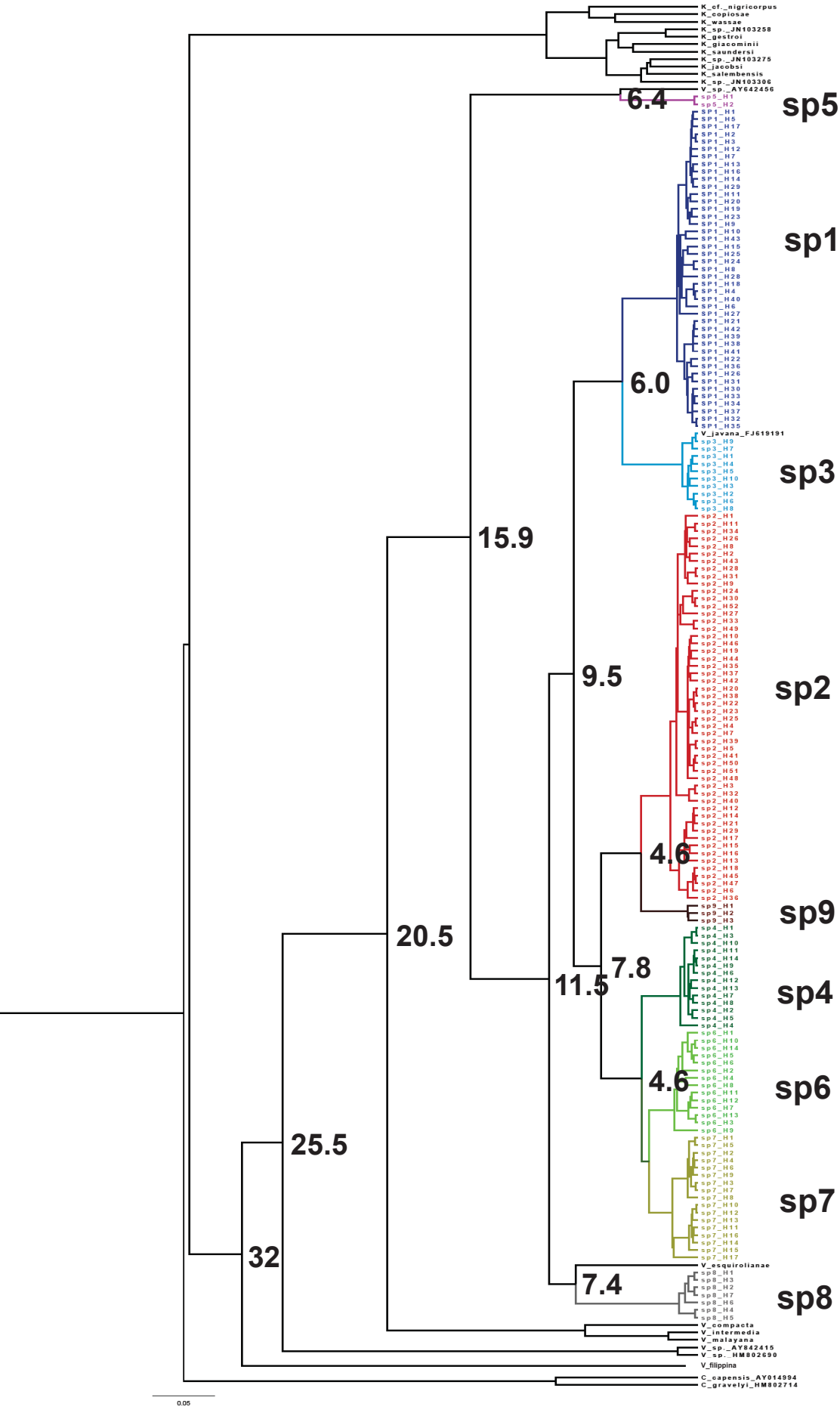


Table S3. Pollinating wasp COI cytoplasmic gene sequence differences (Kimura-2-parameter) within (diagonal) and between species (below diagonal). Within species differences are low (highlighted in green), confirming separation into different species. Note the limited difference between sp2 and sp9 (highlighted in blue) and the limited difference between sp4, sp6 and sp7 (highlighted in yellow). Sp5 stands out as highly divergent suggesting that it does not belong to the species group of *V. javana* (differences highlighted in red).

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9
sp1	0.008								
sp2	0.135	0.014							
sp3	0.113	0.113	0.005						
sp4	0.130	0.113	0.118	0.006					
sp5	0.266	0.266	0.243	0.265	0.001				
sp6	0.124	0.110	0.126	0.068	0.281	0.012			
sp7	0.138	0.117	0.123	0.058	0.266	0.054	0.014		
sp8	0.122	0.121	0.123	0.137	0.243	0.133	0.123	0.009	
sp9	0.134	0.064	0.109	0.114	0.272	0.119	0.111	0.104	0.006

Table S4. Pollinating wasp ITS2 nuclear gene sequence differences (Kimura-2-parameter) within (diagonal) and between species (below diagonal). Within species differences are equal to zero. Note, as for COI sequences, the limited difference between sp2 and sp9 (highlighted in blue) and the limited difference between sp4, sp6 and sp7 (highlighted in yellow). Sp5 stands out as highly divergent suggesting that it does not belong to the species group of *V. javana* (highlighted in red).

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9
sp1	0								
sp2	0.1	0							
sp3	0.09	0.053	0						
sp4	0.104	0.029	0.057	0					
sp5	0.37	0.368	0.369	0.368	0				
sp6	0.105	0.036	0.06	0.025	0.383	0			
sp7	0.104	0.033	0.059	0.023	0.381	0.003	0		
sp8	0.077	0.075	0.068	0.079	0.399	0.08	0.079	0	
sp9	0.093	0.012	0.052	0.031	0.381	0.038	0.035	0.072	0

Figure S2. Pollinating wasps, ITS2 maximum likelihood phylogenetic tree. Number of individuals genotyped: 53, 51, 9, 14, 18, 13, 25, 11, 9 for sp1, sp2, sp3, sp4, sp5, sp6, sp7, sp8, sp9 respectively.
Note the clade comprising sp2, sp4, sp 6, sp7, sp9, the clade comprising sp2 and sp9 and the clade comprising sp4, sp6 and sp7 and the strong divergence of sp5. All these properties were also obtained with the COI data.

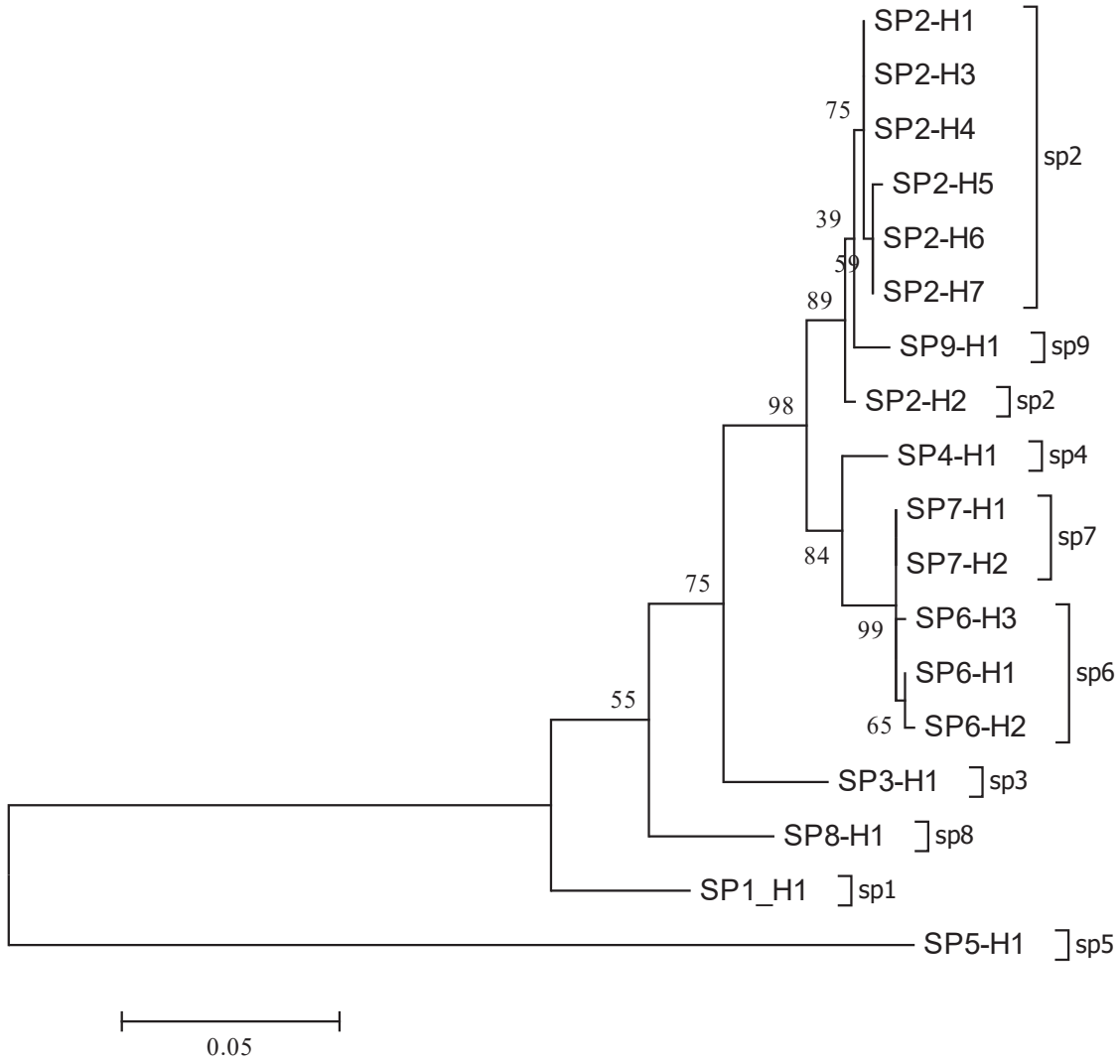
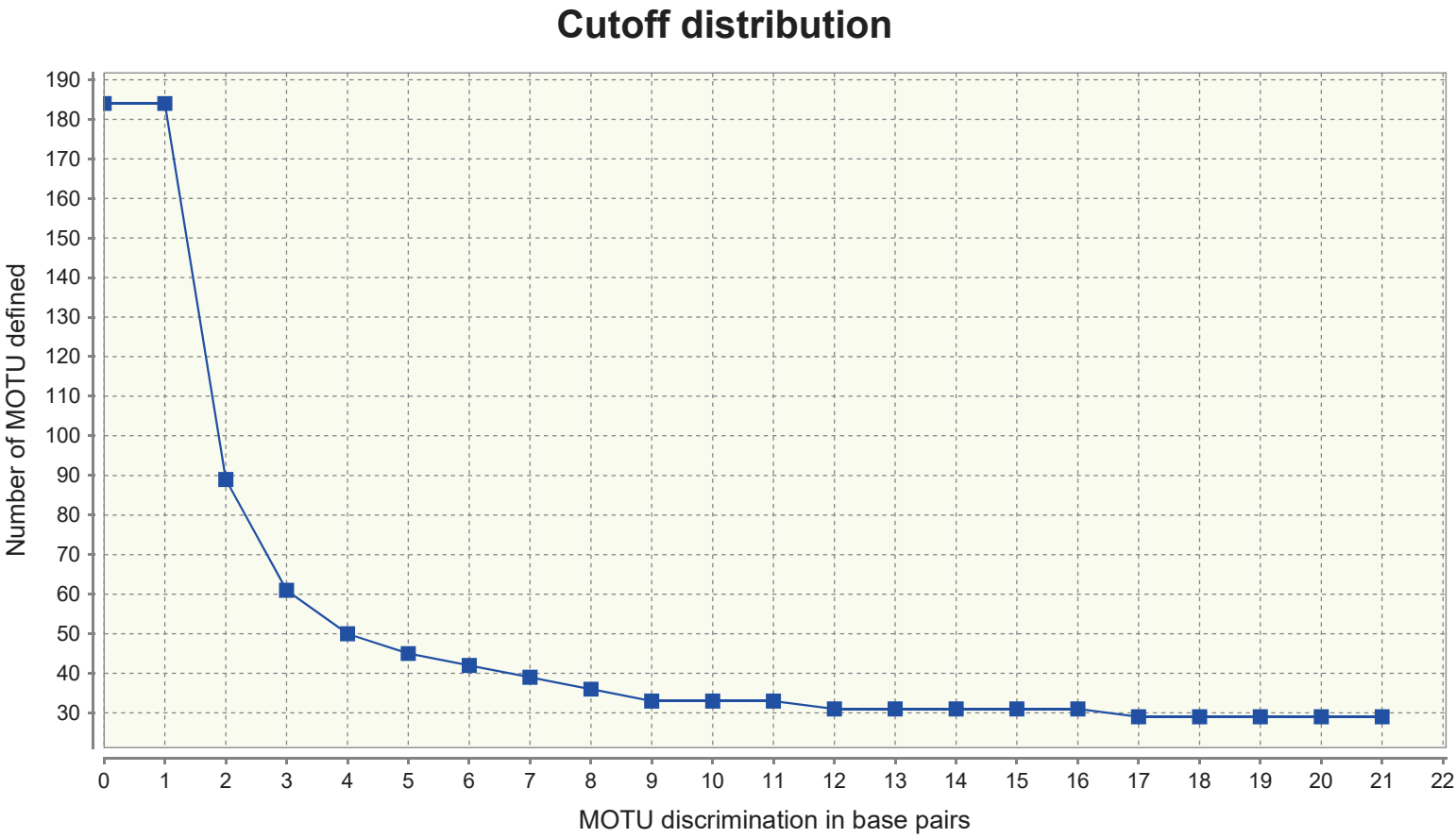


Table S5. Pollinating wasps, ITS2, haplotype data. Sample size (N), number of haplotypes (K), and ITS2 haplotypes with number of individuals.

	Pop.	N	K	ITS2 haplotype (number of individuals)
Sp1	Ning	3	1	Sp1-H1(3)
	Sui	3	1	Sp1-H1(3)
	Xiang	5	1	Sp1-H1(5)
	Huo	4	1	Sp1-H1(4)
	DHS	9	1	Sp1-H1(9)
	Hu	3	1	Sp1-H1(3)
	Sand	2	1	Sp1-H1(2)
	Nan	4	1	Sp1-H1(4)
	Ding	6	1	Sp1-H1(6)
	Wan	5	1	Sp1-H1(5)
	VH	7	1	Sp1-H1(7)
	CP	1	1	Sp1-H1(1)
	NP	2	1	Sp1-H1(1)
Sp2	XI	3	1	H4(3)
	MH	6	2	H3(1), H4(5)
	ML	13	6	H1(1),H2(1), H3(5), H4(4), H5(1), H6(1)
	Tai	12	6	H1(3), H2(1), H3(1), H4(5), H5(1), H7(1)
	CS	9	5	H1(2), H2(1), H3(3), H4(2), H5(1)
	QMS	8	4	H1(1), H3(2), H4(4), H5(1)
Sp3	TNP	8	1	Sp3-H1(8)
	CS1.12	1	1	Sp3-H2(1)
Sp4	HB	14	1	Sp4-H1(14)
Sp5	SNP	18	1	Sp5-H1(18)
Sp6	DAL	4	1	Sp6-H1(4)
	KT	3	1	Sp6-H2(3)
	Wu	6	1	Sp6-H3(6)
Sp7	CI	8	1	Sp7-H1(8)
	JA	7	1	Sp7-H1(7)
	CH	10	1	Sp7-H2(10)
Sp8	ST	11	1	Sp8-H1(11)
Sp9	JP	9	1	Sp9-H1(9)

Figure S3. Pollinating wasps, COI data, number of MOTUs obtained with jMOTU depending on cutoff value.



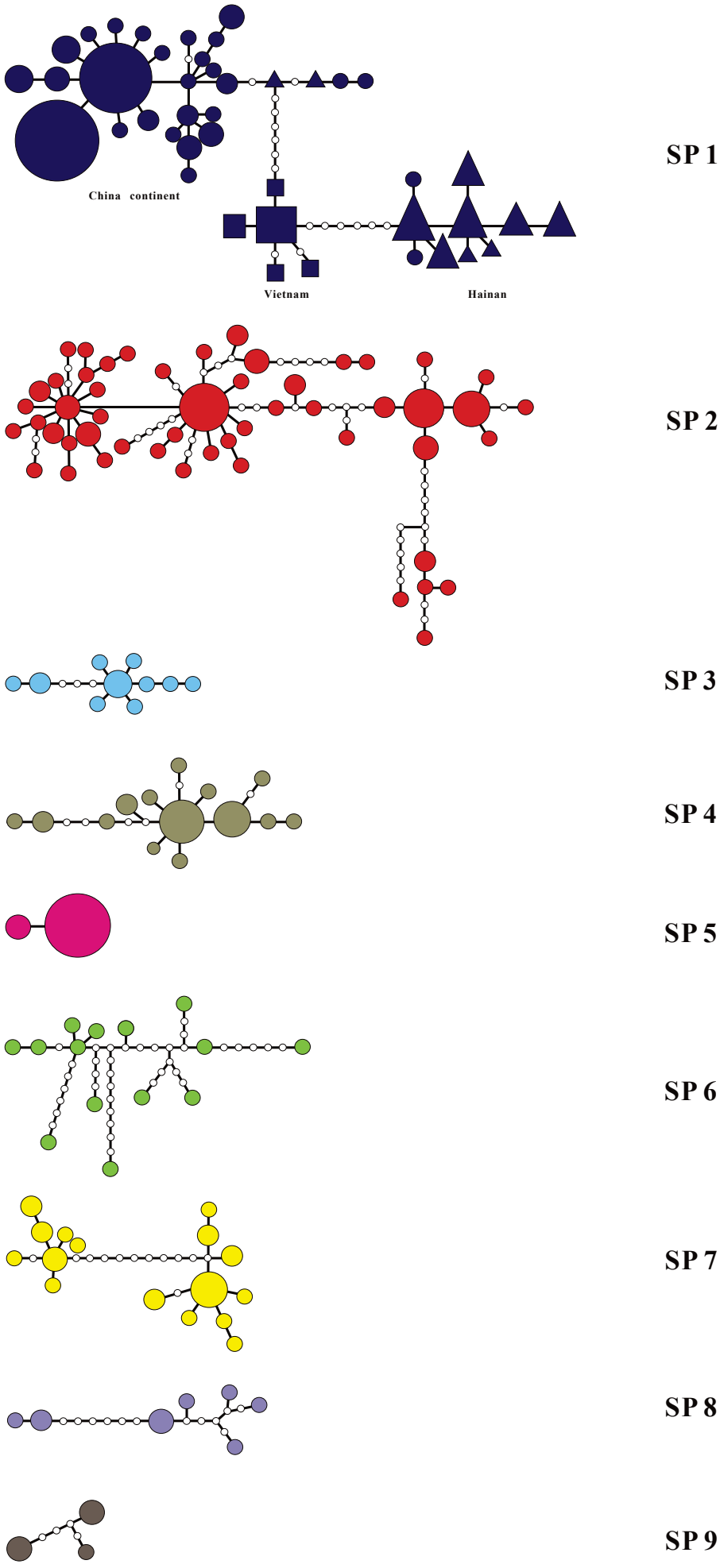


Figure S4. Pollinating wasps, within species COI haplotype networks. For sp1 the network is structured into three entities, South-East China (rounds), Vietnam (squares) and Hainan (triangles), the same structuring as for nuclear microsatellites. Generally, the species are highly diversified even within population, with sp6 an extreme with no repeated haplotype. Sp5 exhibits very limited diversity.

Table S6. Pollinating wasps, COI, AMOVA analysis comparing within and among population sequence variation. There is strong significant structuring among populations within pollinator species.

Species	number of samples	source of variation among populations	significance
sp1	122	44.61	<0.001
sp2	86	17.24	<0.001
sp3	--	--	--
sp4	--	--	--
sp5	--	--	--
sp6	14	12.64	0.00391
sp7	30	82.42	<0.001
sp8	--	--	--
sp9	--	--	--

Table S7. Pollinating wasps, microsatellite data. Number of alleles (Na), allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficients (Fis).

Species	Pop	Na	Ar	PAr	Ho	He	Fis
sp1	Ning	5.333	1.53	0.11	0.483	0.528	0.102
	Sha	5.556	1.56	0.1	0.393	0.562	0.323
	Sui	5.667	1.55	0.09	0.340	0.551	0.356
	Xiang	5.111	1.49	0.1	0.229	0.494	0.529
	Huo	6.444	1.64	0.15	0.367	0.632	0.441
	DHS	6.889	1.58	0.21	0.447	0.589	0.235
	Hu	6.778	1.61	0.22	0.472	0.601	0.222
	Sand	7.111	1.69	0.20	0.412	0.683	0.418
	Nan	6.667	1.55	0.16	0.538	0.557	0.021
	Ding	8.222	1.67	0.43	0.430	0.651	0.391
	Wan	5.222	1.59	0.41	0.344	0.539	0.321
	VH	4.667	1.49	0.14	0.457	0.495	0.073
	CP	4.000	1.45	0.18	0.408	0.446	0.049
	NP	2.556	1.67	0.19	0.667	0.486	-0.367
sp2	MH	4.556	1.46	0.14	0.273	0.408	0.390
	ML	4.667	1.38	0.10	0.207	0.345	0.420
	Tai	5.556	1.54	0.13	0.415	0.529	0.259
	QMS	5.222	1.45	0.11	0.387	0.442	0.103
	CS	6.556	1.51	0.16	0.431	0.481	0.190
sp3	TNP	5.000	1.50	0.36	0.421	0.512	0.169
sp4	HB	6.667	1.48	0.27	0.381	0.517	0.338
sp5	SNP	3.889	1.43	0.33	0.349	0.402	0.091
sp6	DAL	3.778	1.46	0.14	0.284	0.460	0.480
	KT	1.889	1.26	0.08	0.083	0.257	0.504
	Wu	5.333	1.11	0.13	0.365	0.469	0.166
sp7	CI	3.667	1.41	0.15	0.327	0.369	0.045
	JA	4.778	1.48	0.12	0.348	0.443	0.209
	CH	5.222	1.51	0.15	0.402	0.516	0.189
sp8	ST	7.111	1.59	0.59	0.521	0.607	0.093
sp9	JP	3.333	1.40	0.33	0.317	0.399	0.161

Table S8. Pollinating wasps, microsatellite data, pairwise genetic differentiation between locations (F_{ST}). The border colours represent the different species. In green, $F_{ST}<0.1$, in blue $0.1<F_{ST}<0.2$, in yellow $0.2<F_{ST}<0.3$. Sp1 (dark blue) and sp2 (red) are highly homogeneous. Sp7 (yellow) is also highly homogeneous despite its disjoint distribution. Sp4 (dark green) and sp6 (light green) form a slightly less homogeneous group. Within sp1, continental China locations for a very homogeneous entity (locations Ning to Nan), the Hainan Island locations (Ding and Wan) form a slightly different homogenous entity, and Vietnamese locations (VH, CP, NP) form a third homogenous entity.

	Ning	sha	Sui	Hu	Sand	Xiang	SCBG	DHS	Nan	Ding	Wan	VH	CP	NP	MH	ML	QMS	Tai	CS	TNP	KT	Wu	HB	DAL	JP	CH	ST	SNP	CI	JA
Ning	0.000																													
Sha	0.049	0.000																												
Sui	0.037	0.057	0.000																											
Hu	0.049	0.045	0.056	0.000																										
Sand	0.053	0.064	0.042	0.062	0.000																									
Xiang	0.026	0.070	0.042	0.053	0.052	0.000																								
SCBG	0.054	0.046	0.036	0.047	0.045	0.052	0.000																							
DHS	0.055	0.039	0.061	0.040	0.067	0.055	0.051	0.000																						
Nan	0.088	0.081	0.082	0.074	0.092	0.080	0.034	0.066	0.000																					
Ding	0.141	0.128	0.120	0.121	0.110	0.151	0.110	0.112	0.124	0.000																				
Wan	0.175	0.156	0.166	0.161	0.141	0.187	0.127	0.157	0.138	0.059	0.000																			
VH	0.204	0.197	0.166	0.201	0.139	0.195	0.187	0.176	0.254	0.226	0.308	0.000																		
CP	0.241	0.239	0.201	0.249	0.173	0.232	0.224	0.224	0.305	0.251	0.329	0.047	0.000																	
NP	0.211	0.194	0.162	0.191	0.147	0.197	0.181	0.176	0.258	0.237	0.310	0.080	0.112	0.000																
MH	0.384	0.373	0.369	0.350	0.302	0.388	0.330	0.349	0.373	0.331	0.392	0.289	0.343	0.333	0.000															
ML	0.436	0.416	0.415	0.390	0.341	0.442	0.368	0.387	0.405	0.364	0.429	0.326	0.387	0.370	0.043	0.000														
QMS	0.372	0.358	0.355	0.334	0.290	0.379	0.316	0.334	0.351	0.313	0.366	0.297	0.320	0.324	0.038	0.037	0.000													
Tai	0.325	0.309	0.310	0.289	0.252	0.334	0.274	0.289	0.306	0.267	0.322	0.300	0.326	0.315	0.071	0.086	0.050	0.000												
CS	0.356	0.343	0.341	0.320	0.276	0.362	0.301	0.319	0.337	0.299	0.352	0.271	0.293	0.304	0.028	0.029	0.019	0.046	0.000											
TNP	0.347	0.332	0.326	0.315	0.260	0.351	0.291	0.314	0.333	0.294	0.340	0.348	0.376	0.344	0.347	0.365	0.309	0.250	0.301	0.000										
KT	0.496	0.472	0.465	0.449	0.387	0.500	0.421	0.448	0.465	0.429	0.490	0.484	0.516	0.485	0.416	0.463	0.431	0.315	0.330	0.324	0.000									
Wu	0.344	0.330	0.327	0.315	0.266	0.355	0.289	0.310	0.327	0.302	0.344	0.356	0.392	0.348	0.275	0.312	0.246	0.184	0.213	0.217	0.168	0.000								
HB	0.346	0.333	0.328	0.311	0.259	0.347	0.291	0.312	0.332	0.276	0.326	0.351	0.368	0.344	0.261	0.290	0.223	0.181	0.199	0.285	0.305	0.180	0.000							
DAL	0.353	0.340	0.331	0.318	0.268	0.357	0.297	0.325	0.342	0.279	0.341	0.354	0.367	0.338	0.297	0.340	0.279	0.204	0.237	0.238	0.212	0.082	0.188	0.000						
JP	0.383	0.363	0.363	0.344	0.297	0.395	0.323	0.351	0.361	0.317	0.378	0.398	0.426	0.398	0.352	0.395	0.341	0.298	0.318	0.321	0.385	0.293	0.313	0.328	0.000					
CH	0.295	0.286	0.285	0.272	0.228	0.305	0.252	0.266	0.294	0.277	0.320	0.308	0.347	0.302	0.340	0.369	0.315	0.254	0.302	0.212	0.297	0.165	0.287	0.212	0.250	0.000				
ST	0.289	0.277	0.268	0.260	0.215	0.295	0.230	0.260	0.257	0.238	0.280	0.304	0.331	0.301	0.270	0.296	0.247	0.215	0.231	0.221	0.302	0.205	0.210	0.228	0.228	0.222	0.000			
SNP	0.290	0.298	0.281	0.265	0.245	0.295	0.268	0.278	0.334	0.326	0.401	0.194	0.230	0.229	0.403	0.452	0.364	0.372	0.333	0.424	0.581	0.452	0.433	0.440	0.465	0.406	0.348	0.000		
CI	0.394	0.375	0.368	0.366	0.308	0.405	0.328	0.363	0.377	0.331	0.380	0.411	0.455	0.397	0.403	0.426	0.363	0.301	0.354	0.254	0.339	0.190	0.331	0.243	0.338	0.108	0.283	0.485	0.000	
JA	0.354	0.336	0.330	0.325	0.270	0.360	0.296	0.324	0.339	0.291	0.346	0.368	0.410	0.361	0.388	0.416	0.351	0.290	0.339	0.245	0.330	0.201	0.318	0.227	0.282	0.084	0.262	0.456	0.034	0.000

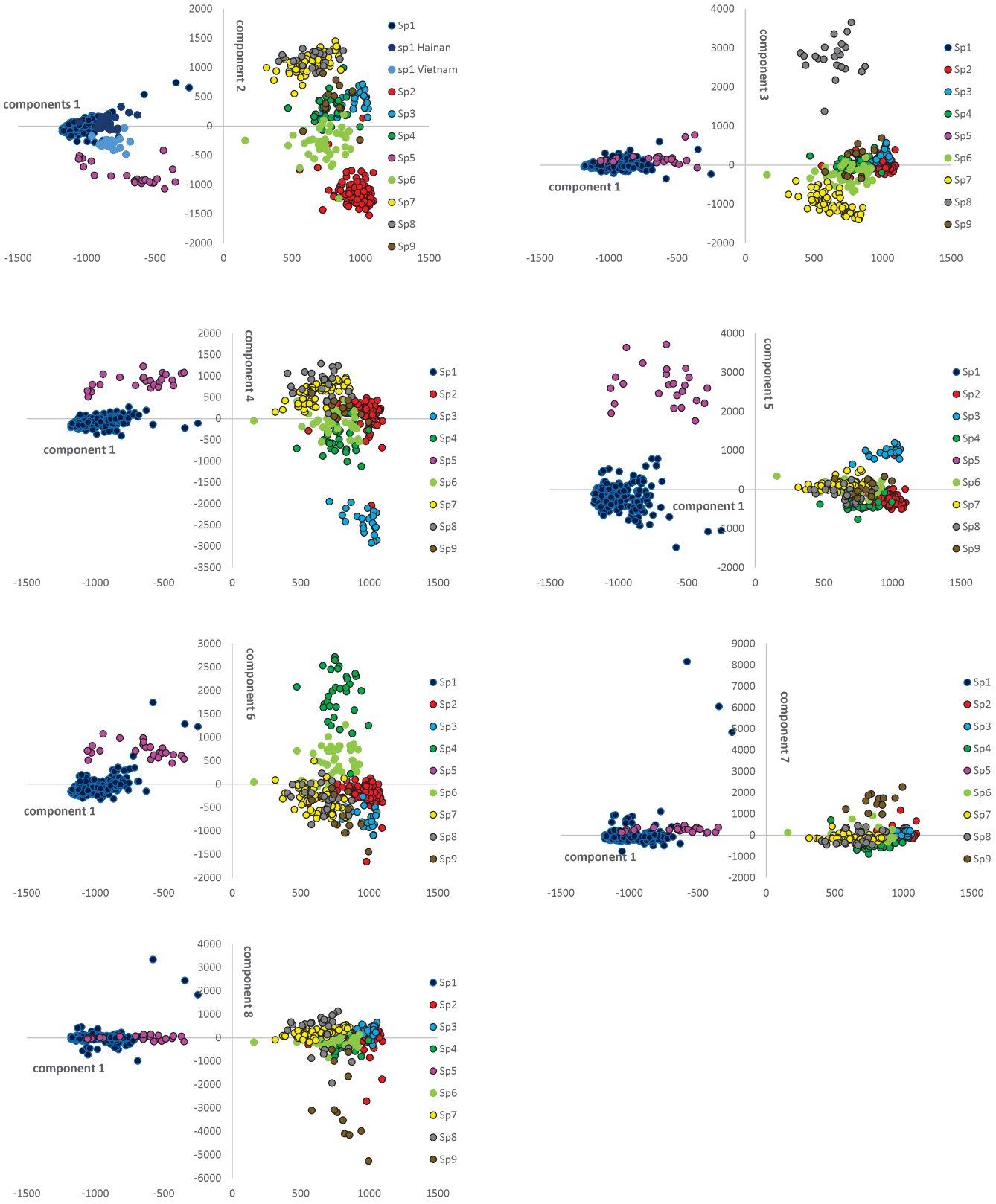


Figure S5. Pollinating wasps, Factorial Component Analysis of individual mutilocus microsatellite genotypes. The first 6 components are informative for species separation. Every species is individualised in the analysis, e.g. sp2 on axis 2, sp7 and sp8 on axis 3, sp3 on axis 4, sp5 on axis 5, and sp4 is distinguished from sp6 on axis 6.

Figure S6a. Assignments of pollinating wasp individuals to clusters using STRUCTURE for K = 2 to K =30
Sp4 is separated from sp6 only for for K values of 20 or above. Sp1, is separated into 3 entities, Continental China locations (Ning to Nan), Hainan locations (Ding and Wan), and Vietnam locations (VH, CP, NP). Sp2 appears as a homogeneous entity (MH, ML, QMS, Tai, CS). Sp3 (TNP) is individualized for K>10.

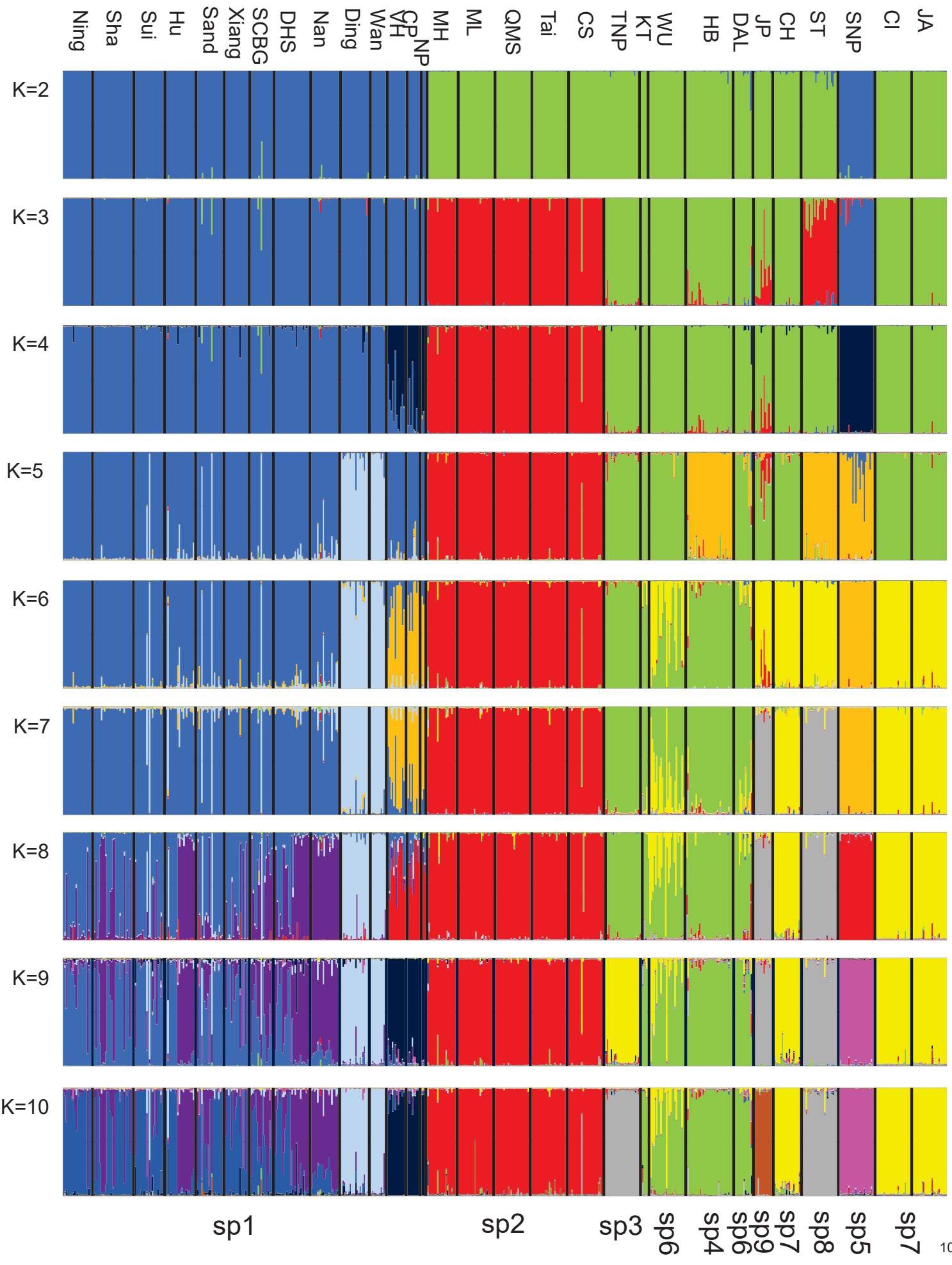


Figure S6a continued. Assignments of pollinating wasp individuals to clusters using STRUCTURE for K = 2 to K =30

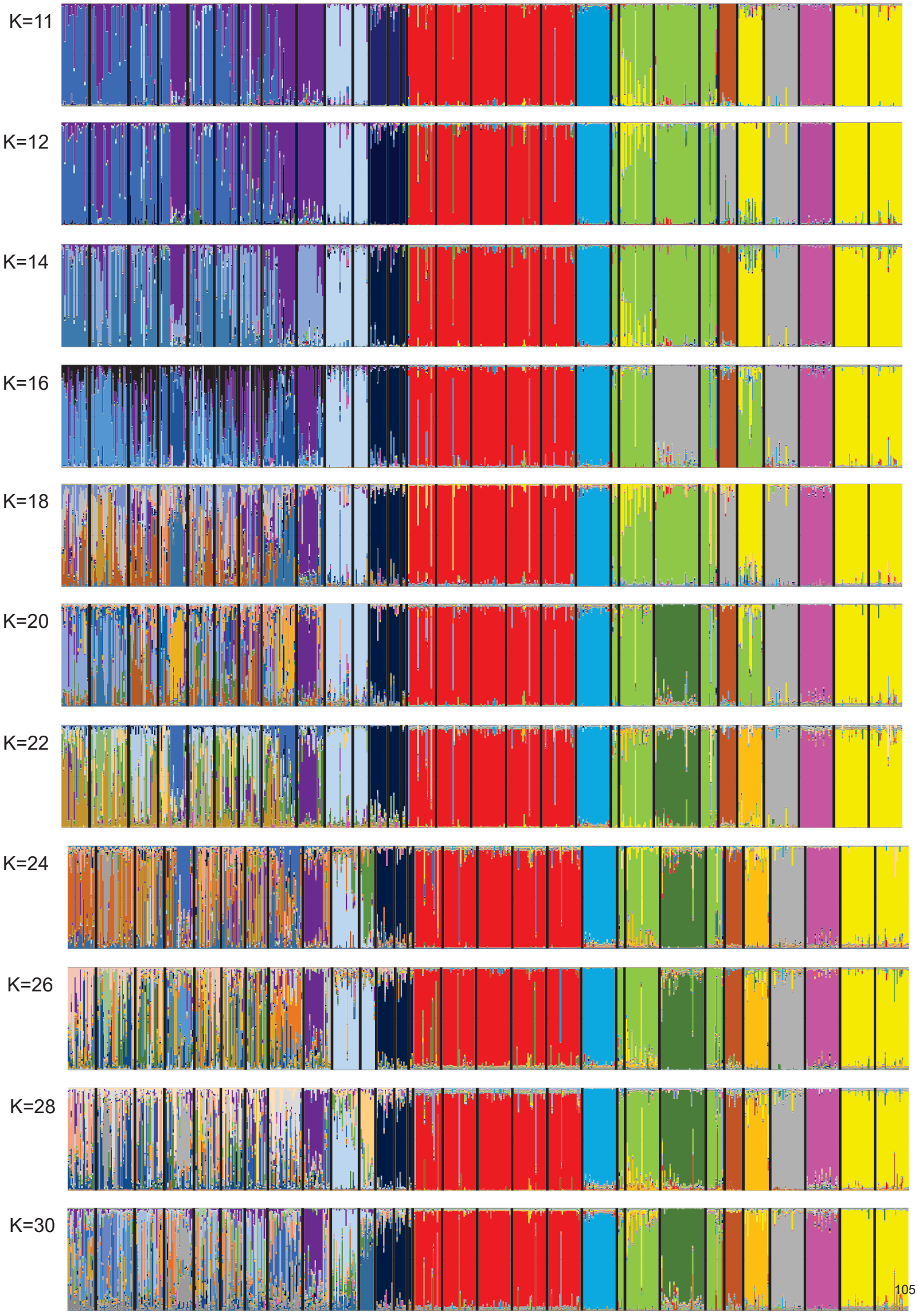


Figure S6b. Assignments of *Ficus hirta* individuals to clusters using STRUCTURE for K = 2 to K =21. For K=2, a north south gradient is apparent. Singapore belongs with the northern locations. At higher values of K, the peninsular location (grey) and the Singapore location (rosa) are separated from the other locations. The two Java locations (yellow) are also distinctive, but close to the central locations. In the north, the Hainan locations (Ding, Wan) are somewhat separated from the other locations.

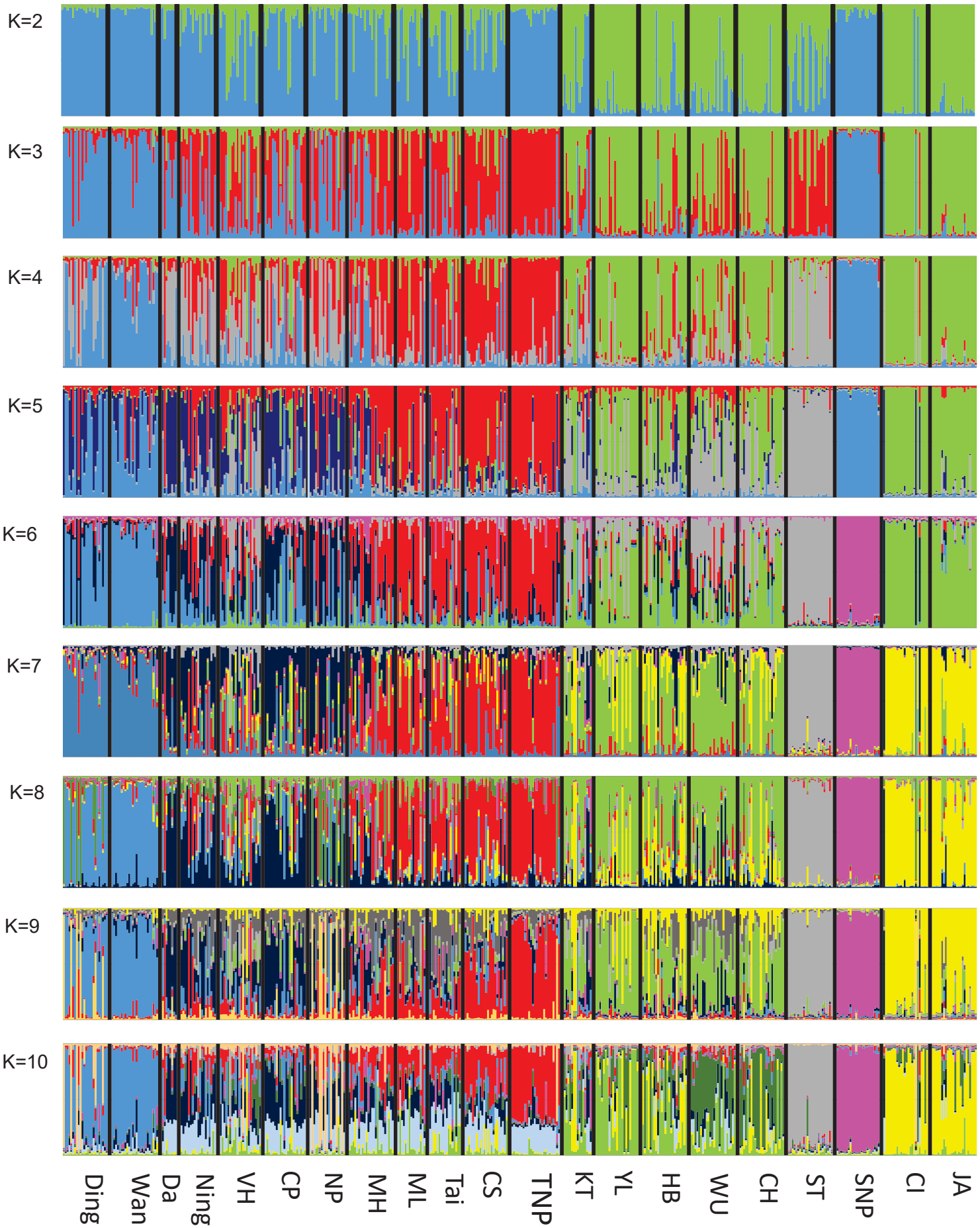


Figure S6b, continued. Assignation of *Ficus hirta* individuals to cluster using STRUCTURE for K=12-21.

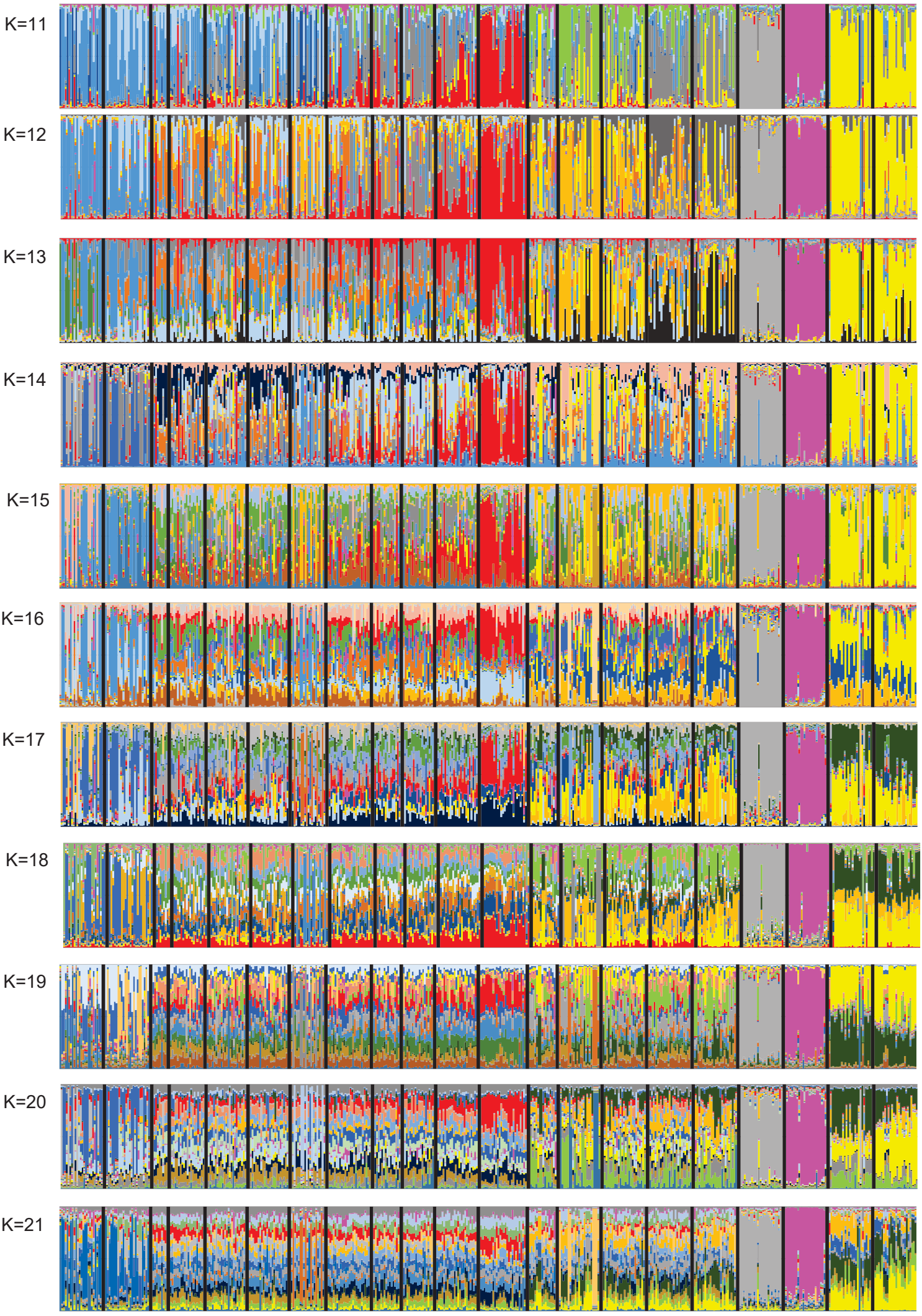
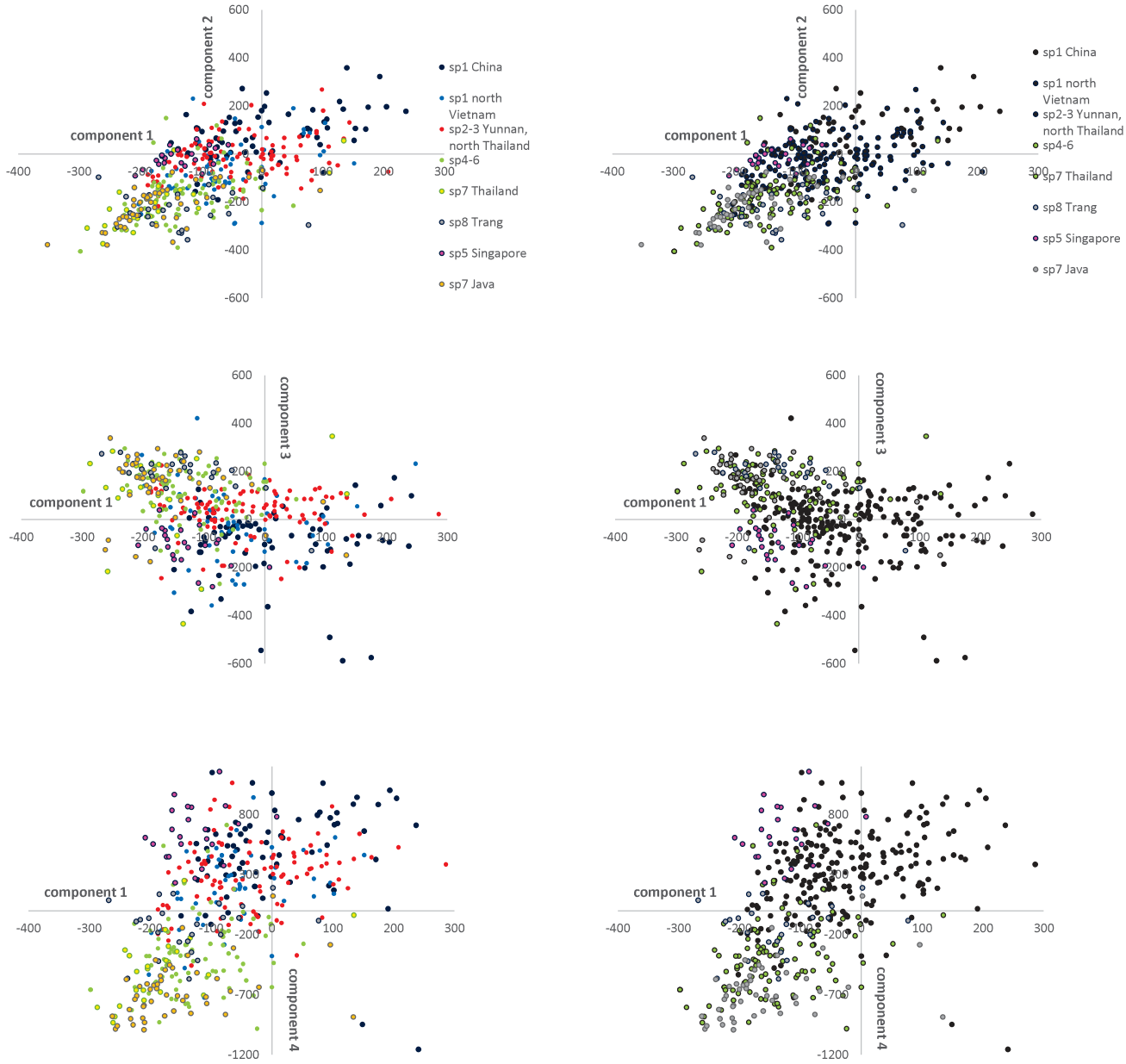


Table S9. *Ficus hirta*, trnLF and trnSG data. Sampled populations with their abbreviations. Sample size (N), number of haplotypes (K), haplotype diversity (Hd), nucleotide diversity (π), and cpDNA haplotypes with number of individuals.

Pop.	N	K	Hd	π	cpDNA haplotype (number of individuals)
Da	10	1	0	0	H1(10)
Xia	10	1	0	0	H1(10)
Sha	9	2	0.556	0.00082	H1(4),H3(5)
Ning	4	1	0	0	H1(4)
Xiang	6	1	0	0	H1(6)
Ao	10	3	0.511	0.00049	H1(1),H2(2),H3(7)
Sui	9	1	0	0	H3(9)
Sand	10	1	0	0	H10(10)
Gui	11	2	0.182	0.00013	H8(10),H11(1)
Huo	9	2	0.389	0.00028	H3(2),H8(7)
DHS	10	2	0.2000	0.00015	H3(9),H8(1)
Nan	9	1	0	0	H3(9)
Hai	2	1	0	0	H12(2)
Wan	12	1	0	0	H8(12)
XI	9	3	0.6667	0.00286	H4(4),H8(1),H12(4)
MH	4	1	0	0	H5(4)
ML	3	2	0.6667	0.00294	H18(1),H19(2)
Tai	18	5	0.4052	0.00051	H4(14),H5(1),H6(1),H7(1),H8(1)
Note: same population of Tai (9samples） in Yu et al. 2010					
CS	10	1	0	0	H20(10)
TNP	14	2	0.3626	0.00027	H7(11),H21(3)
Wu	7	1	0	0	H8(7)
CH	9	1	0	0	H8(9)
ST	22	2	0.4848	0.00036	H8(14),H9(8)
Note: same population of ST (10 samples） in Yu et al. 2010					
VH	10	2	0.3556	0.00079	H13(8),H14(2)
CP	12	2	0.545	0.00121	H15(6),H16(6)
NP	12	2	0.5303	0.00236	H13(7),H17(5)
HB	10	2	0.5556	0.00041	H22(5),H13(5)
KT	12	1	0	0	H13(9)
YL	12	2	0.4848	0.00036	H13(8),H23(4)
Note: the population is near to DAL					
SNP	12	1	0	0	H24(12)
JA	12	1	0	0	H8(12)
TOTAL	309	24	0.716	0.00134	

Table S10. *Ficus hirta*, microsatellite data. Number of alleles (Na), allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficients (Fis).

Pop	Na	Ar	PAr	Ho	He	Fis
Da	5.3	2.9	0.2	0.804	0.712	-0.307
Ning	8.0	3	0.21	0.781	0.766	-0.053
Ding	8.4	2.89	0.4	0.747	0.747	-0.200
Wan	7.1	2.75	0.25	0.704	0.713	-0.167
MH	7.7	2.96	0.2	0.771	0.763	-0.175
ML	6.6	2.93	0.1	0.738	0.749	-0.158
Tai	7.7	2.92	0.15	0.756	0.752	-0.011
CS	8.1	2.91	0.14	0.769	0.744	-0.233
TNP	7.1	2.84	0.15	0.761	0.745	-0.159
Wu	6.9	2.73	0.05	0.789	0.711	-0.023
CH	6.3	2.6	0.07	0.642	0.659	0.146
ST	7.1	2.44	0.23	0.606	0.582	-0.028
VH	7.6	2.98	0.18	0.629	0.759	-0.371
CP	8.1	2.92	0.15	0.747	0.760	-0.165
NP	9.1	3.05	0.58	0.614	0.778	-0.093
HB	5.9	2.44	0.09	0.616	0.590	-0.081
KT	6.4	2.83	0.08	0.800	0.728	-0.295
DAL	6.9	2.68	0.21	0.696	0.690	-0.064
SNP	5.4	2.32	0.21	0.553	0.571	-0.030
CI	6.7	2.39	0.14	0.576	0.599	0.237
JA	5.4	2.38	0.11	0.551	0.597	0.514
Mean	7.0			0.698	0.701	-0.066



Fi ur S Ficus irta, Factorial Component Analysis on multilocus microsatellite genotypes. Left side, colours indicate main pollinating wasp species of the collection location. The pollinator species legend is arranged according to their distribution along the north-south direction. Right side, northern Ficus irta gene pool in black, central gene pool in green, Singapore genotypes in fuschia, southern genotypes in grey. Some points fall outside the graphs, probably representing genotyping errors. On all the axes north-south variation is present, with genotypes from Singapore presenting variable positioning. A northern gene pool (in black on the right) is somewhat individualised, while the distribution of southern gnenotypes overlaps very largely with the distribution of central genotypes.

Table S11 Pairwise F_{ST} values, *Ficus hirta*. The locations are ordered from north to south. Values below 0.08 are highlighted in yellow.

	Ding	Wan	Da	Ning	VH	CP	NP	MH	ML	Tai	CS	TNP	KT	YL	HB	Wu	CH	ST	SNP	CI	JA
Ding	0.000																				
Wan	0.033	0.000																			
Da	0.062	0.064	0.000																		
Ning	0.055	0.062	0.065	0.000																	
VH	0.052	0.047	0.062	0.038	0.000																
CP	0.057	0.045	0.060	0.033	0.027	0.000															
NP	0.052	0.053	0.063	0.053	0.044	0.043	0.000														
MH	0.054	0.055	0.050	0.043	0.038	0.044	0.045	0.000													
ML	0.047	0.044	0.050	0.042	0.034	0.040	0.038	0.031	0.000												
Tai	0.071	0.064	0.077	0.051	0.048	0.048	0.047	0.042	0.032	0.000											
CS	0.041	0.038	0.066	0.049	0.034	0.047	0.034	0.039	0.021	0.040	0.000										
TNP	0.065	0.070	0.075	0.055	0.057	0.060	0.048	0.044	0.040	0.042	0.036	0.000									
KT	0.075	0.075	0.066	0.070	0.048	0.058	0.062	0.047	0.043	0.045	0.058	0.058	0.000								
YL	0.107	0.111	0.115	0.083	0.064	0.072	0.090	0.067	0.065	0.055	0.079	0.083	0.035	0.000							
HB	0.117	0.114	0.134	0.097	0.064	0.079	0.100	0.082	0.080	0.089	0.078	0.102	0.057	0.047	0.000						
Wu	0.091	0.095	0.089	0.052	0.056	0.047	0.066	0.045	0.052	0.037	0.068	0.071	0.035	0.031	0.057	0.000					
CH	0.114	0.115	0.126	0.094	0.090	0.077	0.109	0.094	0.088	0.077	0.102	0.107	0.049	0.038	0.078	0.041	0.000				
ST	0.162	0.178	0.153	0.123	0.144	0.149	0.133	0.142	0.134	0.115	0.141	0.130	0.114	0.149	0.202	0.117	0.158	0.000			
SNP	0.112	0.105	0.158	0.150	0.139	0.128	0.120	0.128	0.124	0.130	0.125	0.132	0.127	0.172	0.198	0.156	0.161	0.179	0.000		
CI	0.110	0.117	0.136	0.111	0.076	0.085	0.098	0.090	0.090	0.096	0.077	0.117	0.067	0.051	0.039	0.074	0.071	0.205	0.181	0.000	
JA	0.105	0.111	0.137	0.112	0.082	0.093	0.089	0.101	0.087	0.095	0.071	0.105	0.072	0.071	0.067	0.087	0.084	0.187	0.158	0.031	0.000

Received: 26 May 2020 | Accepted: 26 February 2021

DOI: 10.1111/1365-2656.13483

RESEARCH ARTICLE

Journal of Animal Ecology | BRITISH ECOLOGICAL SOCIETY

Low host specificity and broad geographical ranges in a community of parasitic non-pollinating fig wasps (Sycoryctinae; Chalcidoidea)

Xiaoxia Deng[1,2,3] | Lianfu Chen[1,2,3] | Enwei Tian[1] | Dayong Zhang[4] | Tanming Wattana[5] | Hui Yu[1,2,3] | Finn Kjellberg[6] | Simon T. Segar[7]

[1]Key Laboratory of Plant Resource Conservation and Sustainable Utilization, South China Botanical Garden, CAS, Guangzhou, China; [2]Centre for Plant Ecology, CAS Core Botanical Gardens, Guangzhou, China; [3]Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, China; [4]State Key Laboratory of Earth Surface Processes and Resource Ecology and MOE Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing, China; [5]Queen Sirikit Botanic Garden, Chiang Mai, Thailand; [6]CEFE, University of Montpellier, CNRS, University of Paul Valéry Montpellier, EPHE, IRD, Montpellier Cedex 5, France and [7]Agriculture and Environment Department, Harper Adams University, Newport, UK

Correspondence
Hui Yu
Email: yuhui@scib.ac.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 31630008, 31670395 and 31971568

Handling Editor: Jenny Dunn

Abstract

1. Plants, phytophagous insects and their parasitoids form the most diverse assemblages of macroscopic organisms on earth. Enclosed assemblages in particular represent a tractable system for studying community assembly and diversification. Communities associated with widespread plant species are especially suitable as they facilitate a comparative approach. Pantropical fig-wasp communities represent a remarkably well-replicated system, ideal for studying these historical processes.
2. We expect high dispersal ability in non-pollinating fig wasps to result in lower geographical turnover in comparison to pollinating fig wasps. The ability of non-pollinating wasps to utilise a number of hosts (low host specificity) is a key determinant of overall geographical range, with intraspecific competition becoming a constraining factor should diet breadth overlap among species. Finally, we expect conserved community structure throughout the host range. We aim to test these expectations, derived from population genetic and community studies, using the multi-trophic insect community associated with *Ficus hirta* throughout its 3,500 km range across continental and insular Asia.
3. We collect molecular evidence from one coding mitochondrial gene, one non-coding nuclear gene and multiple microsatellites across 25 geographical sites. Using these data, we establish species boundaries, determine levels of host specificity among non-pollinating fig wasps and quantify geographical variation in community composition.
4. We find low host specificity in two genera of non-pollinating fig wasps. Functional community structure is largely conserved across the range of the host fig, despite limited correspondence between the ranges of non-pollinator and pollinator species. While nine pollinators are associated with *Ficus hirta*, the two non-pollinator tribes developing in its figs each contained only four species.

Xiaoxia Deng and Lianfu Chen contributed equally.

5. Contrary to predictions, we find stronger isolation by distance in non-pollinators than pollinators. Long-lived non-pollinators may disperse more gradually and be less reliant on infrequent long-distance dispersal by wind currents. Segregation among non-pollinating species across their range is suggestive of competitive exclusion and we propose that this may be a result of increased levels of local adaptation and moderate, but regular, rates of dispersal. Our findings provide one more example of lack of strict codiversification in the geographical diversification of plant-associated insect communities.

KEYWORDS

beta-diversity, community assembly, mutualism, parasitoid, population genetics

1 | INTRODUCTION

Herbivorous insects, their hosts and their carnivores (including parasitoids) likely represent over 75% of all macroscopic terrestrial biodiversity (Price, 2002). In addition to being of high economic relevance, both trophic levels contribute substantially to biodiversity and ecosystem function (Hawkins et al., 1997). Herbivores and parasitoids are also model systems for studying community assembly and coevolution. One of the most fundamental questions in ecology is how multitrophic communities and networks assemble across time and space. Under the expectation of classical cospeciation, entire communities might codiversify such that interactions are inherited across speciation events resulting in predictable and replicated community structure. Widespread evidence suggests that such a process is rare and that coevolution in most networks is diffuse and context-dependent (Thompson, 1994), but still a key driver of network structure (Segar et al., 2020). It is, therefore, necessary to study species across their entire range to fully understand the process behind community patterns. More generally, geographical variation in the specialisation of host–parasitoid networks can be driven by contrasting patterns of beta-diversity among trophic levels, making it important to quantify both local and regional network structure (Galiana et al., 2019).

A major determinant of parasitoid network structure is host specificity because this will determine the number of trophic links formed. Indeed, Hawkins (1994) points to host concealment as a major predictor of parasitoid specificity: concealed hosts are predicted to host generalist ectoparasitic idiobionts and exposed hosts specialist endoparasitic koinobionts. In natural communities of herbivores, parasitoids have been found to be host-specific (an average of 1.8 hosts in a New Guinean caterpillar community; Hrcek et al., 2013), but this can vary in contrast to expectations between host guilds with parasitoids of semi-concealed hosts being the most host-specific. Parasitoids of the miner guild can also be host-specific (an average of 2.8 hosts in one Belizean web) but vary in this degree (Leppänen et al., 2013; Lewis et al., 2002). Evidence for 'Host Associated Differentiation' (the evolution of specialists across host genotypes) suggests that parasitoids of highly concealed galling insects (Nicholls et al., 2018; Stireman et al., 2006) can even be among the most host-specific of insects. However, realised host breadth of parasitoids can be determined by multiple additional factors including evolutionary history (of both host and parasitoid), host abundance and geographical range (Hawkins, 1994).

Prevailing evidence points to low levels of ecologically equivalent species in herbivores (Butterill & Novotny, 2015; Hrcek et al., 2013; Novotny et al., 2012) but a high frequency of morphologically highly similar (cryptic) species among their parasitoids (Li et al., 2010; Smith et al., 2006, 2008) which only in some cases stems from host-associated differentiation (Hernández-López et al., 2012). Indeed, cryptic species are common in parasitoid networks and their inclusion is crucial to inform our understanding of network structure and turnover (Hrcek & Godfray, 2015; Van Veen et al., 2008).

Meso-diverse insect communities in enclosed microcosms, such as those associated with oak (*Quercus*) galls, have become model systems for studying host specificity (Nicholls et al., 2018) and community assembly (Bunnefeld et al., 2018) due to their replicated nature and intercontinental distributions. The current population genetic structure among *Quercus* species and their associated insect fauna has been highly dependent on glacial cycles (Stone et al., 2012). The multi-trophic wasp communities associated with plants of the genus *Ficus* have great potential to serve as a complimentary system to gall wasps. Pollinating fig wasps (Agaonidae, Chalcidoidea) coexist with members of several other chalcid (sub) families. Some fig wasps, including the pollinating wasps, gall fig ovules, other fig-wasps parasitise pollinators and/or other gall formers (as parasitoids or cleptoparasites) while a third trophic level of hyper-parasitoids can also be present (Cook & Rasplus, 2003). Being largely tropical in distribution they offer a tractable counterpoint to the temperate oak galling communities. For example, functional community structure has been shown to be retained across the paleotropics, despite turnover of every species in the community of the canopy hemi-epiphyte *F. benjamina* (Darwell et al., 2018).

While figs and their pollinators provide one of the strongest examples of codiversification on a global scale (Cruaud et al., 2012), regional and local dynamics can demonstrate multiple trajectories. Accumulating evidence for multiple pollinators per host fig and even

pollinator sharing is emerging (Cook & Segar, 2010) with recent examples demonstrating both continuity (Bain et al., 2016) and turnover in pollinator species over islands (Rodriguez et al., 2017) continents (Yu et al., 2019) and elevational gradients (Souto-Vilarós et al., 2019). Our increased understanding of speciation dynamics in pollinators has also shed more light on their diversification. For instance, the pollinators associated with small dioecious hosts are thought to be particularly prone to genetic isolation due to their limited dispersal capabilities when compared to the above canopy dispersers associated with monoecious fig species (Ahmed et al., 2009; Compton et al., 2000; Harrison & Rasplus, 2006; Yu et al., 2019). The concept of high pollinator host specificity, however, has survived scrutiny in most cases.

The discovery of multiple pollinators per host has often been driven by molecular evidence (Darwell et al., 2014; Molbo, 2003; e.g. Yu et al., 2019) and has gradually become reconciled with a 'split-and-sort' mechanisms for diversification (Cook & Segar, 2010). Diversification dynamics of parasitoids are often linked to those of their hosts (Forister & Feldman, 2011). Broad scale studies (at the level of the *Ficus* section) demonstrate congruence among host, pollinator and non-pollinator phylogenies for African fig species (Jousselin et al., 2008). Putative pollinator cleptoparasites in the genus *Philotrypesis* (Joseph, 1958) and two lineages of *Otitesella* ('uluzi' and 'sesquinianellata'), one a galler, the other a cleptoparasite of the galler (Segar et al., 2013, figure 4) all showed high levels of host specificity. Both codiversification and host tracking are likely to have occurred throughout the diversification of the parasitoid fig wasps (Segar et al., 2012). Within this general picture of host tree specialists, several species of neotropical *Idarnes* wasps, including gallers and cleptoparasites (Farache et al., 2018) and a few African parasitoid species of genus *Arachonia* (McLeish et al., 2012) are generalists, developing in the figs of several local *Ficus* species.

Our knowledge at finer scales is more limited, and to date only two studies have addressed the phylogeography and population genetics of non-pollinating fig wasps. Wide-ranging fig species, for example the Australian *Ficus rubiginosa*, are associated with distinct species of non-pollinators throughout their range (Darwell & Cook, 2017). *Ficus rubiginosa* hosts two parapatric species of *Sycoscapter* 'short' and *Philotrypesis* 'black'. Population genetic data from the whole range (Sutton et al., 2016) reveal that another species, *Sycoscapter* 'long', demonstrate higher levels of population connectivity and/or higher levels of dispersal than in one pollinating wasp, which has a disjunct distribution (but see Kjellberg & Proffit, 2016).

1.1 | Expectations and predictions

Here we conduct extensive sampling of the wasp community associated with the small shrub, *F. hirta*. Previous genetic data (Yu et al., 2019) have shown that with nine parapatric species it has the largest set of pollinators reported to date for any *Ficus* species. We connect this large number with the small size of the plant which may lead to localised wasp dispersal in comparison to the large strangling *Ficus* species, and with the seemingly elevated rate of speciation in pollinating fig wasps in comparison to fig trees (Moe et al., 2012). We use a range of genetic markers to establish the taxonomic equivalence of this spatially structured genetic differentiation and test the hypothesis of high connectivity between populations and/or high levels of dispersal in non-pollinating fig wasps. According to existing evidence from a single other gall/parasitoid wasp community (Sutton et al., 2016), we expect lower species diversity and turnover within parasitic fig wasps than for the pollinators, with parasites capable of utilising most members of the pollinating wasp complex as hosts. Due to high dispersal ability and a resilience to founder effects (conferred by the limited necessity for outbred males), we expect that species ranges of parasites will not be shaped by historical contingencies but rather by interspecific competition and species' ecological traits. Following on from range disparities and reduced parasite specificity, we predict that pairwise comparisons across sites will reveal multiple examples of parasites spread across several pollinator hosts.

Furthermore, in systems presenting a linear distribution (as is the case for *F. rubiginosa*) different species may abut on the same ecological barrier, incidentally leading to statistical association between parasites and between parasites and hosts. Such incidental correlations are expected to be less frequent in systems presenting two-dimensional distributions (as is the case for *F. hirta*), as ecological obstacles and ecological gradients are more diversified and different species groups may respond differently to these factors. At the community scale, we expect conserved ecological function throughout the species' range (as appears to be the case in other fig-wasp systems), with the same niches existing and being filled throughout the range of *F. hirta*. This is driven by an underlying expectation that fig-wasp communities should be generally saturated and structured largely by competition for limited resources. Indeed, published data suggest that while not saturated at the individual tree-crop level, fig-wasp communities are saturated when several crops are sampled more widely (Compton & Hawkins, 1992; Hawkins & Compton, 1992); in other words, there is saturation at the regional level.

2 | MATERIALS AND METHODS

2.1 | Study species

Ficus hirta Vahl is a shrub of secondary vegetation (Figure S1). Its distribution extends from the island of Java in the south to China in the north and westwards into northeast India and Nepal (Yu et al., 2019). *Ficus hirta* is pollinated by the host-specific species complex of *Valisia javana* Mayr (Agaonidae, Chalcidoidea, Hymenoptera; Yu et al., 2019). Pollinating wasps enter the fig (a closed urn-shaped receptacle), pollinate and oviposit in flowers.

Three non-pollinating chalcid fig-wasp species have been reported from *F. hirta* (Yu et al., 2018). *Philotrypesis josephi* and *Sycoscapter hirticola* in northeast India (Nair et al., 1981) and *Sycoryctes simplex* in Java (Mayr, 1885). *Philotrypesis* (tribe Philotrypesini), *Sycoscapter* and *Sycoryctes* (tribe Sycoryctini) are monophyletic genera belonging to the subfamily Sycoryctinae (Pteromalidae; Segar et al., 2012). They oviposit into ovaries

containing *Valisia* larvae by inserting their long ovipositor through the fig wall. *Philotrypesis* are cleptoparasites while Sycoryctini are parasitoids (Conchou et al., 2014). *Philotrypesis*, Sycoryctini and pollinating *Valisia* are morphologically remarkably different (Figure S1). Offspring of fig wasps mate in the fig, often between siblings, before dispersal from their natal fig, a feature that facilitates the initial establishment of rare colonisers (Yu et al., 2019).

2.2 | Sampling

Samples were collected from South China to Java (Supporting Information Table S1). Wasp collection protocols follow (Yu et al., 2019). Spatial structuring has been investigated for the pollinators of *F. hirta*, *Valisia* spp. (Yu et al., 2019) and microsatellite data and cytochrome c oxidase I (COI) data have been obtained for *Philotrypesis* and *Sycoscapter* for samples from South-East China (Yu et al., 2018). We sampled representatives of each fig-wasp species emerging from individual figs at each location; however, we did not record complete fig contents or individual fig level occurrence data at all sites.

2.3 | Gene sequence data

Cytoplasmic Cytochrome b (Cytb) was sequenced as preliminary results gave inconsistent results for COI. Cytb sequences were obtained, generally for a single wasp per fig, for a total of 124 *Philotrypesis* from 18 sites and 76 Sycoryctini from 13 sites (Table S1). We sequenced the ITS2 gene for a total of 133 individuals from 15 sites for *Philotrypesis* and 54 individuals from 11 sites for Sycoryctini (Table S1). Molecular procedures were the same as in Yu et al. (2018), except for Cytb amplification (see Table S2). Sequences were aligned as in Yu et al. (2018). A 628 fragment of the Cytb gene and a 180 bp fragment of the ITS2 gene were sequenced for *Philotrypesis*, while for the Sycoryctini the fragment lengths were 652 and 234 bp, respectively.

The within genus phylogenetic positions of *Philotrypesis*, *Sycoscapter* and *Sycoryctes* species associated with *Ficus* section *Eriosycea* have not been ascertained. Therefore, we used our Sycoryctini samples as an outgroup of our *Philotrypesis* and conversely. Maximum likelihood trees were constructed using MEGA 6.0 (Tamura et al., 2013) for Cytb and ITS2 separately, and node supports were assessed based on 2,000 bootstrap replicates. We calculated Kimura-2-parameter (K2P) distances for Cytb haplotypes and ITS2 between all individuals and within and between clades evidenced by the Maximum Likelihood tree, using MEGA 6.0.

2.4 | Microsatellite data

For *Philotrypesis*, 262 individuals, each from a different fig, were genotyped at six unlinked microsatellite loci. For Sycoryctini, 203 individuals, each from a different fig, were genotyped at seven unlinked microsatellite loci. Molecular techniques follow Yu et al. (2018).

Classical indices of genetic diversity were estimated using GenALEx 6.1 (Peakall & Smouse, 2006). F_{IS} values were calculated separately for each location. To provide a global representation of the data, we performed a factorial correspondence analysis as implemented in GENETIX (Belkhir et al., 2004). We used Bayesian clustering to assign multilocus microsatellite genotypes to clusters using STRUCTURE 2.2 (Pritchard et al., 2000). The admixture ancestry and correlated allele frequencies model was used with three independent runs each of 500,000 MCMC iterations and 500,000 burn-in steps. We ran STRUCTURE varying *K* (the number of clusters) from 2 to 13 for *Philotrypesis* and for *Sycoscapter-Sycoryctes*.

2.5 | Co-occurrence analysis

We analysed the co-occurrence (a) of species across sites, (b) between *Philotrypesis* and *Sycoscapter* and (c) between hosts and parasites. First, we plotted the occurrences of the different species on a map and made visual comparisons of the differences in the range limits between the species groups. Second, we constructed co-occurrence matrices for each species of each genus for all sites from which they were recorded (e.g. a 9×32 matrix for *Valisia*) and tested for aggregation or segregation by comparing the observed *V*-ratio (Variance ratio; mean pairwise covariance in association) to the distribution obtained from a set of 1,000 randomised matrices created by shuffling matrix fill (retaining row sums and allowing column totals to vary randomly and equiprobably). We discarded 500 randomisations as 'burnin'. We implemented this analysis using co-occurrence null models in the R package EcoSimR (Gotelli et al., 2015) by setting the metric to '*V*-ratio' and the algorithm to 'Sim 2'. We repeated this analysis for a matrix of co-occurrence between *Philotrypesis* species and *Sycoscapter* species.

Third, we calculated the observed Czekanowski niche overlap index for *Philotrypesis* and *Valisia* species (a 4×9 matrix) and all *Sycoscapter* and *Valisia* species (a 3×9 matrix). The observed niche overlap index was compared to a distribution obtained from 1,000 randomised matrices created by shuffling matrix fill generated by reshuffling row values obtaining the observed number of interactions per species. We use only binary and not abundance-based data. This analysis was also implemented in R using the package EcosimR by running niche overlap null models, we used the Czekanowski and RA3 algorithm.

3 | RESULTS

3.1 | Sequence data

The geographical distribution of the different haplotypes and associated diversity parameters are given in Tables S2–S5. A Cytb maximum likelihood tree separated the non-pollinating wasps into four clades of *Philotrypesis* and four clades of Sycoryctini (Figure 1). Individuals originating from locations monomorphic for *Philotrypesis* or *Sycoryctini* Cytb clades were sequenced for ITS2. Each of the seven sequenced Cytb clades was monomorphic for ITS2, presenting a

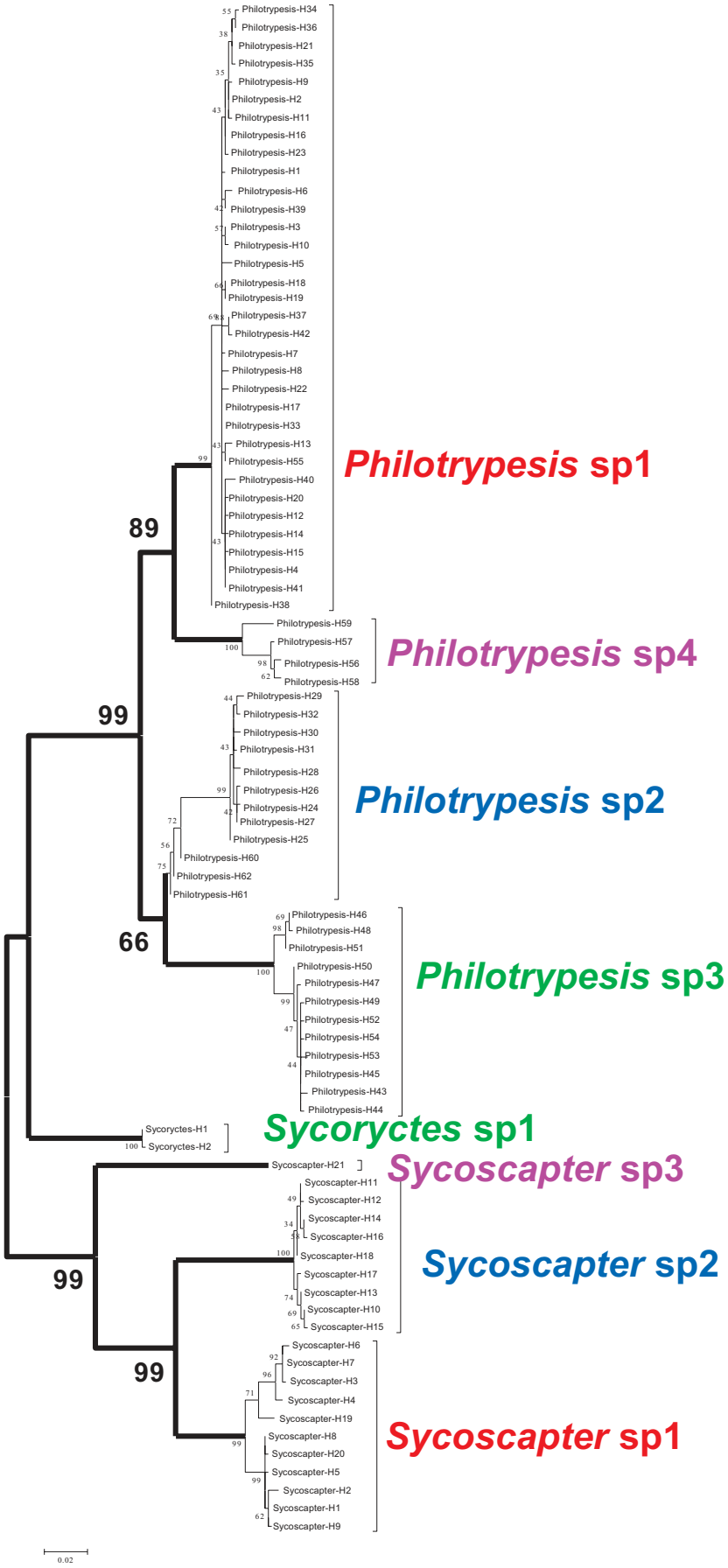


FIGURE 1 Maximum likelihood phylogenetic tree based on Cytb sequences. Genus *Philotrypesis* belongs to the monophyletic tribe Phylotrypesini while genera *Sycoryctes* and *Sycoscapter* belong to the monophyletic tribe Sycoryctini

distinctive haplotype (Figure S2). The distribution of K2P distances within and among clades shows low within clade and large between clade distances for both Cytb and ITS2 (Tables S6 and S7). The cumulative distribution of K2P distances for Cytb presents a marked barcoding gap between clades for both *Philotrypesis* and Sycoryctini (Figure 2). Therefore, in the following, we will accept each clade as a separate species. We made the conservative choice of considering *Philotrypesis* samples from Sand as belonging to sp2 pending deeper sequencing. Genetic distances were smaller between *Philotrypesis* species than between Sycoryctini species (Figure 1; Figures S6 and S7). Among all the data we have only one case where *Philotrypesis* species co-occurred in a single location while

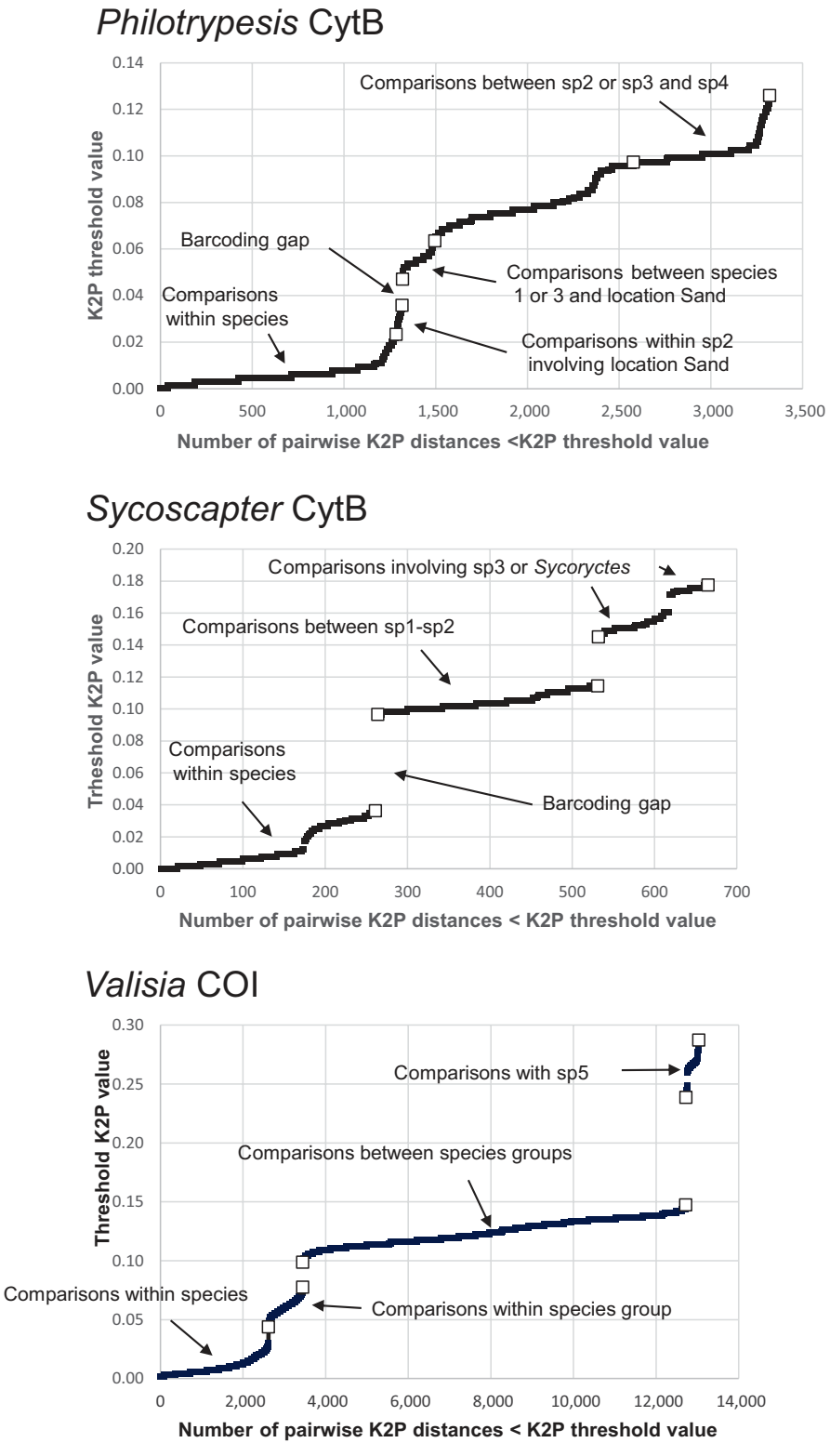


FIGURE 2 Cumulative distribution of Kimura pairwise genetic distances (K2P) for Cytb for *Philotrypesis*, Sycoryctini and *Valisia* wasps associated with *Ficus hirta*. There is a marked barcoding gap between clades in the Sycoryctini. *Philotrypesis* clades are less differentiated, and the barcoding gap is less marked. In *Valisia*, there is almost no barcoding gap due to the presence of two complexes of closely related species

there was no co-occurrence of Sycoryctini species (Tables S2–S5). The Sycoryctini sequences from Java are highly divergent for both Cytb and ITS2 (Tables S6–S7). Morphological inspection showed that samples from the continent belonged to genus *Sycoryctes* while samples from the continent belonged to genus *Sycoscapter* as currently circumscribed (Segar et al., 2012). Critical inspection of the description of *Sycoscapter hirticola* (Nair et al., 1981) confirmed assignment to genus.

3.2 | Microsatellite data

Diversity indices are given in Tables S8–S9. For *Philotrypesis*, there was too much variation within species and too little variation among species to allow clear species separation (Figure S3), while factorial correspondence analysis confirmed the differentiation between species in Sycoryctini (Figure S4). Four genotypes from location Xi grouped with sp1 genotypes while three genotypes grouped with sp2

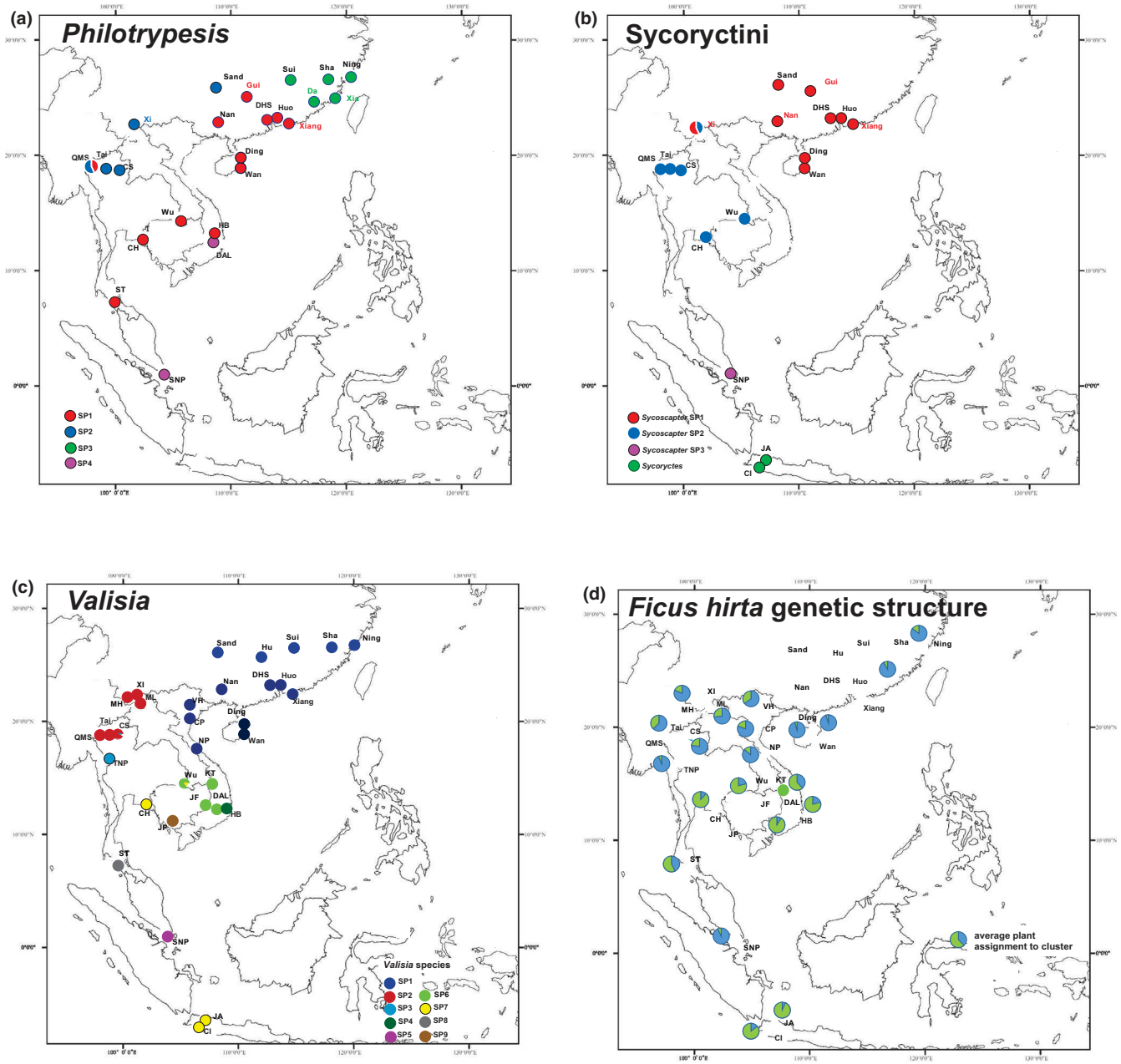


FIGURE 3 Geographic distribution of the three sets of fig wasps associated with *Ficus hirta* and genetic variation of the plant. Panel (a) cleptoparasitic wasp-species distribution, genus *Philotrypesis*; panel (b) parasitoid wasp-species distribution, tribe Sycoryctini; panel (c) pollinating wasp-species distribution, genus *Valisia*; panel (d) host plant spatial genetic structure. Locations indicated with coloured lettering are those for which species assignment was based on SSR genotypes only. *Philotrypesis* are assumed to be cleptoparasites of *Valisia*; *Sycoscapter* and *Sycoryctes* are assumed to be parasitoids of *Valisia*. *Valisia* are the pollinators of *Ficus hirta* and feed on galled plant ovules. For the plant, genetic variation is clinal and is illustrated here by percentage of assignment to two extreme gene pools

TABLE 1 Results of co-occurrence analysis between (i) species within each wasp genus at each site and (ii) the two main parasite wasp genera and each *Valisia* pollinator species

Co-occurrence	*V*-ratio	Mean of simulated data	SES	*p* value
Philotrypesis at each site	0.06	1.005	−3.534	<0.001
Sycoscapter at each site	0.11	1.003	−3.121	<0.001
Valisia at each site	0.07	1.001	−3.964	<0.001
Philotrypesis and *Sycoscapter*	0.33	1.022	−0.837	ns
Czekanowski niche overlap	**Czekanowski index**	**Mean of simulated data**	**SES**	***p* value**
Philotrypesis and *Valisia*	0.22	0.164	0.727	ns
Sycoscapter and *Valisia*	0.11	0.145	−0.305	ns

Abbreviation: SES, standardised effect size.

genotypes (Figure S4). One genotype from Java came out with sp2 genotypes. Bayesian assignment to cluster gave the same insights as the correspondence analysis (Figures S5 and S6). For *Sycoscapter*, the presence in location Xi of both sp1 and sp2, and the presence of an individual of sp2 on Java was supported by the Bayesian assignment.

Pollinators presented higher F_{IS} values than *Philotrypesis* and *Sycoryctini* demonstrating more frequent brother–sister mating (Figure S7). Sample sizes and distances among locations allowed investigating genetic isolation by distance (IBD) for *Valisia* spp 1 and 2, *Philotrypesis* spp. 1, 2, 4 and *Sycoscapter* sp1. In *Valisia* there was no IBD, but differentiation within sp1 between Hainan island, southeast China and Vietnam (Tian et al., 2015). IBD was present in *Philotrypesis* and *Sycoscapter*, except for *Philotrypesis* sp4 (Figure S8).

3.3 | Host specificity and co-occurrence

The geographical distributions of the different species are shown in Figure 3. The limits of the species distributions for the three types of wasps are visually different. For instance, the distribution of *Valisia* sp1 was established by sequencing 176 individuals for COI or for ITS2. In all locations where *Valisia* sp 1 was found, not a single sequence from any other *Valisia* species was found. Reciprocally, locations where *Valisia* sp1 was not found were established by sequencing 358 individuals either for COI or for ITS2. The distribution of *Valisia* sp1 was totally cohesive, and formed one block. *Philotrypesis* sp 1 was found in locations alongside *Valisia* sp1 (123 individuals of *Philotrypesis* sp1 sequenced either for CytB or for ITS2) and in locations where *Valisia* sp1 was not present (31 individuals of *Philotrypesis* sp1 sequenced either for CytB or for ITS2). In Figure 3, it can be seen that *Philotrypesis* sp4 uses *Valisia* sp6 in DAL and *Valisia* sp5 in SNP while *Sycoscapter* sp2 uses *Valisia* sp2 in QMS, Tai and likely CS versus *Valisia* sp6/7 in Wu and *Valisia* sp7 in CH.

As well as *Valisia*, both *Philotrypesis* and *Sycoscapter* segregated across sites (Table 1), such that the entire geographical range was divided into largely non-overlapping blocks occupied by a single species for each genus. Our co-occurrence analysis provides statistical support for this striking pattern presented in Figure 3 and outlined above. It is clear that the geographical limits of the species belonging to the two tribes of parasites do not correspond (the mean and simulated mean are not statistically different, Table 1). In other words, there is no broad congruence between species ranges among members of different genera. Furthermore, there is no niche partitioning between either *Sycoscapter* or *Philotrypesis* across their *Valisia* hosts (Czekanowski niche overlap is no different from random in either case, Table 1). Neither parasite genus shows greater levels of specialisation than expected by chance.

4 | DISCUSSION

While at least eight species of *Valisia* are associated with *Ficus hirta* throughout its range, we found four species of *Philotrypesis*, three species of *Sycoscapter* and one species of *Sycoryctes*. Our expectation of lower parasite diversity and limited host specificity was confirmed. *Philotrypesis* sp1 parasitises at least four species of the pollinating *Valisia* and *Sycoscapter* sp2 at least two species of *Valisia*. These parasitic wasp species are not specialists of host insect species. Ultimately, direct confirmation of host associations will only be obtained by sequencing gall content to determine the host–parasite association present in individual galls (e.g. Sow et al., 2019). Do non-pollinators exist locally but fail to develop, for example? Nevertheless, the non-overlapping distributions of pollinator species gives strength to our correlative conclusion. It is also important to note here that our sampling design did not allow for detailed (e.g. at the level of the individual fig) pairwise comparisons of co-occurrence across fig-wasp species.

Within most parasitic species, we found evidence for genetic isolation by distance. Such a pattern of well delimited sister taxa displaying within species isolation by distance suggests an older colonisation of the range by extant parasite species or more limited dispersal than in the sole other non-pollinating fig wasp for which genetic data are available, a *Sycoscapter* associated with *F. rubiginosa* (Sutton et al., 2016). An alternative explanation is lower population density in the parasites than in the pollinators of *Ficus hirta*. However, there is only a limited difference in density between the two groups, which is probably not sufficient to explain the lack of IBD among pollinator populations coupled with strong IBD among parasite populations. Indeed, the slope of genetic differentiation is predicted to

be simply inversely proportional to density, all other factors remaining constant (Rousset, 1997). The difference may relate to differences in fruiting phenology and distribution between *Ficus hirta*, a small dioecious shrub (continuous fruiting, growing in patches) and *F. rubiginosa*, a monoecious hemiepiphytic figs (synchronised crop on a tree, more dispersed individuals). Several of the sycoryctine species studied here had larger geographical ranges than pollinators. For example, *Philotrypesis* sp 1 occurs from southern China to southern Thailand. Competitive exclusion could be a major driver in conjunction with some ecological differentiation of the parapatric distribution across sites within *Philotrypesis* and *Sycoscapter*. Indeed, the geographical distributions of diversity do not appear to be the same for each trophic group, with little correspondence seen between the ranges of *Philotrypesis* and *Sycoscapter*, and between either and *Valisia*. Climatic modelling would outline the role of environmental gradients versus competition, but the evidence presented here suggests that they are not the sole drivers of species distributions. While host-associated differentiation is largely absent at higher trophic levels some parasites are restricted to single pollinator species, possibly because of their restricted range. The northern populations of *F. hirta* that host the more restricted *Philotrypesis* sp3 are phenotypically distinct and produce larger figs with thick walls (Yu et al., 2018). Perhaps the observed pattern is contingent on encounter frequency and asymmetric coevolution across trophic levels (Lapchin, 2002; diffuse coevolution). Finally, while the community of fig wasps associated with *Ficus hirta* is not diverse, the community structure recorded here (one pollinator, one cleptoparasite and one parasitoid) is largely conserved across the host range. This is in line with our expectations derived from other fig-wasp systems. Indeed, while Hawkins and Compton (1992) stressed a lack of saturation in fig-wasp communities reared from individual crops, their extensive data set showed that both community structure and species richness remain constant for several *Ficus* sampled at a regional scale.

Sympatric, congeneric fig wasps in the same trophic guild tend to diverge in ovipositor length which is a proxy for fig development stage at oviposition (Segar et al., 2014; Weiblen & Bush, 2002). This divergence is highly suggestive of divergent selection driven by competition on a shared host. In our study, sister species are largely allopatric reflecting a situation of competitive exclusion. Certainly, the congeneric individuals studied here are largely similar with respect to ovipositor length (apart from *Philotrypesis* sp3.) and hard to distinguish morphologically. More detailed morphological appraisal of each molecular entity would certainly be highly valuable for determining potential niche overlap. In a similar vein, formal tests of phylogenetic congruence between these various taxonomic groups would be an important next step.

Despite being absent in high canopy sampling efforts (Harrison, 2003), the single species of non-pollinating fig wasp studied to date appears to be a reasonable disperser (Sutton et al., 2016). Sutton et al. (2016) report no or almost no IBD in a wide-ranging *Sycoscapter* wasp. A general point might be the longevity of these wasps, *Philotrypesis* is known to live for as long as 40 days when fed sugar water (Compton et al., 1994; Joseph, 1958). In contrast to non-feeding pollinators and other wasps that enter monoecious figs and which disperse by wind, externally ovipositing wasps can wait for suitable clutches to become locally available. Gradual, but regular, inter-generational dispersal of these generalist parasitoids is likely given their strategy of laying small clutches of offspring across multiple figs (Cook et al., 2017). We might expect broad geographical ranges across parasitoid and cleptoparasitic fig wasps in general. Among the species studied here, some *Philotrypesis* species have remarkable ranges, but display higher levels of IBD than *Valisia* pollinators. These lines of evidence suggest a long-term host association between non-pollinating fig wasps and *F. hirta* and moderate levels of dispersal in these 'slow' wasps (Venkateswaran et al., 2017), or lower densities. Furthermore, *Ficus hirta* is a dioecious species with a clumped distribution in the landscape, a population structure that is likely to offset some long-distance dispersal in wasps (Kjellberg & Proffit, 2016). We suggest that strong gene flow in pollinating wasps, offset by a degree of local dispersal and in combination with limited phenotypic variation in *F. hirta*, will result in multiple phenotypically homogeneous pollinator species. Each species will be easily excluded by another species at its margins. In contrast, parasites display clinal genetic variation, likely reflecting local adaptation, and are hence more resistant to competition at the limits of their range.

4.1 | A generalised pattern of geographical species turnover in fig-wasp community

The geographical variation of associated communities of fig wasps specialised on a single *Ficus* host has now been investigated using molecular markers for two *Ficus* species (Darwell et al., 2014; Darwell & Cook, 2017; Yu et al., 2018; this study, 2019). In both cases, the wasps are mainly structured into groups of parapatric, ecologically equivalent species. In both communities, pollinating wasp species diversity is twice that within parasite group, and the different parasite functional groups on a fig host present similar numbers of parapatric species (4 on *Ficus hirta*, 1–2 on *F. rubiginosa*). A direct correlate is that these parasitoids and cleptorasites are not host specialists, as they parasitise several host species. Such heterogeneous diversification patterns may be general. Indeed, in communities of oak galling wasps, galling species present more divergent populations through their range than their parasitoids (Stone et al., 2012). The pattern observed here could fit within the general scenario where parasitoids of endophytic herbivores (e.g. gallers and leaf miners) are less specialised than parasitoids of exophytic herbivores (Askew & Shaw, 1986). Endophytic parasitoids are generally idiobionts and more subject to interspecific competition (Hawkins et al., 1990), a process which underpins our hypothesis. Another more general example is that of leaf mining insects, a group of endophytes attacked in some cases by rather generalist parasitoids whose host use better correlates to plant than host phylogeny (Ives & Godfray, 2006; Leppänen et al., 2013; Lopez-Vaamonde et al., 2003). The age of divergence among *Valisia* host species is estimated at over 10 Ma with some subgroups splitting as recently as 2.6 Ma (Yu et al., 2019), the age of Western Palearctic cynipid wasp communities is around 3.5 Ma. The progenitor oak gall wasp community likely assembled before the

expansion of oaks into from Asia around 5–7 Ma (Stone et al., 2012). The accumulation of species in these last two systems has operated over similar timescales. While climactic history clearly differs, there are strong parallels in the ecology of the parasites in both systems. These studies highlight the need for range-wide studies for understanding host specificity.

The diversity within group and among locations (beta diversity) was more than twice as high in *Ficus hirta* than in *F. rubiginosa* while the number of groups (alpha diversity) was much higher in *F. rubiginosa* resulting in a total of at least 17 non-pollinating wasp species (Darwell & Cook, 2017; Segar et al., 2014). A simple explanation could be that a shared factor, more limited wasp dispersal, reduces the number of species that can survive locally while facilitating geographical speciation. In agreement, alpha-diversity of fig-wasp communities has been shown to correlate with tree height and breeding system (Compton & Hawkins, 1992). Comparative studies will allow unravelling the causal factors involved.

4.2 | A generalised pattern of geographical stability in fig-wasp community structure

The geographical stability of community structure reported here is frequent in fig-wasp communities. Indeed, there was no, or almost no, latitudinal turnover in parasite species number for 26 *Ficus* species in southern Africa (Hawkins & Compton, 1992). The structure of the community associated with *Ficus rubiginosa* in Australia is constant throughout its range despite geographical turnover in species composition (Darwell & Cook, 2017; Segar et al., 2014). Similarly, the communities associated with *F. benjamina* in Hainan Island and in Australia, while sharing no species, presented highly similar structures (Darwell et al., 2018). Such geographical stability suggests a role for deterministic processes in which host-plant traits determine the structure of their associated fig-wasp communities. Indeed, fig-wasp communities present independently evolved convergent structure across continents (Segar et al., 2013), and the complexity of fig-wasp communities in southern Africa is determined by ecological factors such as tree height rather than by phylogenetic history (Compton & Hawkins, 1992).

4.3 | A generalised pattern of limited spatial turn over in community structure of plant-associated insects

As for fig wasps, the structure of the communities constituted by deciduous-oak galling wasps and their parasitoids across the Western Palearctic is remarkably stable. Indeed, species turnover between refugia in Iran, the Balkan and Iberia is extremely limited (Stone et al., 2012), and this is achieved despite contrasting biogeographical patterns for the different species. However, if we accept long-distance dispersal to be frequent, as documented for the oak associated communities and for fig wasps, then we may expect current genetic structure to reflect the history of the individual species, with occasional regional extinctions of some taxa followed by re-colonisation, but also range-wide invasions with species replacements. Homogeneous spatial genetic structure and age of colonisation within a community is not a prediction (Alvarez et al., 2010). Nevertheless, stability of community structure without geographical species turnover was observed in the most extensive survey of geographical variation in tropical herbivorous insects, in the New Guinea lowlands, over distances greater than 500 km (Novotny et al., 2007). Species turnover should probably be scaled relative to geographical distances, to variation in ecological conditions and to age of the examined communities.

5 | CONCLUSIONS

Comprehensive data on geographical variation in fig-wasp community structure, spatial genetic structure and phylogenetic history are providing new insights into the ecological and evolutionary determinants of community structure and composition. In the case of *F. hirta*, fewer cleptoparasite and parasitoid species are found than obligate pollinator species. These non-pollinating fig wasps are generalist, in the sense that they can utilise several pollinator species as hosts. Furthermore, while range size varies among similar non-pollinating fig wasp species, it is generally large and non-overlapping. As the two types of parasitic wasps are non-specialists and present different geographical patterns, co-diversification is not a process shaping local and regional communities. Isolation by distance is lower in pollinators than non-pollinators. Finally, most populations of *F. hirta* are utilised by a single pollinator species along with one cleptoparasite and one parasitoid. Our results highlight the links between ecology, life history and evolutionary history in shaping community structure, patterns of genetic diversity and host specificity. Fig-wasp communities are becoming a major biological model to understand the diversification of insect communities feeding on plants in the tropics, with real hopes of establishing a set of general rules.

ACKNOWLEDGEMENTS

We thank Prof. Xia Nianhe, Peng Yanqiong, Vu Quang Nam, Tien Tran, Nong Van Duy, Zhang Xianchun (collecting permit number in Indonesia: No. 1799IPH.1.02/KS.01/XI/2014), Harry Wiriadinata, Arief Hidayat, Wei Ran, Chen Wenli, Mashhor Mansor, Nora Sikin, Serena Lee (provided Singapore samples), Khin Me Aung, Chuck Cannon, Thitima Tharawoot and Liang Dan for sample collection and help in the field. This work was supported by the National Natural Science Foundation of China (31630008; 31670395), Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou; GML2019ZD0408), the National Natural Science Foundation of China (31971568), Province Natural Science Foundation of Guangdong (c20140500001306) and the CAS President's International Fellowship Initiative (2018VBA0040). Simon Segar is grateful for departmental support from Harper Adams University. Finn Kjellberg is grateful for support from LIA MOST.

AUTHORS' CONTRIBUTIONS

H.Y. designed the research, collected samples, analysed data and co-wrote the manuscript; L.C. analysed data; S.T.S. co-wrote the manuscript; F.K. contributed to the data analyses and co-wrote the manuscript; X.D. performed the laboratory work and analysed data; E.T. performed the laboratory work; D.Z. designed the research; T.W. collected the samples in Thailand.

DATA AVAILABILITY STATEMENT

Microsatellite genotype data for all individuals of *Philotrypesis* and *Syocryctini* are archived in the Dryad Digital Repository at https://doi.org/10.5061/dryad.5x69p8d0x (Yu, 2021). Haplotype sequences have been deposited in GenBank under Accession No: MK955672–MK955733 for Cytb, and no: MK955734–MK955756 for ITS2.

ORCID

Hui Yu https://orcid.org/0000-0003-0074-9153
Finn Kjellberg https://orcid.org/0000-0001-6708-9538
Simon T. Segar https://orcid.org/0000-0001-6621-9409

REFERENCES

Ahmed, S., Compton, S. G., Butlin, R. K., & Gilmartin, P. M. (2009). Wind-borne insects mediate directional pollen transfer between desert fig trees 160 kilometers apart. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(48), 20342–20347. https://doi.org/10.1073/pnas.0902213106

Alvarez, N., McKey, D., Kjellberg, F., & Hossaert-McKey, M. (2010). Phylogeography and historical biogeography of obligate specific mutualisms. In S. Morand & B. R. Krasnov (Eds.), *The biogeography of host–parasite interactions* (pp. 31–39). Oxford University Press.

Askew, R., & Shaw, M. R. (1986). Parasitoid communities: Their size, structure and development. J. Waage, & D. Greathead (Eds.), *Insect Parasitoids*, *13th Symposium of Royal Entomological Society of London* (pp. 225–264). Academic Press: Now Elsevier.

Bain, A., Borges, R. M., Chevallier, M. H., Vignes, H., Kobmoo, N., Peng, Y. Q., Cruaud, A., Rasplus, J. Y., Kjellberg, F., & Hossaert-Mckey, M. (2016). Geographic structuring into vicariant species-pairs in a wide-ranging, high-dispersal plant–insect mutualism: The case of *Ficus racemosa* and its pollinating wasps and. *Evolutionary Ecology*, *30*(4), 663–684. https://doi.org/10.1007/s10682-016-9836-5

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., & Bonhomme, F. (2004). *GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations*. Université de Montpellier II.

Bunnefeld, L., Hearn, J., Stone, G. N., & Lohse, K. (2018). Whole-genome data reveal the complex history of a diverse ecological community. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(28), E6507–E6515. https://doi.org/10.1073/pnas.1800334115

Butterill, P. T., & Novotny, V. (2015). Gall-forming insects in a lowland tropical rainforest: Low species diversity in an extremely specialised guild. *Ecological Entomology*, *40*(4), 409–419. https://doi.org/10.1111/een.12198

Compton, S. G., Ellwood, M. D. F., Davis, A. J., & Welch, K. (2000). The flight heights of chalcid wasps (Hymenoptera, Chalcidoidea) in a lowland Bornean rain forest: Fig wasps are the high fliers. *Biotropica*, *32*(3), 515–522.

Compton, S. G., & Hawkins, B. A. (1992). Determinants of species richness in southern African fig wasp assemblages. *Oecologia*, *91*(1), 68–74. https://doi.org/10.1007/BF00317243

Compton, S. G., Rasplus, J.-Y., & Ware, A. B. (1994). African fig wasp parasitoid communities. In B. Hawkins & W. Sheehan (Eds.), *Parasitoid community ecology* (pp. 323–348). Oxford University Press.

Conchou, L., Ciminera, M., Hossaert-McKey, M., & Kjellberg, F. (2014). The non-pollinating fig wasps associated with *Ficus guianensis*: Community structure and impact of the large species on the fig/pollinator mutualism. *Acta Oecologica*, *57*, 28–37. https://doi.org/10.1016/j.actao.2013.07.004

Cook, J. M., & Rasplus, J.-Y. (2003). Mutualists with attitude: Coevolving fig wasps and figs. *Trends in Ecology & Evolution*, *18*(5), 241–248. https://doi.org/10.1016/S0169-5347(03)00062-4

Cook, J. M., Reuter, C., Moore, J. C., & West, S. A. (2017). Molecular markers reveal reproductive strategies of non-pollinating fig wasps: Molecular revelation of fig wasp behaviour. *Ecological Entomology*, *42*(6), 689–696. https://doi.org/10.1111/een.12433

Cook,J.M.,&Segar,S.T.(2010).Speciationinfigwasps.*EcologicalEntomology*, *35*(s1), 54–66. https://doi.org/10.1111/j.1365-2311.2009.01148.x

Cruaud, A., Rønsted, N., Chantarasuwan, B., Chou, L. S., Clement, W. L., Couloux, A., Cousins, B., Genson, G., Harrison, R. D., Hanson, P. E., Hossaert-Mckey, M., Jabbour-Zahab, R., Jousselin, E., Kerdelhué, C., Kjellberg, F., Lopez-Vaamonde, C., Peebles, J., Peng, Y.-Q., Pereira, R. A. S., … Savolainen, V. (2012). An extreme case of plant–insect codiversification: Figs and fig-pollinating wasps. *Systematic Biology*, *61*(6), 1029–1047. https://doi.org/10.1093/sysbio/sys068

Darwell, C. T., Al-Beidh, S., & Cook, J. M. (2014). Molecular species delimitation of a symbiotic fig-pollinating wasp species complex reveals extreme deviation from reciprocal partner specificity. *BMC Evolutionary Biology*, *14*(1), 189. https://doi.org/10.1186/s12862-014-0189-9

Darwell, C. T., & Cook, J. M. (2017). Cryptic diversity in a fig wasp community-morphologically differentiated species are sympatric but cryptic species are parapatric. *Molecular Ecology*, *26*(3), 937–950. https://doi.org/10.1111/mec.13985

Darwell, C. T., Segar, S. T., & Cook, J. M. (2018). Conserved community structure and simultaneous divergence events in the fig wasps associated with *Ficus benjamina* in Australia and China and. *BMC Ecology*, *18*(1), 13. https://doi.org/10.1186/s12898-018-0167-y

Farache, F. H. A., Cruaud, A., Rasplus, J.-Y., Cerezini, M. T., Rattis, L., Kjellberg, F., & Pereira, R. A. S. (2018). Insights into the structure of plant-insect communities: Specialism and generalism in a regional set of non-pollinating fig wasp communities. *Acta Oecologica*, *90*, 49–59. https://doi.org/10.1016/j.actao.2018.02.006

Forister, M. L., & Feldman, C. R. (2011). Phylogenetic cascades and the origins of tropical diversity. *Biotropica*, *43*(3), 270–278. https://doi.org/10.1111/j.1744-7429.2010.00702.x

Galiana, N., Hawkins, B. A., & Montoya, J. M. (2019). The geographical variation of network structure is scale dependent: Understanding the biotic specialization of host–parasitoid networks. *Ecography*, *42*(6), 1175–1187. https://doi.org/10.1111/ecog.03684

Gotelli, N. J., Hart, E. M., & Ellison, A. M. (2015). EcoSimR-Alpha. *Zenodo*, https://doi.org/10.5281/zenodo.16522

Harrison, R. D. (2003). Fig wasp dispersal and the stability of a keystone plant resource in Borneo. *Proceedings of the Royal Society of London Series B: Biological Sciences*, *270*, S76–S79.

Harrison, R. D., & Rasplus, J. Y. (2006). Dispersal of fig pollinators in Asian tropical rain forests. *Journal of Tropical Ecology*, *22*(06), 631. https://doi.org/10.1017/S0266467406003488

Hawkins, B. A. (1994). *Pattern and process in host-parasitoid interactions* (pp. 1–190). Cambridge University Press.

Hawkins, B. A., Askew, R. R., & Shaw, M. A. (1990). Influences of host feeding-niche and foodplant type on generalist and specialist parasitoids. *Ecological Entomology*, *15*(3), 275–280. https://doi.org/10.1111/j.1365-2311.1990.tb00809.x

Hawkins, B. A., & Compton, S. G. (1992). African fig wasp communities—Undersaturation and latitudinal gradients in species richness. *Journal of Animal Ecology*, *61*(2), 361–372. https://doi.org/10.2307/5328

Hawkins, B. A., Cornell, H. V., & Hochberg, M. E. (1997). Predators, parasitoids, and pathogens as mortality agents in phytophagous insect populations. *Ecology*, *78*(7), 2145–2152. https://doi.org/10.1890/0012-9658(1997)078[2145:PPAPAM]2.0.CO;2

Hernández-López, A., Rougerie, R., Augustin, S., Lees, D. C., Tomov, R., Kenis, M., Çota, E., Kullaj, E., Hansson, C., Grabenweger, G., Roques, A., & López-Vaamonde, C. (2012). Host tracking or cryptic adaptation? Phylogeography of *Pediobius saulius* (Hymenoptera, Eulophidae), a parasitoid of the highly invasive horse-chestnut leafminer: Parasitoid host races. *Evolutionary Applications*, *5*(3), 256–269. https://doi.org/10.1111/j.1752-4571.2011.00220.x

Hrcek, J., & Godfray, H. C. J. (2015). What do molecular methods bring to host–parasitoid food webs? *Trends in Parasitology*, *31*, 30–35. https://doi.org/10.1016/j.pt.2014.10.008

Hrcek, J., Miller, S. E., Whitfield, J. B., Shima, H., & Novotny, V. (2013). Parasitism rate, parasitoid community composition and host specificity on exposed and semi-concealed caterpillars from a tropical rainforest. *Oecologia*, *173*(2), 521–532. https://doi.org/10.1007/s00442-013-2619-6

Ives, A. R., & Godfray, H. C. J. (2006). Phylogenetic analysis of trophic associations. *The American Naturalist*, *168*, 1–14. https://doi.org/10.1086/505157

Joseph, J. K. (1958). Recherches sur les chalcidiens, *Blastophaga psenes* (L.) et *Phylotrypesis caricae* (L.) du figuier (*Ficus carica*L.). *Annales Des Sciences Naturelles Zoologie et Biologie Animale*, *20*, 197–260.

Jousselin, E., van Noort, S., Berry, V., Rasplus, J.-Y., Ronsted, N., Erasmus, C. J., & Greeff, J. M. (2008). One fig to bind them all: Host conservatism in a fig wasp community unraveled by cospeciation analyses among pollinating and nonpollinating fig wasps. *Evolution*, *62*(7), 1777–1797. https://doi.org/10.1111/j.1558-5646.2008.00406.x

Kjellberg, F., & Proffit, M. (2016). Tracking the elusive history of diversification in plant-herbivorous insect-parasitoid food webs: Insights from figs and fig wasps. *Molecular Ecology*, *25*(4), 843–845. https://doi.org/10.1111/mec.13533

Lapchin, L. (2002). Host-parasitoid association and diffuse coevolution: When to be a generalist? *The American Naturalist*, *160*(2), 245–254. https://doi.org/10.1086/341020

Leppänen, S. A., Altenhofer, E., Liston, A. D., & Nyman, T. (2013). Ecological versus phylogenetic determinants of trophic associations in a plant–leafminer–parasitoid food web. *Evolution*, *67*(5), 1493–1502. https://doi.org/10.1111/evo.12028

Lewis, O. T., Memmott, J., Lasalle, J., Lyal, C. H. C., Whitefoord, C., & Godfray, H. C. J. (2002). Structure of a diverse tropical forest insect–parasitoid community. *Journal of Animal Ecology*, *71*(5), 855–873. https://doi.org/10.1046/j.1365-2656.2002.00651.x

Li, Y., Zhou, X., Feng, G., Hu, H., Niu, L., Hebert, P. D. N., & Huang, D. (2010). COI and ITS2 sequences delimit species, reveal cryptic taxa and host specificity of fig-associated *Sycophila* (Hymenoptera, Eurytomidae). *Molecular Ecology Resources*, *10*(1), 31–40. https://doi.org/10.1111/j.1755-0998.2009.02671.x

Lopez-Vaamonde, C., Godfray, H. C. J., & Cook, J. M. (2003). Evolutionary dynamics of host-plant use in a genus of leaf-mining moths. *Evolution*, *57*(8), 1804–1821. https://doi.org/10.1111/j.0014-3820.2003.tb00588.x

Mayr, G. (1885). Fig insecten. *Verhandlungen Der Zoologische Botanische Gesellschaft Wien (B)*, *35*, 147–250.

McLeish, M. J., Beukman, G., van Noort, S., & Wossler, T. C. (2012). Host-plant species conservatism and ecology of a parasitoid fig wasp genus (Chalcidoidea; Sycoryctinae; *Arachonia*). *PLoS ONE*, *7*(9), e44804. https://doi.org/10.1371/journal.pone.0044804

Moe, A. M., Clement, W., & Weiblen, G. D. (2012). Rapid evolution of pollinator-mediated plant reproductive isolation. In R. S. Singh, X. Jianping, & R. Kulathinal (Eds.), *Rapidly evolving genes and genetic systems* (p. 312). Oxford University Press.

Molbo, D., Machado, C. A., Sevenster, J. G., Keller, L., & Herre, E. A. (2003). Cryptic species of fig-pollinating wasps: Implications for the evolution of the fig-wasp mutualism, sex allocation, and precision of adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(10), 5867–5872. https://doi.org/10.1073/pnas.0930903100

Nair, P. B., Abdurahiman, U. C., & Joseph, J. M. (1981). Two new Torymidae (Hymenoptera, Chalcidoidea) from *Ficus hirta*. *Oriental Insects*, *15*(4), 433–442.

Nicholls, J. A., Schönrogge, K., Preuss, S., & Stone, G. N. (2018). Partitioning of herbivore hosts across time and food plants promotes diversification in the *Megastigmus dorsalis* oak gall parasitoid complex. *Ecology and Evolution*, *8*(2), 1300–1315. https://doi.org/10.1002/ece3.3712

Novotny, V., Miller, S. E., Hrcek, J., Baje, L., Basset, Y., Lewis, O. T., Stewart, A. J. A., & Weiblen, G. D. (2012). Insects on plants: Explaining the paradox of low diversity within specialist herbivore guilds. *The American Naturalist*, *179*(3), 351–362. https://doi.org/10.1086/664082

Novotny, V., Miller, S. E., Hulcr, J., Drew, R. A. I., Basset, Y., Janda, M., Setliff, G. P., Darrow, K., Stewart, A. J. A., Auga, J., Isua, B., Molem, K., Manumbor, M., Tamtiai, E., Mogia, M., & Weiblen, G. D. (2007). Low beta diversity of herbivorous insects in tropical forests. *Nature*, *448*(7154), 692–695.

Peakall, P., & Smouse, P. E. (2006). GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, *6*, 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Price, P. W. (2002). Resource-driven terrestrial interaction webs. *Ecological Research*, *17*(2), 241–247. https://doi.org/10.1046/j.1440-1703.2002.00483.x

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*, 945–959.

Rodriguez, L. J., Bain, A., Chou, L.-S., Conchou, L., Cruaud, A., Gonzales, R., Hossaert-McKey, M., Rasplus, J.-Y., Tzeng, H.-Y., & Kjellberg, F. (2017). Diversification and spatial structuring in the mutualism between *Ficus septica* and its pollinating wasps in insular South East Asia. *BMC Evolutionary Biology*, *17*(1), 207. https://doi.org/10.1186/s12862-017-1034-8

Rousset, F. (1997). Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, *145*(4), 1219–1228.

Segar, S. T., Dunn, D. W., Darwell, C. T., & Cook, J. M. (2014). How to be a fig wasp down under: The diversity and structure of an Australian fig wasp community. *Acta Oecologica*, *15*, 17–27.

Segar, S. T., Fayle, T. M., Srivastava, D. S., Lewinsohn, T. M., Lewis, O. T., Novotny, V., Kitching, R. L., & Maunsell, S. C. (2020). The role of evolution in shaping ecological networks. *Trends in Ecology & Evolution*, *35*(5), 454–466. https://doi.org/10.1016/j.tree.2020.01.004

Segar, S. T., Lopez-Vaamonde, C., Rasplus, J.-Y., & Cook, J. M. (2012). The global phylogeny of the subfamily Sycoryctinae (Pteromalidae): Parasites of an obligate mutualism. *Molecular Phylogenetics and Evolution*, *65*, 116–125. https://doi.org/10.1016/j.ympev.2012.05.030

Segar, S. T., Pereira, R. A. S., Compton, S. G., & Cook, J. M. (2013). Convergent evolution of multitrophic community structure over three continents. *Ecology Letters*, *16*(12), 1436–1445.

Smith, M. A., Rodriguez, J. J., Whitfield, J. B., Deans, A. R., Janzen, D. H., Hallwachs, W., & Hebert, P. D. N. (2008). Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(34), 12359. https://doi.org/10.1073/pnas.0805319105

Smith, M. A., Woodley, N. E., Janzen, D. H., Hallwachs, W., & Hebert, P. D. N. (2006). DNA barcodes reveal cryptic host-specificity within

the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proceedings of the National Academy of Sciences of the United States of America*, *103*(10), 3657–3662. https://doi.org/10.1073/pnas.0511318103

Souto-Vilarós, D., Machac, A., Michalek, J., Darwell, C. T., Sisol, M., Kuyaiva, T., Isua, B., Weiblen, G. D., Novotny, V., & Segar, S. T. (2019). Faster speciation of fig-wasps than their host figs leads to decoupled speciation dynamics: Snapshots across the speciation continuum. *Molecular Ecology*, *28*(17), 3958–3976. https://doi.org/10.1111/mec.15190

Sow, A., Brévault, T., Benoit, L., Chapuis, M.-P., Galan, M., Coeur d'acier, A., Delvare, G., Sembène, M., & Haran, J. (2019). Deciphering host-parasitoid interactions and parasitism rates of crop pests using DNA metabarcoding. *Scientific Reports*, *9*(1), 3646. https://doi.org/10.1038/s41598-019-40243-z

Stireman, J. O., Nason, J. D., Heard, S. B., & Seehawer, J. M. (2006). Cascading host-associated genetic differentiation in parasitoids of phytophagous insects. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1586), 523–530.

Stone, G. N., Lohse, K., Nicholls, J. A., Fuentes-Utrilla, P., Sinclair, F., Schönrogge, K., Csóka, G., Melika, G., Nieves-Aldrey, J.-L., Pujade-Villar, J., Tavakoli, M., Askew, R. R., & Hickerson, M. J. (2012). Reconstructing community assembly in time and space reveals enemy escape in a western palearctic insect community. *Current Biology*, *22*(6), 532–537. https://doi.org/10.1016/j.cub.2012.01.059

Sutton, T. L., Riegler, M., & Cook, J. M. (2016). One step ahead: A parasitoid disperses farther and forms a wider geographic population than its fig wasp host. *Molecular Ecology*, *25*(4), 882–894. https://doi.org/10.1111/mec.13445

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Thompson, J. N. (1994). *The coevolutionary process* (pp. 1–383). The University of Chicago Press.

Tian, E., Nason, J. D., Machado, C. A., Zheng, L., Yu, H., & Kjellberg, F. (2015). Lack of genetic isolation by distance, similar genetic structuring but different demographic histories in a fig-pollinating wasp mutualism. *Molecular Ecology*, *24*(23), 5976–5991. https://doi.org/10.1111/mec.13438

Van Veen, F. J. F., Müller, C. B., Pell, J. K., & Godfray, H. C. J. (2008). Food web structure of three guilds of natural enemies: Predators, parasitoids and pathogens of aphids. *Journal of Animal Ecology*, *77*(1), 191–200. https://doi.org/10.1111/j.1365-2656.2007.01325.x

Venkateswaran, V., Shrivastava, A., Kumble, A. L. K., & Borges, R. M. (2017). Life-history strategy, resource dispersion and phylogenetic associations shape dispersal of a fig wasp community. *Movement Ecology*, *5*(1), 25. https://doi.org/10.1186/s40462-017-0117-x

Weiblen, G. D., & Bush, G. L. (2002). Speciation in fig pollinators and parasites. *Molecular Ecology*, *11*(8), 1573–1578. https://doi.org/10.1046/j.1365-294X.2002.01529.x

Yu, H. (2021). Low host specificity and broad geographic ranges in a community of parasitic non-pollinating fig wasps (Sycoryctinae; Chalcidoidea) [Dataset]. *Dryad*, https://doi.org/10.5061/dryad.5x69p8d0x

Yu, H., Liang, D., Tian, E., Zheng, L., & Kjellberg, F. (2018). Plant geographic phenotypic variation drives diversification in its associated community of a phytophagous insect and its parasitoids. *BMC Evolutionary Biology*, *18*(1), 134. https://doi.org/10.1186/s12862-018-1239-5

Yu, H., Tian, E., Zheng, L., Deng, X., Cheng, Y., Chen, L., Wu, W., Tanming, W., Zhang, D., Compton, S. G., & Kjellberg, F. (2019). Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation. *Molecular Ecology*, *28*, 2391–2405. https://doi.org/10.1111/mec.15046

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Deng X, Chen L, Tian E, et al. Low host specificity and broad geographical ranges in a community of parasitic non-pollinating fig wasps (Sycoryctinae; Chalcidoidea). *J Anim Ecol*. 2021;00:1–13. https://doi.org/10.1111/1365-2656.13483

Supporting Information

Table S1. Sampling sites and sample sizes for sequences and microsatellite data

Site	Latitude, longitude	*Philotrypesis*			*Sycoscapter-Sycoryctes*		
		Cytb	ITS2	SSR	Cytb	ITS2	SSR
Ning	26.664, 119.549	6	6	25	-	-	-
Sha	26.419, 117.818	18	16	20	-	-	-
Xia	24.742, 118.072	-	-	11	-	-	-
Sui	26.476, 114.239	10	12	24	-	-	-
Da	24.258, 116.806	-	-	15	-	-	-
Gui	25.077, 110.306	-	-	12	-	-	8
Huo	23.170, 113.373	12	9	-	7	2	24
DHS	23.166, 112.543	20	21	-	8	12	19
Xiang	22.424, 114.306	-	-	21	-	-	21
Sand	25.984, 107.874	3	6	12	7	3	21
Nan	22.787, 108.389	3	9	20	-	-	9
Ding	19.697, 110.328	12	16	24	1	5	24
Wan	18.795, 110.391	7	14	17	3	-	17
XI	21.913, 101.264	-	-	24	-	-	7
CH	12.774, 102.096	6	5	5	9	3	4
Wu	14.443, 105.273	2	2	2	6	1	2
ST	7.467, 99.639	6	6	12	-	-	-
Tai	18.894, 98.858	2	3	-	1	-	-
CS	18.84, 99.47	5	5	8	1	1	3
QMS	18.809, 98.914	7	3	5	6	6	6
DAL	12.157, 108.137	2	-	2	-	-	-
HB	12.999, 108.230	1	-	1	-	-	-
SNP	1.312, 103.816	2	-	2	19	3	14
CI	-6.566, 106.706	-	-	-	3	7	10
JA	-6.368, 106.830	-	-	-	5	11	14
Total		124	133	262	76	54	203

Table S2. *Philotrypesis* wasps, cytb. Sample size (*N*), number of haplotypes (*K*), haplotype diversity (*Hd*), nucleotide diversity (π), and name of haplotypes (*H*) with number of individuals. A single female wasp per fig was sequenced in most locations (1-2 individuals per fig in CH and CS, 6 individuals from a fig in ST). We used the primers of Jermiin and Crozier (1994) and optimised the amplification as follows: one cycle of denaturation at 94°C for 3 min, 35 cycles of 30 s denaturation at 92°C, 30 s at a 48°C annealing temperature, and 30 s extension at 72˚C, followed by 10 min extension at 72˚C.

	site	N	K	Hd	*Π*	cytb haplotype (number of individuals)
sp 1	DHS	20	13	0.953	0.00521	H1(2);H2(3);H3(2);H4(3);H5(1);H6(1);H7(1);H8(1);H9(1); H10(1);H11(1);H12(1);H13(2)
	Huo	12	8	0.894	0.00267	H4(4);H14(1);H15(2);H16(1);H17(1);H18(1);H19(1);H20(1)
	Nan	3	3	1.000	0.00203	H4(1);H17(1);H39(1)
	Ding	12	7	0.864	0.00403	H17(4);H37(1);H38(1);H39(3);H40(1);H41(1);H42(1)
	Wan	7	4	0.810	0.00246	H17(1);H37(1);H39(2);H55(3)
	CH	6	4	0.800	0.00415	H17(1);H21(3);H22(1);H23(1)
	Wu	2	1	0.000	0.000	H17(2)
	ST	6	4	0.800	0.0037	H33(1);H34(3);H35(1);H36(1)
	HB	1	1	-	-	H34(1)
	QMS	3	2	0.667	0.00507	H17(2);H34(1)
	Total	**72**	**34**	1.000	0.11457	
sp2	Tai	2	1	0.000	0.000	H29(2)
	CS	5	5	1.000	0.00456	H24(1);H25(1);H26(1);H27(1);H28(1)
	QMS	4	4	1.000	0.00507	H29(1);H30(1);H31(1);H32(1)
	Sand	3	3			H60(1);H61(1);H62(1)
	Total	**14**	**10**	**0.945**	**0.00553**	
sp3	Ning	6	5	0.933	0.01145	H43(1);H44 (1);H45(1);H46(2);H47(1)
	Sha	18	6	0.725	0.00835	H45(3);H46 (3);H48(9);H49(1);H50(1);H51(1)
	Sui	10	7	0.933	0.00601	H45(2);H47(1);H48(1);H49(2);H52(2);H53(1);H54(1)
	Total	**34**	**12**	0.866	0.01051	
sp4	SNP	2	2	1.000	0.00608	H56(1);H57(1)
	DAL	2	2	1.000	0.03191	H58(1);H59(1)
	Total	**4**	**4**	**1.000**		

Table S3. *Philotrypesis* wasps, ITS2. Sample size (*N*), number of haplotypes (*K*), and name of haplotypes with number of individuals. Nine individuals from the South China Botanical Garden (Guangzhou, SCBG) were sequenced.

	site	N	K	ITS2 haplotype (number of individuals)
sp1	DHS	21	1	H2(21)
	SCBG	9	1	H2(9)
	Nan	9	1	H2(9)
	Ding	16	1	H2(15)
	Wan	14	1	H2(14)
	CH	5	1	H2(5)
	Wu	2	1	H2(2)
	ST	6	1	H2(6)
	Total	**82**	**1**	
sp2	CS	5	5	H1(5);
	Tai	3	1	H1(3)
	QMS	3	1	H1(3)
	Sand	6	1	H1(6)
	Total	**17**	**1**	
sp3	Ning	6	1	H3(6)
	Sha	16	1	H3(16)
	Sui	12	1	H3(12)
	Total	**34**	**1**	

Table S4. Sycoryctini wasps, Cytb. Sample size (*N*), number of haplotypes (*K*), haplotype diversity (*Hd*), nucleotide diversity (π), and name of haplotypes (*H*) with number of individuals. A single female wasp per fig was sequenced in most locations (1-2 individuals per fig in Wu and SNP). We used the primers of Jermiin and Crozier (1994) and optimised the amplification as follows: one cycle of denaturation at 94°C for 3 min, 35 cycles of 30 s denaturation at 92°C, 30 s at a 48°C annealing temperature, and 30 s extension at 72˚C, followed by 10 min extension at 72˚C.

	site	N	K	Hd	*Π*	Cytb haplotype (number of individuals)
Sycoscapter sp1	DHS	8	6	0.929	0.01883	H1(2);H2(2);H3(1);H4(1);H5(1);H6(1)
	Huo	7	6	0.952	0.0207	H7(1);H2(2);H3(1);H4(1);H5(1);H6(1)
	Sand	7	2	0.286	0.00043	H9(1);H1(6)
	Ding	1	1	---	---	H8(1)
	Wan	3	3	1	0.0152	H8(1);H19(1);H20(1)
	Total	**26**	**11**	**0.880**	**0.01499**	
Sycoscapter sp2	Tai	1	1	0.000	0.000	H11(1)
	QMS	6	3	0.733	0.00511	H10(2);H11(3);H14(1)
	CS	1	1	0.000	0.000	H11(1)
	CH	9	7	0.917	0.00435	H11(3);H12(1);H13(1);H16(1);H14(1);H17(1);H18(1)
	Wu	6	3	0.600	0.00307	H11(1);H10(4);H15(1)
	Total	**23**	**9**	**0.794**	**0.00487**	
Sycoscapter sp3	SNP	**19**	**1**	**0.000**	**0.000**	H21(19)
Sycoryctes sp	CI	3	2	0.667	0.00102	H1(1);H2(2)
	JA	5	1	0.000	0.000	H1(5)
	Total	**8**	**2**	**0.429**	**0.00066**	

Table S5. Sycoryctini wasps, ITS2. Sample size (*N*), number of haplotypes (*K*), and name of haplotypes (*H*) with number of individuals.

	site	N	K	ITS2 haplotype (number of individuals)
Sycoscapter sp 1	DHS	12	1	H1(12) *Sycoscapter*
	Huo	2	1	H1(2) *Sycoscapter*
	Sand	3	1	H1(3) *Sycoscapter*
	Ding	5	1	H1(5) *Sycoscapter*
	Total	**22**	**1**	
Sycoscapter sp2	QMS	6	1	H2(6) *Sycoscapter*
	CS	1	1	H2(1) *Sycoscapter*
	CH	3	1	H2(3) *Sycoscapter*
	Wu	1	1	H2(1) *Sycoscapter*
	Total	**11**	**1**	
Sycoscapter sp 3	SNP	**3**	**1**	H3(3) *Sycoscapter*
Sycoryctes sp	CI	7	1	H1 *Sycoryctes*
	JA	11	1	H1 *Sycoryctes*
	Total	**18**	**1**	

Table S6. Cytb gene sequence differences (Kimura-2-parameter) within (diagonal) and between groups (below diagonal). Within species differences are low (highlighted in green). Within genus differences are highlighted in yellow. Note the limited sequence difference between *Philotrypesis* species.

	Philo1	Philo2	Philo3	Philo4	Sycos1	Sycos2	Sycos3	*Sycoryctes*
Philo1	0.0059							
Philo2	0.072	0.014						
Philo3	0.101	0.077	0.010					
Philo4	0.070	0.085	0.120	0.018				
Sycos1	0.177	0.175	0.183	0.204	0.019			
Sycos2	0.172	0.176	0.197	0.190	0.101	0.0058		
Sycos3	0.175	0.192	0.200	0.198	0.150	0.157	-	
Sycoryctes	0.132	0.132	0.141	0.142	0.147	0.175	0.170	0.0016

Table S7. ITS2 gene sequence difference (Kimura-2-parameter) between groups (below diagonal). Species are monomorphic. Within genus differences (highlighted in yellow) are smaller than between genus differences.

	Philo1	Philo2	Philo3	Sycos1	Sycos2	Sycos3	Sycoryctes
Philo1							
Philo2	0.036						
Philo3	0.044	0.007					
Sycos1	0.149	0.158	0.166				
Sycos2	0.167	0.194	0.203	0.090			
Sycos3	0.185	0.213	0.222	0.106	0.014		
Sycoryctes	0.243	0.242	0.243	0.184	0.222	0.242	

Table S8. *Philotrypesis* wasps, microsatellite data. Number of samples (N), number of alleles (Na), allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficients; In red, locations for which no cytb sequence is available. Additional data for sp1 and sp3 is already published (Yu et al. 2018). In black, locations for which Cytb sequence is available.

The amplification reactions were conducted using a PTC-200 thermal cycler (Bio-Rad, Hercules, CA) in 20 µl volume containing 20 ng of genomic DNA, 0.2 mM of each dNTP, 0.4 µM of fluorescent primer, 1× PCR buffer (Mg2+ free), 2.5 mM Mg2+ and 1 unit of Taq DNA polymerase (Takara, Dalian, China), using the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of (94°C, 30 s; 46 to 50°C for Sycoryctini and 47 to 52 °C for *Philotrypesis*, 60 s; 72°C, 45 s) and a final extension of 72°C for 8 min. The fragment sizes of the PCR products were determined on the ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA) using genotyper 4.0 and LIZ 500 (Applied Biosystems, Foster City, CA) as an internal size standard.

site	N	Na	Ar	Par	Ho	He	F_{IS}
Gui	12	8.3	2.87	0.40	0.704	0.754	0.057
Xiang	21	9.2	2.90	0.42	0.618	0.749	0.147
Sand	12	5.5	2.40	0.39	0.458	0.604	0.026
Nan	20	9.5	2.59	0.33	0.576	0.657	0.317
Ding	24	5.8	2.22	0.09	0.407	0.545	0.182
Wan	17	5.2	2.03	0.05	0.463	0.498	0.129
HB	1	1.8	1.8	0.05	0.833	0.417	-1.00
CH	5	4.7	2.92	0.38	0.692	0.674	0.026
Wu	2	2.0	1.80	0.14	0.500	0.354	-0.013
ST	12	6.0	2.63	0.29	0.633	0.663	0.116
QMS	5	4.5	2.67	0.12	0.658	0.657	0.002
XI	24	14.8	3.25	0.73	0.693	0.853	0.273
CS	8	6	2.79	0.10	0.658	0.725	0.142
Ning	25	6.0	2.18	0.12	0.440	0.557	0.193
Sha	20	7.5	2.40	0.20	0.523	0.613	0.153
Xia	11	5.7	2.34	0.15	0.548	0.599	0.066
Sui	24	6.8	2.30	0.15	0.485	0.593	0.188
Da	15	5.5	2.61	0.18	0.560	0.625	0.125
SNP	2	1.8	2.40	0.23	0.750	0.646	-0.178
DAL	2	2.8	2.8	0.33	0.917	0.583	-0.600

Table S9. *Sycoscapter* wasps, microsatellite data. Number of samples (N), number of alleles (Na), allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficients (F_{IS}). In black, locations for which no Cytb sequence is available. Other colors correspond to color code of main species recorded on the site according to sequence data.

The amplification reactions were conducted using a PTC-200 thermal cycler (Bio-Rad, Hercules, CA) in 20 µl volume containing 20 ng of genomic DNA, 0.2 mM of each dNTP, 0.4 µM of fluorescent primer, 1× PCR buffer (Mg2+ free), 2.5 mM Mg2+ and 1 unit of Taq DNA polymerase (Takara, Dalian, China), using the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of (94°C, 30 s; 46 to 50°C for Sycoryctini and 47 to 52 °C for *Philotrypesis*, 60 s; 72°C, 45 s) and a final extension of 72°C for 8 min. The fragment sizes of the PCR products were determined on the ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA) using genotyper 4.0 and LIZ 500 (Applied Biosystems, Foster City, CA) as an internal size standard.

Site	N	Na	Ar	PAr	Ho	He	F_{IS}
Gui	8	3.7	1.60	0.05	0.429	0.532	0.173
DHS	19	4.1	1.54	0.07	0.421	0.518	0.269
Huo	24	3.6	1.50	0.10	0.393	0.479	0.197
Xiang	21	4.0	1.47	0.12	0.362	0.469	0.174
Sand	21	3.9	1.48	0.06	0.397	0.492	0.163
Nan	9	4.3	1.63	0.08	0.410	0.597	0.290
Ding	24	5.7	1.60	0.18	0.446	0.610	0.238
Wan	17	5.6	1.67	0.16	0.705	0.665	-0.066
XI	7	4.7	1.72	0.36	0.515	0.657	0.285
QMS	6	5.1	1.74	0.21	0.500	0.694	0.296
CS	3	2.0	1.48	0.10	0.429	0.391	-0.047
CH	4	4.7	1.87	0.37	0.607	0.750	0.198
Wu	2	2.1	1.39	0.23	0.500	0.357	-0.387
SNP	14	3.4	1.36	0.97	0.285	0.315	0.193
CI	10	4.4	1.62	0.47	0.422	0.506	0.153
JA	14	4.4	1.56	0.37	0.491	0.466	-0.057

References

Jermiin LS, Crozier RH. 1994 The cytochrome-b region in the mitochondrial DNA of the ant Tetraponera rufoniger – sequence divergence in Hymenopteran may be associated with nucleotide content. *J. Mol. Evol.* **38**, 282-294.

Yu H, Liang D, Tian EW, Zheng LN, Kjellberg F. 2018 Plant geographic phenotypic variation drives diversification in its associated community of a phytophagous insect and its parasitoids. *BMC Evol. Biol.*, **18**, 134.

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

Deng Xiaoxia
CEFE: Centre d'Ecologie Fonctionnelle et Evolutive
Buatois Bruno
CEFE: Centre d'Ecologie Fonctionnelle et Evolutive
Peng Yan-Qiong
XTBG: Xishuangbanna Tropical Botanical Garden
Hui Yu (yuhui@scib.ac.cn)
South China Botanical Garden Chinese Academy of Sciences https://orcid.org/0000-0003-0074-9153
Cheng Yufen
South China Botanical Garden
Kjellberg Finn
CEFE: Centre d'Ecologie Fonctionnelle et Evolutive
Proffit Magali
CEFE

Research article

Keywords: Geographic variation, mutualism, coevolution, volatile organic compounds, Ficus

DOI: https://doi.org/10.21203/rs.3.rs-192226/v1

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 olfactive [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 tract 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 olfactive 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 ontogenic 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) -β-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 ± 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
		O	%	O	%	O	%	O	%	O	%	O	%
Fatty acid derivatives													
(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±0.05	1	n.d.	3	0.17±0.19	1	0.01±0.02	0	n.d.	6	1.41±1.16
Decanal*	1203	0	n.d.	0	n.d.	4	0.27±0.23	1	0.07±0.16	0	n.d.	0	n.d.
Total percent			0.03		0.00		0.44		0.08		0		1.41
Monoterpenes													
α-pinene*	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.
β -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±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
(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**
Linalool*	1101	0	n.d.	1	0.01±0.01	0	n.d.	0	n.d.	0	n.d.	2	0.98±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±0.16
Total percent			0.119		2.26		2.88		0.22		3.81		3.40
Sesquiterpenes													
δ-elemene*	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
α-cubebene	1355	4	3.49±3.35	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
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
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**
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
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**
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**
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
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.
(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**
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
(E)- α-bergamotene*	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
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
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
E-β-farnesene*	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
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**
γ –muurolene*	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
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**
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
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
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
α-muurolene*	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
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

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
Total percent			92.4		82.96		86.94		99.69		96.02		94.87
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.
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.
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**
Total percent			7.28		14.79		9.74				0.15		0.33
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**
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**

* 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	**0.079**								
Sui	**0.103**	0.046							
SCBG	0.022	0.02	0.02						
DHS	0.02	0.02	0.02	**0.344**					
Nan	0.012	0.02	0.014	0.045	0.02				
Ding	**0.081**	0.036	0.028	0.022	0.04	0.02			
Wan	0.023	0.012	0.02	**0.21**	**0.588**	0.014	**0.071**		
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 signaling 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 olfactive 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 ± 4, for southern locations 17 ± 4, and for the south-western location 19 ± 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 terephtalate (Nalophan®, Kalle Nalo GmbH, Wursthüllen, Germany) bag for 30 min. Then, air was pulled out of the bag (flow rate: 200 $mLmin^{-1}$) 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/µl 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 °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 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 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 (Alcanes 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.

Funding

HY was funded by the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2019ZD0408), National Natural Science Foundation of China (31630008; 31971568), and Province Natural Science Foundation of Guangdong (c20140500001306). XD was supported by a grant from the China Scholarship Council No. (2017)3109. This work was also supported by the LIA MOST (France).

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

Acknowledgements

Not applicable.

References

1. Boavida LC, Becker JD, Feijo JA. The making of gametes in higher plants. Int J Dev Biol. 2005;49(5-6):595-614.
2. Vamosi JC, Knight TM, Steets JA, Mazer SJ, Burd M, Ashman T-L. Pollination decays in biodiversity hotspots. Proc Natl Acad Sci. 2006;103(4): 956-961.
3. Morales CL, Traveset A. Interspecific pollen transfer: Magnitude, prevalence and consequences for plant fitness. CRC Crit Rev Plant Sci. 2008;27(4):221-238.
4. Ollerton J, Winfree R, Tarrant S. How many flowering plants are pollinated by animals? Oikos. 2011;120(3):321-326.
5. Sun S, Leshowitz MI, Rychtář J. The signalling game between plants and pollinators. Sci Rep. 2018;8(1):1-8.
6. Schiestl FP. Ecology and evolution of floral volatile-mediated information transfer in plants. New Phytol. 2015;206(1):571-577.
7. Schiestl FP, Dotterl S. The evolution of floral scent and olfactory preferences in pollinators: coevolution or pre-existing bias? Evol. 2012;66(7):2042-2055.
8. Thompson JN, Schwind C, Guimaraes PR, Jr., Friberg M. Diversification through multitrait evolution in a coevolving interaction. Proc Natl Acad Sci U S A. 2013;110(28):11487-11492.
9. Sayers TD, Steinbauer MJ, Farnier K, Miller RE. Dung mimicry in *Typhonium* (Araceae): explaining floral trait and pollinator divergence in a widespread species complex and a rare sister species. Bot J Linn Soc. 2020.
10. Kula AA, Dudash MR, Fenster CB. Choices and consequences of oviposition by a pollinating seed predator, *Hadena ectypa* (Noctuidae), on its host plant, *Silene stellata* (Caryophyllaceae). Am J Bot. 2013;100(6):1148-1154.
11. Gomez JM, Bosch J, Perfectti F, Fernández JD, Abdelaziz M, Camacho JPM. Spatial variation in selection on corolla shape in a generalist plant is promoted by the preference patterns of its local pollinators. Proc Biol Sci. 2008;275(1648):2241-2249.
12. Gómez JM, Zamora R. Generalization vs. specialization in the pollination system of *Hormathophylla spinosa* (Cruciferae). Ecology. 1999;80(3):796-805.
13. Ivey CT, Martinez P, Wyatt R. Variation in pollinator effectiveness in swamp milkweed, *Asclepias incarnata* (Apocynaceae). Am J Bot. 2003; 90(2):214-225.
14. Motten AF, Campbell DR, Alexander DE, Miller HL. Pollination effectiveness of specialist and generalist visitors to a North Carolina population of Claytonia virginica. Ecology 1981, 62(5):1278-1287.
15. Olsen KM. Pollination effectiveness and pollinator importance in a population of Heterotheca subaxillaris (Asteraceae). Oecologia. 1996;109(1):114-121.
16. Thomason JD, Thomson BA. Pollen presentation and viability schedules in animal-pollinated plants: consequences for reproductive success. 1992.
17. Dufaÿ M, Anstett M-C. Conflicts between plants and pollinators that reproduce within inflorescences: evolutionary variations on a theme. Oikos. 2003;100(1):3-14.
18. Dufaÿ M, Hossaert-McKey M, Anstett MC. When leaves act like flowers: how dwarf palms attract their pollinators. Ecol Lett. 2003;6(1):28-34.
19. Okamoto T, Kawakita A, Goto R, Svensson GP, Kato M. Active pollination favours sexual dimorphism in floral scent. Proc Biol Sci. 2013;280:20132280.
20. Hu R, Sun P, Yu H, Cheng Y, Wang R, Chen X, Kjellberg F. Similitudes and differences between two closely related *Ficus* species in the synthesis by the ostiole of odours attracting their host-specific pollinators: A transcriptomic based investigation. Acta Oecol. 2020;105:103554.
21. Toon A, Terry LI, Tang W, Walter GH, Cook LG. Insect pollination of cycads. *Austral Ecol.* 2020.
22. Shi T, Toda MJ, Takano KT, Yafuso M, Suwito A, Wong SY, Shang S-Q, Gao J-J. A review of taxonomy and flower-breeding ecology of the *Colocasiomyia toshiokai* species group (Diptera: Drosophilidae), with description of a new species from Indonesia. Eur J Entomol. 2019;116:341-361.
23. Moog U, Fiala B, Federle W, Maschwitz U. Thrips pollination of the dioecious ant plant *Macaranga hullettii* (Euphorbiaceae) in Southeast Asia. Am J Bot. 2002;89(1):50-59.
24. Wei ZD, Kobmoo N, Cruaud A, Kjellberg F. Genetic structure and hybridization in the species group of Ficus auriculata: can closely related sympatric *Ficus* species retain their genetic identity while sharing pollinators? Mol Ecol. 2014;23(14):3538-3550.
25. Cousins SR, Witkowski ETF. African Cycad ecology, ethnobotany and conservation: A synthesis. Bot Rev. 2017;83(2):152-194.
26. Willmott KR, Willmott JCR, Elias M, Jiggins CD. Maintaining mimicry diversity: optimal warning colour patterns differ among microhabitats in Amazonian clearwing butterflies. Proc Royal Soc B. 2017;284(1855):20170744.
27. Pellmyr O: Yuccas, yucca moths, and coevolution: a review. Ann Mo Bot Gard. 2003, 35-55.
28. Kawakita A, Kato M. Repeated independent evolution of obligate pollination mutualism in the Phyllantheae-*Epicephala* association. Proc Royal Soc B. 2009;276(1656):417-426.

29. Yu H, Tian E, Zheng L, Deng X, Cheng Y, Chen L, Wu W, Tanming W, Zhang D, Compton *SG, et al*. Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation. Mol Ecol. 2019, 28(9):2391-2405.
30. Yoder JB, Nuismer SL. When does coevolution promote diversification? Am Nat. 2010;176(6):802-817.
31. Grison-Pigé L, Bessière J-M, Hossaert-McKey M. Specific attraction of fig-pollinating wasps: role of volatile compounds released by tropical figs. J Chem Ecol. 2002;28(2):283-295.
32. Proffit M, Johnson SD. 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. 2009;75(4):771-777.
33. Proffit M, Lapeyre B, Buatois B, Deng X-X, Arnal P, Gouzerh F, Carrasco D, Hossaert-McKey M. Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction. Sci Rep.2020;10(1):1-11.
34. Soler C, Hossaert-McKey M, Buatois B, Bessiere JM, Schatz B, Proffit M. Geographic variation of floral scent in a highly specialized pollination mutualism. Phytochemistry. 2011;72:74-81.
35. Rodriguez LJ, Bain A, Chou L-S, Conchou L, Cruaud A, Gonzales R, Hossaert-McKey M, Rasplus J-Y, Tzeng H-Y, Kjellberg F. Diversification and spatial structuring in the mutualism between *Ficus septica* and its pollinating wasps in insular South East Asia. BMC Evol Biol. 2017;17(1):207.
36. Yu H, Nason JD. Nuclear and chloroplast DNA phylogeography of *Ficus hirta*: obligate pollination mutualism and constraints on range expansion in response to climate change. New Phytol. 2013;197(1):276-289.
37. Tian E, Nason JD, Machado CA, Zheng L, Yu H, Kjellberg F. Lack of genetic isolation by distance, similar genetic structuring but different demographic histories in a fig-pollinating wasp mutualism. Mol Ecol.2015;24(23):5976-5991.
38. Bain A, Borges RM, Chevallier MH, Vignes H, Kobmoo N, Peng YQ, Cruaud A, Rasplus JY, Kjellberg F, Hossaert-Mckey M. Geographic structuring into vicariant species-pairs in a wide-ranging, high-dispersal plant-insect mutualism: the case of *Ficus racemosa* and its pollinating wasps. Evol Ecol. 2016;30(4):663-684.
39. Harrison RD, Rasplus J-Y. Dispersal of fig pollinators in Asian tropical rain forests. J Trop Ecol. 2006;22:631-639.
40. Leebens-Mack J. Patterns of Genetic Structure among Populations of an Oligophagous Pollinating Yucca Moth (*Tegeticula yuccasella*). J Hered.2004;95(2):127-135.
41. Svensson GP, Michael O. Hickman J, Bartram S, Boland W, Pellmyr O, Raguso RA. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). Am J Bot. 2005;92(10):1624-1631.
42. Massey LK, Hamrick JL. Genetic diversity and population structure of *Yucca filamentosa* (Agavaceae). Am J Bot. 1998;85(3):340-345.
43. Svensson GP, Raguso RA, Flatz R, Smith CI. Floral scent of Joshua trees (*Yucca brevifolia* sensu lato): Divergence in scent profiles between species but breakdown of signal integrity in a narrow hybrid zone. Am J Bot. 2016;103(10):1793-1802.
44. Powell JA. Longest insect dormancy: Yucca moth larvae (Lepidoptera: Prodoxidae) metamorphose after 20, 25, and 30 years in diapause. Ann Entomol Soc Am. 2001;94(5):677-680.
45. Kjellberg F, Doumesche B, Bronstein JL. Longevity of a fig wasp (Blastophaga psenes). *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen Series C Biological and medical sciences*. 1988;91(7):117-122.
46. Cooper L, Bunnefeld L, Hearn J, Cook JM, Lohse K, Stone GN. Low-coverage genomic data resolve the population divergence and gene flow history of an Australian rain forest fig wasp. Mol Ecol. 2020.
47. Friberg M, Schwind C, Jr. PRG, Raguso RA, Thompson JN. Extreme diversification of floral volatiles within and among species of *Lithophragma* (Saxifragaceae). Proc Natl Acad Sci. 2019;116(10): 4406-4415.
48. Thompson JN, Rich KA. Range edges and the molecular divergence of *Greya* moth populations. J Biogeogr. 2011;38(3):551-563.
49. Raimundo RLG, Gibert JP, Hembry DH, Jr PRGe. Conflicting selection in the course of adaptive diversification: the interplay between mutualism and intraspecific competition. Am Nat. 2014;183(3):363-375.
50. Suinyuy TN, Donaldson JS, Johnson SD. Geographical matching of volatile signals and pollinator olfactory responses in a cycad brood-site mutualism. Proc Biol Sci. 2015, 282(1816):20152053.
51. Cornille A, Underhill JG, Cruaud A, Hossaert-McKey M, Johnson SD, Tolley KA, Kjellberg F, van Noort S, Proffit M. Floral volatiles, pollinator sharing and diversification in the fig-wasp mutualism: insights from *Ficus natalensis*, and its two wasp pollinators (South Africa). Proc Royal Soc B. 2012, 279(1734):1731-1739.
52. Wang G, Cannon CH, Chen J. Pollinator sharing and gene flow among closely related sympatric dioecious fig taxa. Proc Royal Soc B. 2016;283(1828):20152963.
53. Okamoto T, Kawakita A, Kato M. Interspecific variation of floral scent composition in *Glochidion* and its association with host-specific pollinating seed parasite (*Epicephala*). J Chem Ecol. 2007;33(5):1065-1081.
54. Huang D, Shi F, Chai M, Li R, Li H. Interspecific and intersexual differences in the chemical composition of floral scent in Glochidion species (Phyllanthaceae) in South China. J Chem. 2015;2015:1-14.
55. Yu H, Zhao N-X, Chen Y-Z, Deng Y, Yao J-Y, Ye H-G. Phenology and reproductive strategy of a common fig in Guangzhou. Bot Stud. 2006;47:435-441.
56. Yu H, Zhao N, Chen Y, Herre EA. Male and female reproductive success in the dioecious fig, *Ficus hirta* Vahl. in Guangdong Province, China: Implications for the relative stability of dioecy and monoecy. Symbiosis (Rehovot). 2008;45(1):121.
57. Dötterl S, Wolfe LM, Jürgens A. Qualitative and quantitative analyses of flower scent in *Silene latifolia*. Phytochemistry. 2005;66(2):203-213.

58. Hossaert-McKey M, Proffit M, Soler CC, Chen C, Bessiere JM, Schatz B, Borges RM. How to be a dioecious fig: Chemical mimicry between sexes matters only when both sexes flower synchronously. Sci Rep.2016;6(1):1-11.
59. Souto-Vilarós D, Proffit M, Buatois B, Rindos M, Sisol M, Kuyaiva T, Isua B, Michalek J, Darwell CT, Hossaert-McKey M, et al. Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. J Ecol. 2018;106(6):2256-2273.
60. Yu H, Liang D, Tian E, Zheng L, Kjellberg F. Plant geographic phenotypic variation drives diversification in its associated community of a phytophagous insect and its parasitoids. BMC Evol Biol. 2018;18(1):134.
61. Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. Carol Stream, IL: Allured publishing corporation. 2007;456:1-698.
62. Team R.C. R: A language and environment for statistical computing. 2013.
63. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, et al. Package 'vegan'.Community Ecology Package. R package version. 2013;2:1-295.
64. Hervé M, Hervé MM. Package 'RVAideMemoire'. *See https://CRAN R-project org/package= RVAideMemoire* 2020.
65. Clarke, K.R. Non-parametric multivariate analyses of changes in community structure. Aust J Ecol. 1993;117-143.

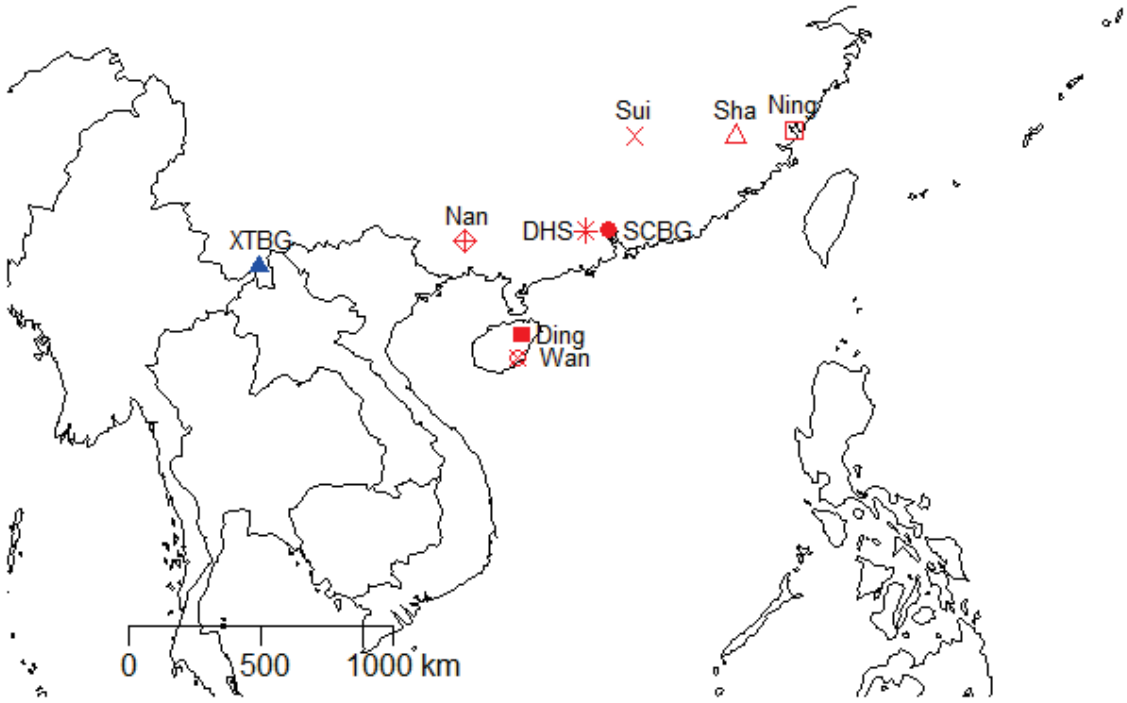


Fig. 1 Receptive fig odour collection sites. In red, locations where *F. hirta* is pollinated by *Valisia javana* sp1, in blue locations where it is pollinated by *V. javana* sp2. Locations Ding and Wan are pollinated by a distinct population of *V. javana* sp1. Map drawn with the package 'rworldmap' using the public domain Natural Earth map dataset https://naturalearthdata.com.

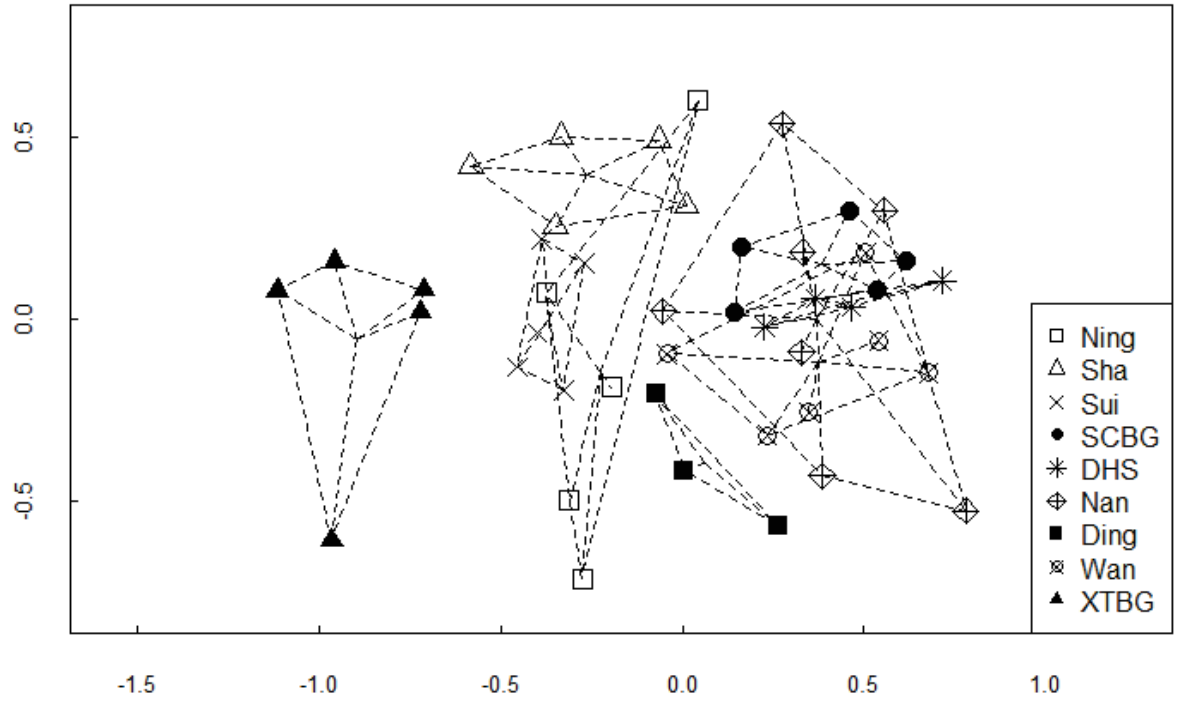


Fig.2 Non-metric multi-dimensional scaling representation of the relative proportions of VOCs in the odours emitted by individual plants based on the Bray-Curtis dissimilarity Index (stress=0.173). Three geographic groups are visualised: a south-eastern one (Ning-Sha-Sui), a southern one (SCBG-DHS-Nan-Wan)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

16 Table 1 Occurrence and relative proportion (% mean ± SD) of volatile compounds from three classes, and total amount, detected in the bouquets

17 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*	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*	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*	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*	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*	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*	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*	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

α-cubebene	1355	4	3.49±3.35	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±8.8**	**5**	**7.69±5.09**	**7**	**11.95±4.1**	**3**	**28.67±2.3**	**6**	**11.82±6.3**	**6**	**1.94±2.05**
									9				**1**		**1**		**4**		
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±10.9**	**6**	**2.91±3.03**	**3**	**2.23±0.21**	**6**	**6.05±5.54**	**0**	**n.d.**
											6								
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-	**1430**	**5**	**30.8±18.69**	**5**	**46.45±8.0**	**5**	**36.38±5.9**	**5**	**56.89±8.7**	**5**	**45.11±8.9**	**7**	**56.98±9.1**	**3**	**31±2.4**	**6**	**46.6±15.5**	**6**	**20.57±9.8**
Caryophyllene*					**9**		**9**		**1**		**4**		**3**				**8**		**7**
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-	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
bergamotene*																			
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*	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*	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±4.8**	**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**
							1												
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*	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*	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**

* 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.

Table 2 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	**0.079**								
Sui	**0.103**	0.046							
SCBG	0.022	0.02	0.02						
DHS	0.02	0.02	0.02	**0.344**					
Nan	0.012	0.02	0.014	0.045	0.02				
Ding	**0.081**	0.036	0.028	0.022	0.04	0.02			
Wan	0.023	0.012	0.02	**0.21**	**0.588**	0.014	**0.071**		
XTBG	0.022	0.021	0.02	0.02	0.02	0.012	0.03	0.03	

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

SCIENTIFIC REPORTS
nature research

Check for updates

OPEN

Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction

Magali Proffit[1] ✉, Benoit Lapeyre[1], Bruno Buatois[1], Xiaoxia Deng[1], Pierre Arnal[1], Flora Gouzerh[1,2], David Carrasco[2] & Martine Hossaert-McKey[1]

In several highly specialized plant-insect interactions, scent-mediated specificity of pollinator attraction is directed by the emission and detection of volatile organic compounds (VOCs). Although some plants engaged in such interactions emit singular compounds, others emit mixtures of VOCs commonly emitted by plants. We investigated the chemical ecological bases of host plant recognition in the nursery pollination mutualism between the dioecious *Ficus carica* and its specific pollinator *Blastophaga psenes*. Using Y-tube olfactometer tests, we show that *B. psenes* females are attracted by VOCs of receptive figs of both sexes and do not exhibit preference for VOCs of either male or female figs. Electrophysiological tests and chemical analysis revealed that of all the VOCs emitted by receptive figs, only five were found to be active on female antennae. Behavioural tests show that, in contrast to VOCs presented alone, only a blend with a particular proportion of four of these VOCs is as attractive as the odour of receptive figs, and that if there is a very small change in this blend proportion, the pollinator is no longer attracted. This study revealed that in highly specialized mutualistic interactions specificity could be mediated by a particular blend of common compounds emitted by plants.

About two-thirds of all flowering plants depend on insects for pollination[1]. Plant-pollinator encounters are mediated by the different cues plants display, generally visual and olfactory, that constitute signals for their pollinators. For instance, floral volatile organic compounds (VOCs) are generally involved in the attraction of pollinators independently of the degree of interaction specialization[2,3]. Within the complex VOCs mixtures emitted by plants, pollinators only detect a part of the compounds and use a portion of them as a signal to find their resource[2,4,5]. In specialized plant-insect interactions, partner encounter should be mediated by particular floral signals that allow pollinators to unambiguously identify their host-plants. Therefore, it has been hypothesized that scent-mediated specificity of pollinator attraction to plants is directed by the emission and detection of either i) uncommon compounds emitted by plants or ii) a blend of common compounds emitted in unique proportions[2,4,6]. For instance, the interaction between diverse groups of floral oil-secreting plants and oil-collecting bees around the world is mediated by one rare VOC, diacetin[7]. Unique compounds have also been documented as pollinator attractants in sexually deceptive orchids, for example in several orchid species of the genus *Chiloglottis*[8,9]. However, other sexually deceptive orchid species emit blends of commonly occurring hydrocarbons to attract their specific pollinators[10]. While the nature of the chemical signals responsible for the specific attraction of pollinators has been well studied in the case of sexually deceptive Australian and European orchids[9–14], there are still major gaps in our understanding of the signals involved in plant-pollinator encounter in most specialized interactions.

Nursery pollination mutualisms, in which larvae of the pollinators feed on floral tissue, are among the most specialized plant-pollinator interactions. In several of these interactions, empirical studies have pointed out the determinant role of floral VOCs for the attraction of the highly specialized and obligate pollinators[3,6,15–17]. Behavioural studies conducted in the laboratory have shown for three different nursery pollination systems that pollinators are significantly attracted by one or two major VOCs emitted by their respective host plants, which are uncommon compounds[6,16,17]. However, chemical analysis of floral VOCs indicates that not all plants involved in nursery pollination mutualisms emit rare compounds[3]. This therefore suggests that the specific attraction of the pollinator in these interactions is mediated by common plant-emitted VOCs.

In the 800 interactions between *Ficus* species (Moraceae) and their pollinating fig wasps (Hymenoptera, Chalcidoidea, Agaonidae) pollinators reproduce within the flowers of the inflorescence, *i.e.* the fig, they pollinate.

[1]CEFE, Univ Montpellier, CNRS, EPHE, IRD, Univ Paul Valéry Montpellier 3, Montpellier, France. [2]MIVEGEC, Univ Montpellier, IRD, CNRS, Montpellier, France. ✉e-mail: magali.proffit@cefe.cnrs.fr

Inflorescences of *Ficus* species emit complex species-specific mixtures of VOCs that attract specifically their pollinators[3,6,18–25]. However, except for one species[6], the composition of the chemical signal responsible for the specific attraction of pollinating fig wasps to figs of their host species is still unidentified.

Pollinators of all dioecious *Ficus* species (roughly 50% of all *Ficus* species) suffer a conflict of interest with their host plant because they cannot reproduce within female figs, which are therefore pollinated by deceit[3,26]. From a theoretical point of view, selection should favour those wasps that are able to distinguish between female and male tree figs, so that they enter solely into the latter. However, if wasps avoid female tree figs (*i.e.* no pollination), this would lead to the end of fig seed production and to a potential breakdown of the dioecious system in figs.

Different hypotheses have been proposed to explain the persistence of the mutualism in dioecious fig species and widely discussed in other papers[3,26]. One of the hypotheses proposes that pollinating fig wasps cannot choose between male and female tree figs because they are not able to differentiate between them[26,27]. Indeed, a recent study describing the VOCs emitted by several dioecious *Ficus* species revealed an apparent intersexual similarity in those species in which male and female figs are receptive to pollinators at the same time[28]. This could explain why pollinators seem unable to discriminate between male and female receptive figs[26,27]. However, appropriate behavioural bioassays to test this hypothesis are still lacking.

In the present study, we investigate the basis of plant-pollinator chemical communication in the specific interaction between the pollinating wasp, *Blastophaga psenes*, and the dioecious Mediterranean fig tree, *Ficus carica*. Volatile odour profiles of male and female receptive figs of *F. carica* have already been described[29,30]. As in most *Ficus* species, these profiles are constituted by numerous compounds (26, in the case of *F. carica*) commonly occurring in floral scents[29–31]. Based on these results, we hypothesized that receptive figs of *F. carica* emit a blend of common VOCs in a particular proportion to attract *B. psenes*. *Ficus carica* is a dioecious species that displays an unusual phenology: in summer, both sexes flower partly synchronously, whereas in spring only male trees flower (see Fig. 1). Previous chemical analyses conducted on the odour of receptive figs reported that chemical profiles of summer male figs resemble those of the co-flowering females, and are different from those of spring male figs, when female figs are absent. Based on these results we hypothesized that the pollinator-attractive blend of VOCs is similar between male and female figs in summer, so that pollinators cannot discriminate between these two types of inflorescences. In order to test our hypotheses, we combined chemical and electrophysiological analysis with behavioural tests using both natural and synthetic VOCs. We addressed the following questions: (1) Does *B. psenes* discriminate VOCs emitted by male and female receptive figs of *F. carica*? (2) Does *B. psenes* use a combination of common VOCs to find its host plant? (3) Is the proportion of the different VOCs emitted important for host plant recognition?

Results

Does the pollinator discriminate between odours of male and female receptive figs? In our olfactometer bioassays, females of *B. psenes* were significantly more attracted by VOCs released by both summer male (binomial test, $N = 38$, $P = 0.04$) and female receptive figs (binomial test, $N = 45$, $P = 0.04$), than by the control (empty container) (Fig. 2). In addition, pollinators did not show any significant preference for either summer male or female figs when these were presented at the same time on different arms of the Y-tube olfactometer (Fig. 2, binomial test, $N = 40$, $P = 0.87$).

Among the VOCs emitted by receptive figs, which compounds are active on *B. psenes* antennae? In order to identify which compounds are detected by the pollinator antennae in the complex mixture of VOCs emitted by receptive figs[29,30], we performed experiments of electroantennographic detection coupled with gas chromatography (GC-EAD). These analyses conducted on *B. psenes* revealed that antennae of the pollinator responded consistently to five compounds present in the headspace of receptive figs of *F. carica*: one shikimic compound, benzyl alcohol; and four monoterpenes, (*S*)-linalool, (*Z*)-linalool-oxide (furanoid), (*E*)-linalool-oxide (furanoid) and (*Z*)-linalool-oxide (pyranoid) (Fig. 3). Reponses to these five VOCs were confirmed with synthetic compounds. Except for (*Z*)-linalool-oxide (pyranoid), these compounds were always present in the volatile profiles of male and female receptive figs. In contrast, (*Z*)-linalool-oxide (pyranoid) was present in fewer than 30% of our samples: in two out of six samples for spring male figs, and only in one out of four samples for male and female summer figs. If a VOC is involved in pollinator attraction it should always be present in the odour emitted by receptive figs. Therefore, (*Z*)-linalool-oxide (pyranoid) was discarded from the rest of our analyses. Proportions of the four other antennal-active VOCs in the different types of figs, as well as the total quantities emitted by receptive figs, are presented in Table 1.

Is the emission of antennal-active VOCs different among the three types of figs? Emissions of the three types of figs (*i.e.* female, summer male and spring male figs) showed no significant variation in the relative proportions of the four antennal-active compounds (NMDS, Fig. 4, stress = 0.03; PERMANOVA, $F_{2,13} = 0.25$, $P = 0.12$). Pairwise comparisons pointed out homogeneity between the odour of spring males and summer males ($P = 0.19$), between spring males and females ($P = 0.10$) and between female and summer male figs ($P = 0.89$). However, the multivariate Levene's test indicated that the dispersion among the three types of figs was significantly heterogeneous ($F_{2,13} = 5.15$, $P = 0.02$). This variation was mainly due to the significant difference in dispersion between spring males and summer males ($P = 0.01$) and between spring males and females ($P = 0.005$). In contrast, the variation of scents emitted by female and summer male figs was homogeneous ($P = 0.78$). Finally, the total quantity of the four antennal-active VOCs emitted (see Table 1) was significantly different among the three types of figs ($F_{2,13} = 9.81$, $P = 0.004$). This effect was mainly due to the significant difference in the total quantity emitted by figs between spring males and summer males ($P = 0.02$) and between spring males and females ($P = 0.005$). In contrast, the variation of total scents emitted by female and summer male figs was not significantly different ($P = 0.73$).

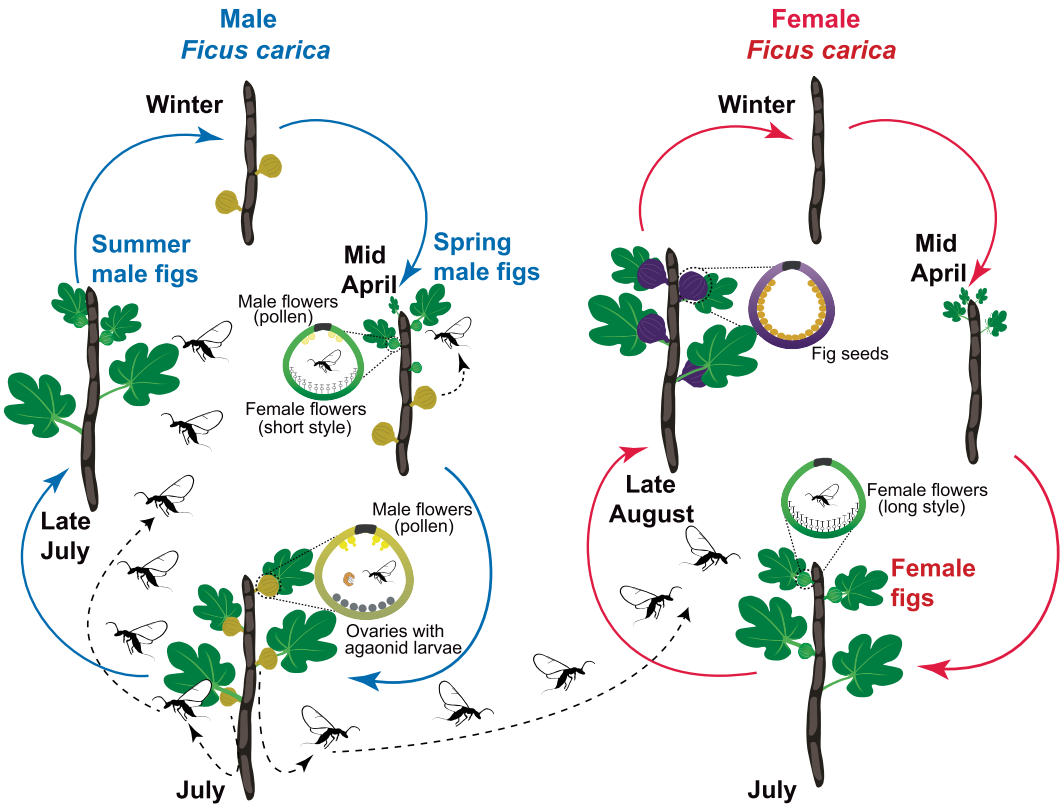


Figure 1. Life cycles of male and female tree of *Ficus carica* (respectively on the left and right side) and *Blastophaga psenes* in southern France. For each type of fig, receptive (green), ripe male (khaki) and ripe female (purple), a schematic representing wasps and flowers inside the fig is presented. Maturing male figs give rise to wasps (grey) and pollen (yellow), whereas female figs produce only seeds (orange) and contain no male flowers. Females and males of *B. psenes* are also represented in black and brown respectively. *Blastophaga psenes* has two generations per year coinciding with the flowering of male trees first in April (spring male figs) and then in July (summer male figs). In contrast, female trees flower only once a year, in July, and thus partially synchronously with summer male figs. The two distinct productions of male figs perform different functions. *Blastophaga psenes* larvae survive winter by staying in diapause within summer-produced male figs that will stay on the tree until the following spring. In spring, the overwintering wasps complete their development and male pollinators emerge in the fig cavity and copulate with female wasps before the latter emerge from their galls. After emerging from the gall within the fig cavity, female wasps exit their natal figs to enter the spring male figs, in which they oviposit. In summer, the new generation of adult female wasps, after having been fertilized, exit from their natal figs loaded with pollen grains. At this point in time, figs of male and female trees have reached receptivity, and female wasps face two scenarios: i) penetrate into figs of female trees, pollinate their flowers and then die without laying eggs due to a morphological incompatibility between the wasp ovipositor and the style of the female flowers, or ii) penetrate into figs of male trees and reproduce by laying eggs within the ovaries. Then, the cycle closes when the female offspring that entered male figs exit from them later in summer and find male figs in which they will oviposit, giving rise to the overwintering generation. This figure was prepared with the help of Jennifer McKey.

Which VOCs are responsible for pollinator attraction? In the bioassays with Y-tube olfactometers, females of *B. psenes* were significantly more attracted by the blend 1 (B1, the blend with VOC proportions are similar to the mean of the three types of receptive figs; Table 1) compared to the control (Fig. 5, binomial test, $N = 49$, $P = 0.02$), whereas wasps did not show any preference when given the choice between B1 and a female receptive fig (Fig. 5, binomial test, $N = 50$, $P = 0.48$). They were not preferentially attracted to either the racemic mixture of linalool (binomial test, $N = 44$, $P = 0.17$) or the mixture of (Z) and (E)-linalool oxide furanoid (binomial test, $N = 45$, $P = 0.16$) when compared to the control (Fig. 5). Whereas wasps were more attracted to benzyl alcohol alone compared to the control (Fig. 5, binomial test, $N = 46$, $P = 0.01$), they nonetheless preferred B1 compared to benzyl alcohol alone (Fig. 5, binomial test, $N = 52$, $P = 0.04$).

When blends were tested against an empty container (control), wasps were significantly attracted to (Fig. 6, binomial test): B1 ($N = 52$, $P = 0.04$), B6 ($N = 31$, $P < 0.001$) and B7 ($N = 40$, $P = 0.02$). None of the other blends (*i.e.* VOC proportions differing substantially from those in receptive figs; Table 1) was more attractive than the control (Fig. 6, binomial test): B2 ($N = 42$, $P = 0.24$), B3 ($N = 42$, $P = 0.46$), B4 ($N = 48$, $P = 0.39$) and B5 ($N = 41$, $P = 0.87$).

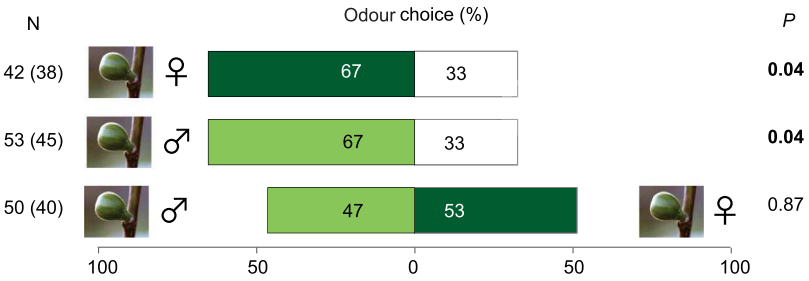


Figure 2. Attraction responses of *Blastophaga psenes* females towards VOCs of summer-male and female receptive figs. Tests were conducted in Y-tube olfactometers where females were allowed to choose between VOCs of either female or male receptive figs in one branch, and control odour in the other. In a second set of tests, female wasps could choose between odours of female and male receptive figs. Number of wasps tested (N), number of individuals that made a choice (in parentheses), and *P*-values (exact binomial test) are indicated for each comparison. Photograph by D. Delgado.

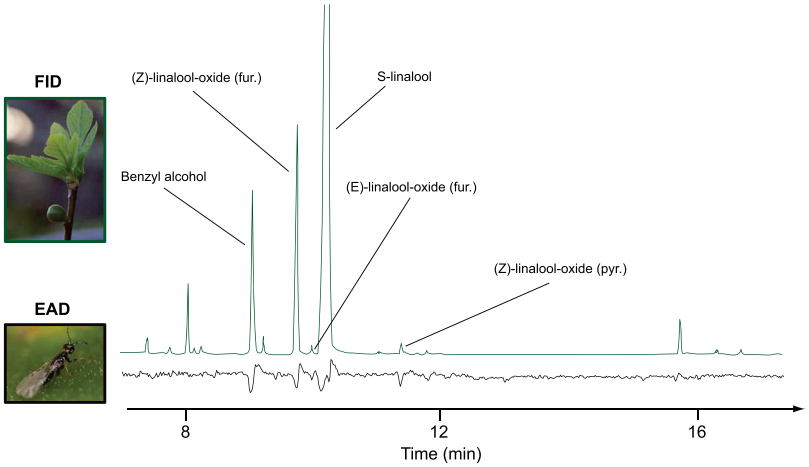


Figure 3. Example of response of antennae of *Blastophaga psenes* females to VOCs from receptive figs. Analyses were carried out using gas chromatography coupled with electroantennographic detection (GC-EAD). Bottom, antennal signal (EAD); top, GC trace (FID). Photographs by D. Delgado and F. Kjellberg.

Discussion

This study provides new insights into the chemical ecological basis of host plant recognition in interactions between figs and their pollinating fig wasps, and in other highly specialized plant-pollinator interactions. We showed that, as hypothesized, *B. psenes*, the pollinator of the Mediterranean *Ficus* species, *F. carica*: 1) does not show any preference for the odour of either male or female receptive figs in summer when they are partly co-flowering; 2) detects only five of the 26 VOCs emitted by receptive figs; 3) is attracted by the blend of four of these VOCs in a specific set of proportions.

Blastophaga psenes uses to locate its obligatory host a blend of four VOCs commonly emitted by plants [benzyl alcohol, (*S*)-linalool, (*Z*)-linalool-oxide (furanoid), and (*E*)-linalool-oxide (furanoid)]. The results of our behavioural tests revealed that the presence of these four VOCs in the blend is crucial for wasp attraction and a decrease of the proportions of benzyl alcohol or of the linalool-oxides in the blend reduces its attractiveness to the pollinator. Therefore *B. psenes* needs a particular proportion of these four common VOCs to locate its host plant. Interestingly, similar mechanisms are well known in another kind of extremely specialized interactions, sexual recognition between insects. For instance, in several moth species, females emit species-specific pheromones that attract conspecific males over long distances while inhibiting the attraction of sympatric heterospecific males[32,33]. This mechanism therefore plays a significant role in interspecific reproductive isolation. Such specificity is achieved by a combination of VOCs, generally fatty-acid compounds, emitted by females in a unique blend with particular proportions[32]. Males are attracted by this signal and a small change in the proportion of these VOCs can have a critical impact on male behaviour[34,35]. In contrast, little is known about the coding of the chemical signal mediating most plant-insect interactions. Based on studies conducted on model systems (*e.g. Drosophila melanogaster*, *Apis mellifera* or *Manduca sexta*) it has been suggested that insects in general use particular proportions of multiple VOCs to find their resources in the chemical landscape[4,36,37]. However, this hypothesis has been rarely tested empirically, as most of the studies investigating the signal mediating plant-insect interactions compare insect responses to blends of different VOCs[38–40] but rarely carry out bioassays with different proportions

	Odour Source	Benzyl alcohol[a]	(*S*)-linalool[b]	(*Z*)-linalool[c] oxide (fur.)	(*E*)-linalool[c] oxide (fur.)	Emission rate[d] (ng. min^{-1})
Figs	Spring male	7.58 ± 0.73	90.22 ± 0.64	2.14 ± 0.30	0.06 ± 0.001	115.84 ± 20.50
	Summer male	27.02 ± 15.31	70.45 ± 14.52	2.44 ± 1.34	0.08 ± 0.05	29.84 ± 17.14
	Female	33.17 ± 9.30	64.26 ± 9.75	2.49 ± 1.77	0.09 ± 0.06	7.41 ± 2.85
	Mean	20.45 ± 5.59	77.15 ± 5.53	2.33 ± 0.59	0.07 ± 0.02	60.32 ± 16.94
Synthetic compounds	Li	0	100	0	0	34.90 ± 4.00
	BA	100	0	0	0	34.43 ± 4.00
	Li Ox	0	0	50	50	20.57 ± 4.11
	B1	22.90	76.34	0.38	0.38	65.92 ± 7.98
	B2	10.53	87.72	0.88	0.88	35.80 ± 5.74
	B3	0	99.01	0.50	0.50	64.35 ± 8.73
	B4	1.94	97.09	0.49	0.49	59.08 ± 10.08
	B5	16.73	83.17	0.05	0.05	126.07 ± 6.62
	B6	18.25	78.85	1.50	1.50	119.55 ± 11.21
	B7	14.82	79.17	3.00	3.00	105.55 ± 8.66

Table 1. Composition of the odour sources used for the behavioural test. Percentages of the different volatile organic compounds (VOCs) in each odour source are presented, as well as the diffusion rate of the odour source. For the odours of receptive figs, for each odour the means are based on the measurements conducted using headspace sampling and GC-MS analyses. In addition, we present the average (±standard errors) of the three odour sources. For the synthetic compounds, the percentage indicated represents the quantity introduced in the vial and the diffusion rates are based on the measures of the weight-loss of the vial. The synthetic mixtures are as follows: Li (linalool alone), BA (benzyl alcohol alone), Li Ox [(Z) and (E)-linalool oxide furanoid] and B1-B7 (seven blends with mixtures of the four VOCs in different proportions). [a]Benzyl alcohol (Fluka, CAS Number: 100-51-6; purity: >99.5). [2](S)-linalool and (R)-linalool racemic (Fluka, Racemic mixture CAS Number: 78-70-6; purity: ~97%). [c]Li Ox: (Z) and (E)-linalool oxide furanoid (Fluka, CAS Number: 68780-91-6; purity: >97%). [d]For the figs, this value corresponds to emission rate per fig.

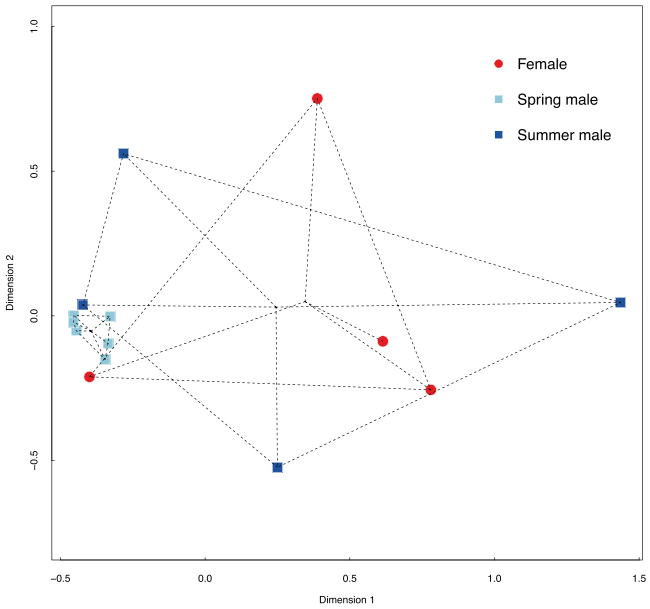


Figure 4. Non-metric multi-dimensional scaling of the relative proportions of VOCs in the global bouquets emitted by the three types of receptive figs of *Ficus carica* based on Bray-Curtis dissimilarity index (stress = 0.03). Male trees flower twice a year, in spring (spring male figs ■) and in summer (summer male figs ■), and females only during summer (females ●). Samples are grouped (dashed lines) by type of fig, and the centroid of each group is indicated.

of these VOCs in the blend[41,42]. A major improvement in the study of signals mediating plant-insect interactions would come if attraction tests with different proportions of VOCs were conducted, as has been done to test conspecific recognition in moths[34,35], in order to characterize the extent to which the proportions of the different constituents could be changed without affecting insect attraction. In addition, in contrast to what we have done in the current study, in almost all previous studies that have tested blends of VOCs, there is a lack of measurement of

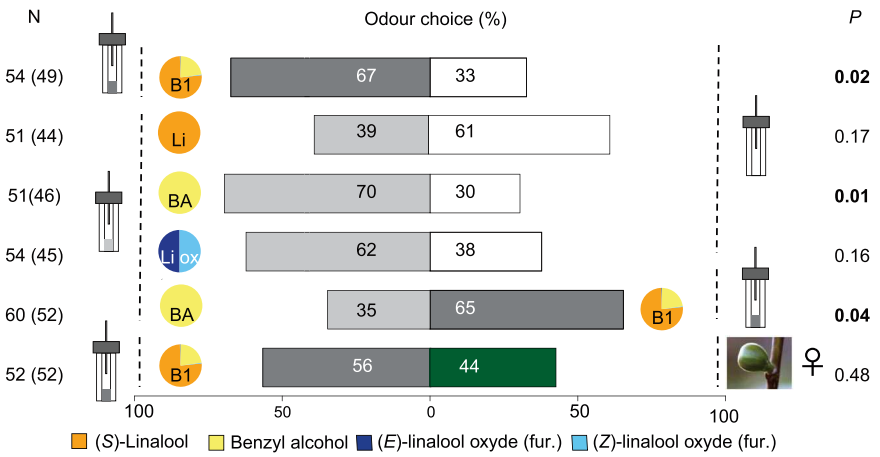


Figure 5. Attraction responses of *Blastophaga psenes* females towards different VOCs alone or in blends. Tests were conducted in Y-tube olfactometers in which females were allowed to choose between synthetic versions of the four VOCs (alone or in a blend) and control odour. In a second set of tests, female wasps could select between a blend of the four VOCs and benzyl alcohol alone, or, a blend of the four VOCs and odour of female receptive figs. For the four VOCs detected by the pollinator, proportions of each in each odour source are indicated in the pie chart and in more details in Table 1. Number of wasps tested (N), number of individuals that made a choice in parentheses, and *P*-values (exact binomial test) are indicated for each comparison.

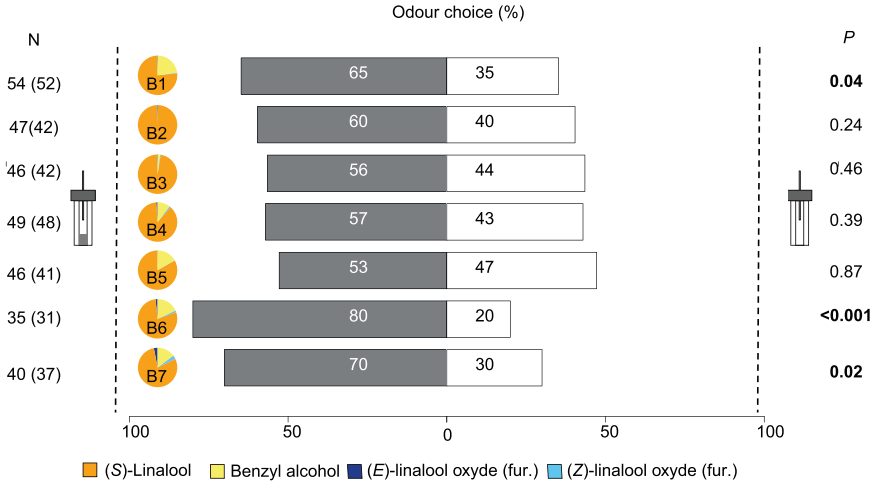


Figure 6. Attraction responses of *Blastophaga psenes* females towards blends with different proportions of the three VOCs. Tests were conducted in Y-tube olfactometers in which female wasps were allowed to choose between synthetic versions of the four VOCs in different proportions, in one branch, and control odour in the other. For the four VOCs detected by the pollinating females, proportions in each odour source are indicated in the pie chart and in Table 1. Number of wasps tested (N), number of individuals that made a choice (in parentheses), and *P*-values (exact binomial test) are indicated for each comparison.

the diffusion rate of the different compounds and of the stability of their proportions in the resultant signal. VOCs have considerably different evaporation rates[43] and as a consequence, in mixtures of different VOCs, their proportions diffused in the odour source should be very different from their proportions initially applied in the diffuser.

Our results indicate that a very small change in the proportions of the four VOCs could impede the attraction of females of *B. psenes*. Scent emitted by flowers has conventionally been viewed as a trait that is highly variable within one species, based on genetic drift, environmental constraints or selection mediated by pollinators or other agents[44,45]. However, in the case of extremely specialized plant-pollinator interactions, the fitness of both partners is strongly dependent on the maintenance of the interaction. Thus, it is expected that the chemical signal responsible for pollinator attraction should be under strong stabilizing selection[12]. To our knowledge, *B. psenes* is the sole pollinator of *F. carica* throughout its distribution. Whereas there should be strong selection to conserve the proportions of the VOCs emitted by *F. carica* receptive figs that contribute to attraction of *B. psenes*, we should observe more variation in the proportions of those VOCs that do not contribute to attraction, as has

been previously reported for some orchid species[12]. Collections and analyses of the VOCs emitted by different populations of *F. carica* would be required in order to test this hypothesis.

Blastophaga psenes is not preferentially attracted by the VOCs emitted by summer male figs compared to those emitted by female figs. To our knowledge, this is the first study that demonstrates clearly that a species of pollinating fig wasp associated with dioecious figs does not show any preference for the VOCs emitted by male figs of its host compared to those emitted by conspecific deceptive female figs. Although our experiments do not allow affirming whether wasps can or cannot differentiate male from female figs, they reveal that *B. psenes* individuals do not prefer one to the other. These results are consistent with the hypothesis that intersexual chemical mimicry of odours of male and rewardless female figs prevents choice by pollinators when they are partly co-flowering during summer[3,26–28].

A previous study analysing the overall scent of receptive figs of *F. carica* reported a significant difference in the relative proportions of all the VOCs emitted by spring male figs compared to summer figs, and more specifically an increase in the relative proportions of several sesquiterpenes in summer[29]. Nonetheless, that study did not investigate whether such a difference exists also for the specific VOCs used by the pollinator as signal. Our study reveals that the mean relative proportions of the four VOCs used by the pollinator (one shikimic compound and four monoterpenes) are not significantly different among the three types of figs. Contrary to the quantity of sesquiterpenes in the overall fig scent[29], these four VOCs are emitted in much higher quantities in spring figs than in summer figs (16 times more than in female figs and 4 times more than in male figs). These inter-seasonal differences in the total quantity of VOCs, both those involved in the attraction of the pollinator and others, could be driven by abiotic or biotic factors, or by the two combined. Indeed, it is well established that environmental conditions, particularly temperature, can affect VOC emission by plants[46,47]. On the one hand, owing to the physicochemical properties of plant VOCs, warming increases the rates of total emissions of VOCs in plants[44,46]. However, this cannot explain the variation in our study, as temperatures in the Mediterranean region are higher in summer than in spring. On the other hand, in response to variable temperature conditions, floral physiology can modify the biosynthetic activity of terpene synthases to regulate the emission of each floral compound, or of multiple compounds simultaneously, depending on synthase specificity[48]. As a consequence, in addition to affecting the overall quantity of VOCs emitted by flowers, temperature can also change the proportions of the compounds that constitute floral scents. For instance, in a community of Mediterranean plants, a species-specific optimum temperature has been reported in the emission of VOCs by flowers, and temperature also affects the relative proportions of different compounds[49]. Another possible explanation for the observed inter-seasonal variation is that it results from pollinator-mediated selection acting differently on floral scent in spring and summer figs. Indeed, pollinator density is considerably lower in spring compared to summer[30,50], leading to greater competition between individual trees for access to pollinators in spring than in summer. As a consequence, selection should favour male fig phenotypes that emit a larger amount of the VOCs to attract pollinators in spring, when pollinators are a more strongly limiting resource. In other plant-pollinator interactions, selection to increase the emission of VOCs detected by pollinators has been reported[51]. In summer, pollinator-mediated selection in relation to VOCs emission by male figs is expected to be different, because (i) pollinator density is higher than in spring and (ii) summer figs are partly co-flowering with rewardless female figs. Each sex may be under vicarious selection, *i.e.* selection acts on each sex to resemble the other, in order to prevent the breakdown of the interaction. Because intersexual mimicry benefits pollination and assures seed production (but see[28,29]), we do not expect that selection will favour an increase in the emission of VOCs by male figs during summer.

Coding of the signal responsible for plant-pollinator encounters has been elucidated in only a limited number of other obligate nursery pollination mutualisms[6,16,17]. The present study reveals that females of *B. psenes* do not use a rare compound to localize the host plant but a specific blend of common VOCs in very particular set of proportions. The wasp family Agaonidae is estimated to include more than 1000 species[52]. Each species of Agaonidae is associated with one or very few *Ficus* spp. and is specifically attracted by the VOCs emitted by the receptive figs of its host(s)[3,6,18,20,21,23]. Chemical analyses conducted so far on more than 30 *Ficus* spp. have revealed that species of this genus emit species-specific mixtures of VOCs that are usually commonly emitted by flowers[3,18,21,23–25,28]. For instance, more than 50% of *Ficus* species emit the monoterpenes (E)-β-ocimene and linalool or the sequiterpenes α-copaene, α-humulene or germacrene D, which are very commonly emitted by plants[3,18,21,23–25,28,31]. Therefore, we can expect that, similarly to *B. psenes*, the majority of species of Agaonidae use a particular combination of common VOCs in unique proportions, and not uncommon compounds, to localize their host plant. Studies similar to the present one, combining chemical and electrophysiological analysis with behavioural tests, should be conducted in the future in order to establish if our findings can be generalized, not only to interactions between other figs and their pollinating fig wasps, but also to other nursery pollination mutualisms.

Materials and Methods

Study system. This study was carried out in the region of Montpellier, southern France, with insects from natural populations collected in fig trees present at the CEFE ("Centre d'Ecologie Fonctionnelle et Evolutive") experimental garden (43°38′19″N, 3°51′49″E) in Montpellier, France, and from natural populations less than 40 km distant from Montpellier.

Our model system is the mutualistic interaction between the agaonid *Blastophaga psenes* L. (Hymenoptera, Chalcidoidea, Agaonidae) and its exclusive host, the Mediterranean *Ficus* species, *i.e. Ficus carica* (subgenus *Ficus*, section *Ficus*, subsection *Ficus*). This pollinating fig wasp species has two generations per year coinciding with the flowering of male trees first in April ("spring male figs") and then in July ("summer male figs") (Fig. 1). In contrast, female trees flower only once a year, in July, and thus partially synchronously with summer male figs (Fig. 1). Detailed life cycles of both *Blastophaga psenes* and *Ficus carica* are presented in Fig. 1 and in[50,53].

Preparation of odour sources. As for all the analysis and behavioural tests, odour preparations were conducted at the "Platform for Chemical Analyses in Ecology" (PACE, Montpellier) technical facilities of the LabEx CeMEB ("Centre Méditerranéen pour l'Environnement et la Biodiversité", Montpellier, France). For the GC-EAD recordings, VOCs of receptive summer male figs and female figs were collected using classical adsorption-desorption headspace technique[30,54,55]. Three groups of 20 to 30 receptive figs were collected haphazardly from both male and female trees and directly enclosed in polyethylene terephthalate bags. For each bag, traps containing 30 mg of Alltech Super Q adsorbent (ARS Inc., Gainesville, FL, USA) were placed at the end of tube from which air was drawn in. Airflow was maintained through the bags by two pumps (KNF, Neuberger, Freiburg, Germany). Air pushed into the bag by a polytetrafluoroethylene (PTFE) tube was filtered using activated charcoal. The entrance and exit flow rates were regulated by flowmeters at 300 and 200 ml·min^{-1}, respectively, to create a positive pressure inside the bag and thereby prevent contamination from the environment. The collection duration was 4 h. Each trap was eluted with 150 µl of hexane (Sigma Aldrich, Munich, Germany, purity >99%) and the three extracted samples were pooled together and stored at −20 °C. In addition to these odour samples, synthetic compounds were used during the electrophysiological recordings to validate the antennal responses.

For the behavioural tests, summer male figs, female figs, and synthetic compounds, singly or in blends, were used. Fresh male and female figs were collected in the field and used within 2 h after collection for the behavioural tests. For these bioassays, a single fig was used for each test. Furthermore, four synthetic compounds were chosen based on their activity on insect antennae (see below) and their constant occurrence in the volatile profile of receptive figs of *F. carica*[29,30]. With these compounds, seven blends were prepared with different compound proportions (Table 1). The composition of the blend 1 (B1) was based on the average proportion and quantities of the four synthetic compounds emitted by the three types of figs (Table 1), with the constraint that some VOCs were not available commercially as pure compound. In order to investigate the importance for pollinator attraction of each compound in the blend, in the following six other blends we reduced or increased the proportions of the different VOCs. To deliver the odours in the experimental set-up (Supplementary, Fig. S1), pure synthetic compounds were added into a glass insert of 400 µl, which was placed into a 1.5 ml vial sealed with a polytetrafluoroethylene/rubber septum (Chromoptic, Courtaboeuf, France). A micro-capillary tube made of fused silica (Agilent technologies, Redmond, USA), 40 mm long and 0.53 mm internal diameter (ID), was inserted through the septum. The diameter and length of the capillary tube were calibrated to release a controlled amount of VOCs corresponding to the mean release rates of one receptive fig. For that, vials were positioned inside glass containers of 500 ml and a diameter of 100 mm that were connected to a continuous airflow of 200 ml.min^{-1} and maintained inside an oven at 25 °C. Vials were weighed regularly during 2 months using a microbalance (MC5, Sartorius, Goettingen, Germany) to determine the diffusion rate (ng.min^{-1}). Preliminary tests revealed that diffusion of the VOCs using these dispensers is only stable after 10 days (Proffit *et al.*, *unpublished data*).

Electrophysiology on *B. psenes*. GC-EAD recordings were conducted on a gas chromatograph-flame ionization detector (GC-FID, CP-3800, Varian, Palo Alto, USA) equipped with an optima 5-MS capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness, Machery-Nagel, Düren, Germany) coupled to an electroantennography detector setup (EAD, Syntech IDAC-2, Kirchzarten, Germany). Four µl of either receptive fig odour or synthetic mix solution were injected into the GC-FID. The injector was heated to 250 °C, with a 1:4 split ratio to inject the compounds into the column. Oven temperature was held at 50 °C for 1 minute, increased from 50 °C to 100 °C at a rate of 9 °C.min^{-1} then from 100 to 140 °C at a rate of 8.1 °C.min^{-1}, then from 140 °C to 190 °C at a rate of 7.2 °C min^{-1}, then from 190 °C to 210 °C at a rate of 20 °C.min^{-1} and finally the temperature was held at 210 °C during 50 seconds. The carrier gas used was helium at 1 ml·min^{-1}. The effluent was split equally into two deactivated fused silica capillary columns (100 cm × 0.25 mm), one leading to the FID (270 °C) and one into a heated EAD port (200 °C) (transfer line, Syntech, Kirchzarten, Germany). For the EAD, wasp heads were cut at their base. Head base and the tip of one antenna were mounted between two glass capillary tubes filled with insect Ringer solution (6.0 g·l^{-1} NaCl, 0.4 g·l^{-1}, KCl, 0.27 g·l^{-1}, $CaCl_2$ and 3.20 g·l^{-1} of sodium lactate) and connected to silver wires. Electrophysiological measurements were conducted separately with the antennae of seven adult female wasps for each odour source tested. A compound was considered to be EAD-active when it elicited an unequivocal depolarization response in four antennae out of seven. In addition, the activity of VOCs on pollinator antennae was confirmed using synthetic standards.

Volatile collections and chemical analysis. Headspace collections of VOCs of spring male (N = 6), summer male and female receptive figs (N = 4 for each), and of all the dispensers used for the behavioural test were conducted. For these collections, either receptive figs or an odour dispenser were placed into a glass container of 500 ml for 30 minutes before collection. Air pushed into the glass container was filtered using activated charcoal at a flow rate of 200 ml·min^{1}. The same amount of air was drawn out of the container through an adsorbent trap compatible with a thermal desorption system, consisting of an external glass tube (length: 60 mm and 6 mm O.D., Gerstell, Mulheim, Germany) filled with 80 mg of Tenax-TA and 40 mg of Carbotrap (60–80 and 20–40 mesh respectively, Sigma-Aldrich, Munich, Germany). Odour collections lasted 10 min for the synthetic compounds and 30 min for the figs. All adsorbent traps were sealed with lids on both side openings and stored at −20 °C until further use.

Chemical analyses were conducted using a method similar to that of Souto-Vilarós *et al.*[18]. We used a gas chromatograph (GC, Trace 1310, Thermo Scientific, Milan, Italy) coupled to a mass spectrometer (ISQ QD Single Quadrupole, Thermo Scientific, Milan, Italy). The column used was an Optima 5-MS capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness, Machery-Nagel, Düren, Germany). Absorbent traps were handled with a Multi Purpose Sampler (Gerstell, Mülheim, Germany) and desorbed with a double stage desorption system, composed of a Thermal Desorption Unit (TDU) and a Cold Injection System (CIS) (Gerstell, Mülheim, Germany).

First, the injector was 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. We used helium at 1 ml·min^{-1} as a carrier gas. Oven temperature was held at 40 °C for 3 minutes, increased from 40 °C to 220 °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 minutes at 250 °C. The temperature of the transfer line and the ion source of the mass spectrometer was 250 °C and 200 °C respectively. The acquisition was at 70 eV ionisation energy, from 38 m/z to 350 m/z. We used Xcalibur TM 266 software (Thermo Scientifc TM, Milan, Italy) for data processing. Retention times of a series of *n*-alkanes (Alkanes standard solution, 04070, Sigma Aldrich, Munich, Germany) were used to convert retention times into a retention index. Compound identification was based on computer matching of mass spectra and retention indices with reference compounds. In addition, to quantify precisely the emission rate of the VOCs detected by the pollinator in each sample, known amounts of these different reference compounds (100, 50, 20 and 2 ng) were injected into an adsorbent trap and analysed in the GC-MS system using the same method. Their mean peak areas were used for calibration. In addition, the stereochemistry of linalool was determined using the same analytic method as indicated above in the same GC-MS equipped with a β-cyclodextrin chiral capillary column (Cyclosil-B, 30 m–0.25 mm i.d., 0.25 μm film thickness, Agilent J&W columns, USA).

Behavioural experiments. We tested whether females of *B. psenes* were attracted to VOCs emitted by receptive figs of *F. carica* and by several synthetic compounds, using a glass Y-tube olfactometer in which only chemical cues were presented to wasps (Supplementary, Fig. S1)[6,18,23]. Bioassays were conducted from 2016 to 2019 under laboratory conditions at the temperature of 25.75 ± 0.10 °C and relative humidity of 51.52 ± 1.60%. Y-tubes were in glass (40 mm in diameter), each lateral arm was 200 mm long and the central arm was 150 mm long. Odour sources were placed in glass containers of 500 ml and connected to each lateral arm of the Y-tube. Air was purified with activated charcoal, humidified with distilled water and blown into the glass containers (200 ml·min^{-1} per arm). Y-tubes and glass containers were changed and cleaned with acetone after each trial in order to remove any chemical traces left by the insects. To avoid a directional bias, the positions of odour sources were inverted between the two arms in each successive trial. Treatments were alternated to be able to compare wasp choice frequencies between treatments. For the bioassays, between 42 and 60 fig wasps were tested per treatment. Air was blown for 1 min prior to insect introduction into the olfactometer. During ten minutes, the choice made by each individual was recorded. We considered that wasps did not choose when they stayed motionless in the departure section and/or the central arm before the bifurcation of the olfactometer after these ten min. These wasps were not taken into account in the statistical analyses.

Newly emerging adult female wasps were collected from mature figs taken haphazardly from different individual male trees. Because of their very short lifespan outside the fig (less than 24 h), individuals of *B. psenes* were tested shortly after their exit. Each day a maximum of 25 individuals were tested per treatment. All tested wasps were naïve to the odour sources presented in our bioassays.

Data analysis. All the data analyses were performed in R (v. 3.5.3; R Development Core Team; http://www.R-project.org) using multivariate analysis incorporated in the Vegan package[56]. As variation in the relative proportions of all the VOCs emitted by receptive figs of *F. carica* was compared in a previous study[29], we only focused on comparison among the three types of receptive figs of relative proportions of the four VOCs used by the pollinator. Data were standardized before the analyses and a data matrix of pairwise Bray-Curtis dissimilarity indices between samples was built. Non-metric multi-dimensional scaling (NMDS) was used to visualize similarities among the samples by finding the best two-dimensional representation of the distance matrix. A Permutational Multivariate Analysis of Variance (PERMANOVA) based on 999 permutations was used to test the null hypothesis of no centroid (*i.e.* mean) difference in on the relative proportions of the four VOCs among the three different types of figs, as well as in pairwise comparisons. The difference of dispersion (*i.e.* variance) in the relative proportions of these VOCs among the three different types of figs, and also in pairwise comparisons, was tested using a multivariate analogue of Levene′s test for homogeneity of variance. Finally, we performed an ANOVA to compare the total amounts of VOCs emitted by receptive figs of the three types. For pairwise comparisons, *P*-values were adjusted for multiple comparisons using the FDR method[57,52].

For all dual-choice bioassays in the Y-tube olfactometer, two-tailed exact binomial tests were used to test the null hypothesis that the same number of wasps was attracted to both odour sources.

Received: 7 February 2020; Accepted: 14 May 2020;
Published: 22 June 2020

References

1. Klein, A. M. *et al.* Importance of pollinators in changing landscapes for world crops. *Proc. R. Soc. B Biol. Sci.* **274**, 303–313 (2007).
2. Raguso, R. A. Wake up and smell the roses: the ecology and evolution of floral scent. *Annu. Rev. Ecol. Evol. Syst.* **39**, 549–569 (2008).
3. Hossaert-McKey, M., Soler, C., Schatz, B. & Proffit, M. Floral scents: their roles in nursery pollination mutualisms. *Chemoecology* **20**, 75–88 (2010).
4. Bruce, T. J. A., Wadhams, L. J. & Woodcock, C. M. Insect host location: a volatile situation. *Trends Plant Sci.* **10**, 269–274 (2005).
5. Milet-Pinheiro, P. *et al.* The chemical basis of host-plant recognition in a specialized bee pollinator. *J. Chem. Ecol.* **39**, 1347–1360 (2013).
6. Chen, C. *et al.* Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*. *Funct. Ecol.* **23**, 941–950 (2009).
7. Schäffler, I. *et al.* Diacetin, a reliable cue and private communication channel in a specialized pollination system. *Sci. Rep.* **5**, 12779, https://doi.org/10.1038/srep12779 (2015).
8. Franke, S. *et al.* The discovery of 2,5-dialkylcyclohexan-1,3-diones as a new class of natural products. *Proc. Natl. Acad. Sci.* **106**, 8877–8882 (2009).
9. Schiestl, F. P. *et al.* The chemistry of sexual deception in an orchid-wasp pollination system. *Science* **302**, 437–438 (2003).

10. Schiestl, F. P. *et al*. Orchid pollination by sexual swindle. *Nature* **399**, 421–422 (1999).
11. Ayasse, M., Schiestl, F. P., Paulus, H. F., Ibarra, F. & Francke, W. Pollinator attraction in a sexually deceptive orchid by means of unconventional chemicals. *Proc. R. Soc. B Biol. Sci.* **270**, 517–522 (2003).
12. Mant, J., Peakall, R. & Schiestl, F. P. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? *Evolution* **59**, 1449–1463 (2005).
13. Peakall, R. & Whitehead, M. R. Floral odour chemistry defines species boundaries and underpins strong reproductive isolation in sexually deceptive orchids. *Ann. Bot.* **113**, 341–355 (2014).
14. Cuervo, M., Rakosy, D., Martel, C., Schulz, S. & Ayasse, M. Sexual deception in the *Eucera*-pollinated *Ophrys leochroma*: a chemical intermediate between wasp- and *Andrena*-pollinated species. *J. Chem. Ecol.* **43**, 469–479 (2017).
15. Dötterl, S. *et al*. Nursery pollination by a moth in *Silene latifolia*: the role of odours in eliciting antennal and behavioural responses. *New Phytol.* **169**, 707–718 (2006).
16. Svensson, G. P., Okamoto, T., Kawakita, A., Goto, R. & Kato, M. Chemical ecology of obligate pollination mutualisms: testing the 'private channel' hypothesis in the *Breynia-Epicephala* association. *New Phytol.* **186**, 995–1004 (2010).
17. Song, B. *et al*. A new pollinating seed-consuming mutualism between *Rheum nobile* and a fly fungus gnat, *Bradysia* sp., involving pollinator attraction by a specific floral compound. *New Phytol.* **203**, 1109–1118 (2014).
18. Souto-Vilarós, D. *et al*. Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. *J. Ecol.* **106**, 2256–2273 (2018).
19. Gu, D., Yang, D.-R., Yang, P., Peng, Y.-Q. & Wang, Z.-J. Work division of floral scent compounds in mediating pollinator behaviours. *Chem. Ecol.* **32**, 733–741 (2016).
20. Chen, C. & Song, Q. Responses of the pollinating wasp *Ceratosolen solmsi marchali* to odor variation between two floral stages of *Ficus hispida*. *J. Chem. Ecol.* **34**, 1536–1544 (2008).
21. Wang, G., Cannon, C. H. & Chen, J. Pollinator sharing and gene flow among closely related sympatric dioecious fig taxa. *Proc. R. Soc. B Biol. Sci.* **283**, 20152963, https://doi.org/10.1098/rspb.2015.2963 (2016).
22. Proffit, M., Bessière, J.-M., Schatz, B. & Hossaert-McKey, M. Can fine-scale post-pollination variation of fig volatile compounds explain some steps of the temporal succession of fig wasps associated with *Ficus racemosa*? *Acta Oecologica* **90**, 81–90 (2018).
23. Proffit, M. *et al*. 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 (2009).
24. Grison-Pigé, L., Hossaert-McKey, M., Greeff, J. M. & Bessière, J.-M. Fig volatile compounds-a first comparative study. *Phytochemistry* **61**, 61–71 (2002).
25. Proffit, M. & Johnson, S. D. 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. South Afr. J. Bot.* **75**, 771–777 (2009).
26. Patel, A., Anstett, M.-C., Hossaert-McKey, M. & Kjellberg, F. Pollinators entering female dioecious figs: why commit suicide? *J. Evol. Biol.* **8**, 301–313 (1995).
27. Grafen, A. & Godfray, H. C. J. Vicarious selection explains some paradoxes in dioecious fig pollinator systems. *Proc. R. Soc. B Biol. Sci*. **245**, 73–76.
28. Hossaert-Mckey, M. *et al*. How to be a dioecious fig: chemical mimicry between sexes matters only when both sexes flower synchronously. *Sci. Rep.* **6**, 21236, https://doi.org/10.1038/srep212361-11 (2016).
29. Soler, C. C. L., Proffit, M., Bessière, J.-M., Hossaert-McKey, M. & Schatz, B. Evidence for intersexual chemical mimicry in a dioecious plant. *Ecol. Lett.* **15**, 978–985 (2012).
30. Grison Pigé, L. *et al*. Limited intersex mimicry of floral odour in *Ficus carica*. *Funct. Ecol.* **15**, 551–558 (2001).
31. Knudsen, J. T., Eriksson, R., Gershenzon, J. & Stahl, B. Diversity and distribution of floral scent. *Bot. Rev.* **72**, 1–120 (2006).
32. Cardé, R. T. & Haynes, K. F. Structure of the pheromone communication channel in moths. in *Advances in insect chemical ecology* (eds. Cardé, R. T. & Millar, J. G.) 283–332 (Cambridge University Press, 2004).
33. Smadja, C. & Butlin, R. K. On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity* **102**, 77–97 (2008).
34. Löfstedt, C. & van der Pers, J. N. C. Sex pheromones and reproductive isolation in four european small ermine moths. *J. Chem. Ecol.* **11**, 649–666 (1985).
35. Mazor, M. & Dunkelblum, E. Role of sex-pheromone components in behavioral reproductive isolation between *Autographa gamma* (L.) and either *Trichoplusia* (Hübner) or *Chrysodeixis chalcites* (Esp) (Lepidoptera, Noctuidae, Plusinae). *J. Chem. Ecol.* **18**, 2373–2384 (1992).
36. de Bruyne, M. & Baker, T. C. Odor detection in insects: volatile codes. *J. Chem. Ecol.* **34**, 882–897 (2008).
37. Andersson, M. N., Löfstedt, C. & Newcomb, R. D. Insect olfaction and the evolution of receptor tuning. *Front. Ecol. Evol.* **3**, 691 (2015).
38. Tasin, M. *et al*. Attraction of female grapevine moth to common and specific olfactory cues from 2 host plants. *Chem. Senses* **35**, 57–64 (2009).
39. Shiojiri, K. *et al*. Herbivore-specific, density-dependent induction of plant volatiles: honest or "cry wolf" signals? *PLoS One* **5**, e12161 (2010).
40. Borrero-Echeverry, F. *et al*. Flight attraction of *Spodoptera littoralis* (Lepidoptera, Noctuidae) to cotton headspace and synthetic volatile blends. *Front. Ecol. Evol.* **3**, e00421 (2015).
41. Beyaert, I. *et al*. Relevance of resource-indicating key volatiles and habitat odour for insect orientation. *Anim. Behav.* **79**, 1077–1086 (2010).
42. Najar-Rodriguez, A. J., Galizia, C. G., Stierle, J. & Dorn, S. Behavioural and neurophysiological responses of an insect to changing ratios of constituents in host plant-derived volatile mixtures. *J. Exp. Biol.* **214**, 162–162 (2011).
43. Andersson, M. N., Schlyter, F., Hill, S. R. & Dekker, T. What reaches the antenna? How to calibrate odor flux and ligand-receptor affinities. *Chem. Senses* **37**, 403–420 (2012).
44. Farré-Armengol, G., Filella, I., Llusia, J. & Peñuelas, J. Floral volatile organic compounds: Between attraction and deterrence of visitors under global change. *J. PPEES Sources* **15**, 56–67 (2013).
45. Delle-Vedove, R., Schatz, B. & Dufaÿ, M. Understanding intraspecific variation of floral scent in light of evolutionary ecology. *Ann. Bot.* **120**, 1–20 (2017).
46. Peñuelas, J. & Staudt, M. BVOCs and global change. *Trends Plant Sci.* **15**, 133–144 (2010).
47. Borghi, M., Perez de Souza, L., Yoshida, T. & Fernie, A. R. Flowers and climate change: a metabolic perspective. *New Phytol.* **224**, 1425–1441 (2019).
48. Pichersky, E., Noel, J. P. & Dudareva, N. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science* **311**, 808–811 (2006).
49. Farré-Armengol, G., Filella, I., Llusia, J., Niinemets, Ü. & Peñuelas, J. Changes in floral bouquets from compound-specific responses to increasing temperatures. *Glob. Change Biol.* **20**, 3660–3669 (2014).
50. Kjellberg, F., Gouyon, P.-H., Ibrahim, M., Raymond, M. & Valdeyron, G. The stability of the symbiosis between dioecious figs and their pollinators: a study of *Ficus carica* L. and *Blastophaga psenes* L. *Evolution* **41**, 693–704 (1987).
51. Parachnowitsch, A. L., Raguso, R. A. & Kessler, A. Phenotypic selection to increase floral scent emission, but not flower size or colour in bee-pollinated. *Penstemon digitalis. New Phytol.* **195**, 667–675 (2012).
52. Lopez-Vaamonde, C. *et al*. Molecular dating and biogeography of fig-pollinating wasps. *Mol. Phylogenet. Evol.* **52**, 715–726 (2009).
53. Kjellberg, F. & Lesne, A. *Ficus carica* and its pollination, https://hal.archives-ouvertes.fr/hal-02516888 (2020).

54. Tholl, D. *et al*. Practical approaches to plant volatile analysis. *Plant J.* **45**, 540–560 (2006).
55. Proffit, M. *et al*. 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 (2008).
56. Oksanen, J. *et al. vegan: Community Ecology Package* (2019).
57. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* (1995).

Acknowledgements
We thank Nicolas Barthes for help during the chemical analysis conducted at the PACE [LabEx CEMEB, the French National Research Agency (ANR) under the "Investissements d'avenir"], Finn Kjellberg and Rumsais Blatrix for providing figs, Candice Dubuisson for help with chemical analysis, Jennifer McKey for her help with the preparation of the figures, Doyle McKey for providing helpful comments on the manuscript including language correction and two anonymous referees for their comments on the manuscript. This research was partly supported by the programs EC2CO-Ecodyn, PEPS-Exomod-CNRS, International Research Project (IRP)-CNRS-MOST, ANR under the "Investissements d'avenir" program (reference ANR-16-IDEX-0006) and the French National Research Program for Environmental and Occupational Health of ANSES (reference 2018/1/138). Xiaoxia Deng's PhD grant was supported by China Scholarship Council [No. (2017)3109].

Author contributions
M.P., B.L. and M.H.M. designed the study. M.P., B.L., X.D., P.A. and F.G. collected the data. M.P., B.L., B.B. and D.C. analysed and interpreted the data. M.P. and M.H.M. obtained the funding. M.P. wrote the manuscript with the assistance of D.C. and M.H.M. All authors contributed considerably to the preliminary versions of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-66655-w.

Correspondence and requests for materials should be addressed to M.P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary file

Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction

Magali Proffit, Benoit Lapeyre, Bruno Buatois, Xiaoxia Deng, Pierre Arnal, Flora Gouzerh, David Carrasco, Martine Hossaert-McKey

Figure legend

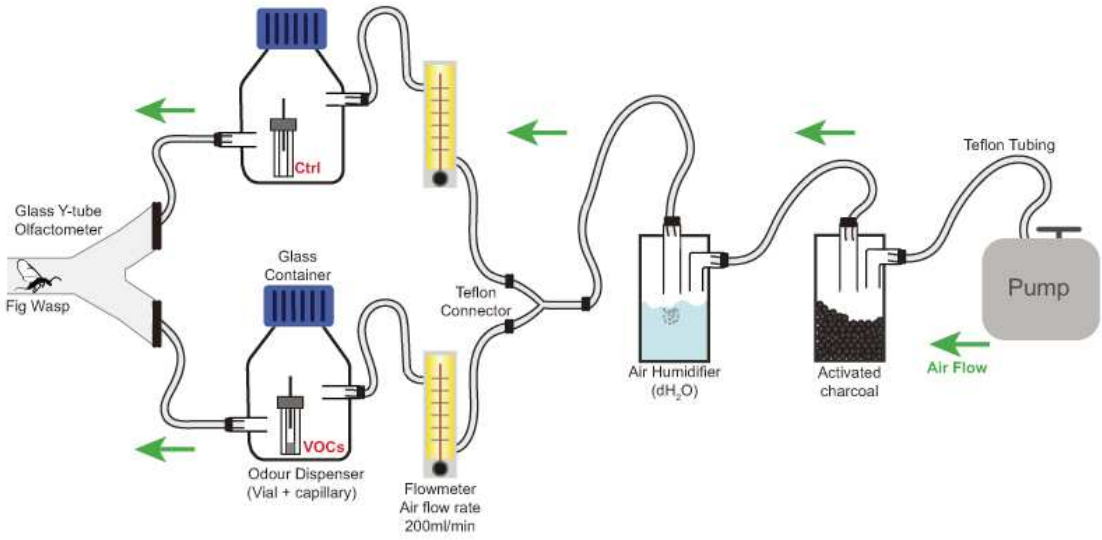


Figure sp1: Schematic of the system used to conduct behavioral tests with Y-tube olfactometer. Details on the equipment and protocol used to test wasp attraction with this set-up are presented in Materials and methods/Behavioral experiments. “VOCs” stands for volatile organic compounds and “ctrl” for control. This figure was prepared with the help of Jennifer McKey.

Original Article

Geographic variation in plant-insect signalling: the Achilles heel of the obligate fig-fig wasp mutualism when exposed to climatic accidents?

Deng Xiaoxia[1,2], Wang Gang[3], Peng Yan-Qiong[3], Yu Hui[1,4,*], Aroonrat Kidyoo[5], Zhang Ting[3], Finn Kjellberg[2], Magali Proffit[2]

[1] Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, the Chinese Academy of Sciences, Guangzhou 510650, China;

[2] CEFE, CNRS, Univ Montpellier, EPHE, IRD, Montpellier, France

[3] CAS Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303

[4] Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou 511458, China

[5] Plants of Thailand Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand

running title of not more than 75 characters, including spaces

Geographic variation in plant-insect signalling: the case of *Ficus hispida*

*

Abstract

- ***Background and Aims***

Odours released at floral receptivity play a central role in attracting pollinators in plant-pollinator mutualisms. This is especially true for pollinators in brood-site pollination mutualisms. In some associations, floral odours vary geographically. This may affect the resilience of the mutualistic association in response to climatic accidents. When pollinators go locally extinct, will migrant pollinators from other locations recognise the local host-plants?

Using the mutualistic association between *Ficus hispida* and its pollinating wasps, we tested the hypothesis that receptive fig (inflorescence) odours vary geographically and that this variation affects pollinator attraction.

- ***Methods***

We used the headspace technique to collect *in situ* receptive fig odours in a series of locations in continental South-East Asia. Under controlled conditions, we tested the attraction of fig pollinating wasps from two locations (South China and South Western China) by local versus non-local receptive figs in Y tube experiments.

- ***Key Results***

Receptive fig odours varied geographically and were clustered into three groups, South China, South West China and South Thailand. Pollinating wasps were only attracted by local receptive fig odours.

- ***Conclusions***

We observe geographic variation in plant signalling and local adaptation of the pollinators to this variation. In case of climatic accidents, the association between *Ficus hispida* and its pollinating wasps may be fragile as non-local wasps may fail to recognise receptive host

plants. Despite a huge range, extending from India to Australia, *Ficus hispida*, as many other species involved in specialised pollination mutualisms, may be sensitive to the predicted increase in extreme climatic events.

Key words: co-evolution; floral scent; *Ficus*; behaviour; pollination; *Ficus hispida*; *Ceratosolen marchali*; *Ceratosolen solmsi*.

Introduction

Out of 300,000 species of vascular plants (Christenhusz and Byng, 2016), 88.7% are angiosperms, and 90% of these are pollinated by insects, such as bees, beetles, moths and flies (Ollerton et al., 2011). Pollination by insects is hypothesized to have been one of the driving forces that has allowed the diversification of angiosperms (Hernandez-Hernandez and Wiens, 2020). Insect-plant interactions are affected by climate variation as it plays an important regulatory role in the timing of both plant and insect development (DeLucia et al., 2012, Jaworski and Hilszczański, 2013, Kharouba et al., 2015). However, if the effect of climate on plants and insects is discordant, mismatches could be generated by rapid global change (Parmesan, 2007). Substantial climate change has occurred since the 1950s according to the consensus of the Intergovernmental Panel on Climate Change (IPCC). It is likely that in the short term (2016–2035), the global mean surface air temperature will increase by 0.3-0.7 °C comparatively to 1986-2005 levels (IPCC 2013). Climate change affects the phenology, local abundance and large-scale distribution of plants and pollinators (Hegland et al., 2009, Forrest, 2015, Gerard et al., 2020). Therefore, global climate change will pose a series of challenges for pollination in this century, especially in specialized interactions.

Because specialized pollination requires the interacting species to locate each other in time and space, climate change can cause phenological decoupling of plant-pollinator associations (Settele et al., 2016, Gerard et al., 2020). Alternatively, differing demographic responses may affect the relative densities of the partners and may turn mutualistic pollinators into parasites feeding on plant resources while providing limited pollination service (Hegland et al., 2009, Krishnan et al., 2014, Rafferty et al., 2015). Ultimately, these effects could result in rapid population declines, potentially leading to local, regional or global extinction of interacting species (Hegland et al., 2009, Jevanandam et al., 2013).

Highly specialized specific pollination systems, in which a plant species has few alternative pollinators, are expected to be more vulnerable to global change than generalized ones (Koh et al., 2004, Gilman et al., 2012). Among these, species-specific reciprocal mutualisms could potentially be the most fragile. In the extreme case of obligate one-to-one relationships (one pollinator exclusively feeding on one plant that has no alternative pollinator), neither species will survive climatic conditions that become unsuitable for the more sensitive partner (Gilman et al., 2012). Brood site pollination mutualisms, *i.e.* systems in which the larvae of the pollinating insects feed on floral organs, represent some of the most specialized systems. They include, among others, figs and fig wasps (Cornille et al., 2012, Rodriguez et al., 2017, Yu et al., 2019), yuccas and yucca moths (Pellmyr, 2003), *Epicephala* moths and Philanthacea (Kawakita and Kato, 2009). Even more than sensitivity to general aspects of climate change, such associations have been shown to be sensitive to climatic accidents. Indeed, long-term observations have documented an instance where *Yucca elata* flowering was strongly delayed following an exceptionally cold winter, while their obligate pollinator was not. Only the very earliest flowers were pollinated by the very latest moths, which overwinter as larvae at the base of the plants (Rafferty et al., 2015). This observation shows that the cues that trigger flowering in yuccas and emergence in yucca moths can be decoupled. Mismatches can occur, even in obligate highly specialized coevolved mutualisms. However, in the Yucca-Yucca moth association, prolonged moth diapause – up to 30 years – may mitigate climatic accidents that are not repeated every year by providing a local reservoir of individuals (Powell, 2001). In other systems there is no diapause and hence no potential local reservoir of quiescent individuals that can rescue a pollinator population. In the fig-fig wasp interaction, during the severe El Niño Southern Oscillation event of 1997-1998, fig trees, that normally fruit all year round, stopped fruiting but survived while their short-lived mutualistic pollinating wasps went regionally extinct (Harrison, 2000). The populations of

more dispersive fig-wasp species recovered instantly, while less dispersive ones had not recolonised 7 months later (Harrison, 2000). Limited or even total lack of spatial genetic structure has been observed in some Yucca moths and in some fig pollinating wasps (Leebens-Mack, 2004, Cooper et al., 2020). This suggests that pollinators have the capacity to disperse over large distances and may be adapted to a broad set of ecological situations. However, dispersing pollinators need to locate receptive host plants.

In brood site pollination mutualisms, the main message used in plant-insect communication is odours released by the plants at anthesis (Hossaert-McKey et al., 2010, Borges, 2016). In some systems, both plants and pollinators present large geographic ranges and plant-insect chemical signalling is constant throughout the geographic range. For example, two *Yucca* species (*Y. filamentosa* and *Y. elata*) show minimal variation in floral scent within and between species, and genetic differentiation among the populations of their pollinator, *Tegeticula yuccasella*, is slight (Leebens-Mack, 2004, Svensson et al., 2005). Such a system that has recently expanded geographically (Cole et al., 2011, Harrower and Gilbert, 2018), should be resilient to climatic accidents and to climate change. However, in other cases, spatial structure is prevalent, and involves within species spatial genetic structuring, geographic pollinator species turnover and geographic variation in floral odours (Suinyuy et al., 2015, Rodriguez et al., 2017, Friberg et al., 2019, Deng et al., 2021). In interactions that are (co)evolving locally, geographic variation in communication may preclude successful unilateral migration of individuals or propagules of one of the interacting species. This could have severe consequences for the resilience of interactions confronted to climatic accidents and to climate change, as pollinators may fail to recolonize from other locations, and associates would need to migrate together. Evaluating geographic variation of plant-insect communication in geographically widespread systems may help to predict whether partners

may disperse independently or whether adaptation of pollinators or plants to new partner populations may be required for successful dispersal.

Ficus provide a model system to investigate geographical variation in an apparently highly resilient biological interaction. There are over 750 species of *Ficus* distributed throughout the tropics and subtropics. They are all involved in a reciprocally obligate pollination mutualism with small, species-specific, short-lived (1–2 days) fig wasps (Agaonidae) that breed within the enclosed inflorescences called figs. *Ficus* generally have very large distributions, so that the local alpha diversity is high while geographic species turnover is low comparatively to other tree genera (Harrison et al., 2012). Large distributions suggest strong resilience of the mutualistic pollination interaction, and indeed, low extinction rates have been inferred from phylogenetic studies (Bruun-Lund et al., 2018). However, small sized animals, especially tropical ones, often have narrow ecological tolerance (García-Robledo et al., 2016). This may explain why many widespread *Ficus* species are associated with different pollinator species in different parts of their range (Bain et al., 2016, Rodriguez et al., 2017, Morgan et al., 2018, Yu et al., 2019). Hence, each pollinating wasp species could have narrower ecological limits than their larger, longer-lived host plants (Jevanandam et al., 2013). The system could be resilient if wasps can be sourced from other populations following climatic accidents or when the local wasp population goes extinct due to changes in local conditions. Many *Ficus* species have continuous receptive fig production throughout the year, so that plant flowering phenology is not an issue, wasps will always find receptive figs (Chiang et al., 2018). However, in some associations the odours of receptive figs, the message used by the wasps to locate their host, vary geographically (Soler et al., 2011, Rodriguez et al., 2017, Deng et al., 2021). If this odour variation affects the capacity of the wasps to locate receptive figs, then the system may be much less resilient to climatic accidents and global change than the large ranges of the host plants would suggest.

A key step toward anticipating how a particular fig–fig wasp association may respond to climatic accidents and rapid climate change is to examine whether receptive fig odours vary across its geographic range, and whether the wasps are attracted by non-local fig odours. Here, we investigate variation in an extremely widely distributed species, *Ficus hispida.* It ranges from Western India and the Himalayan foothills to Australia. It has been reported that receptive fig odours of *F. hispida* differ between Yunnan in China and the Western Ghats in India (Soler et al., 2011). In the current study, we investigate geographic variation in floral odours within a biogeographic region, continental South-East Asia. We establish whether geographic variation in receptive fig odours between two south China locations is biologically significant for the wasps.

2. Materials and methods

Ficus hispida L. (subgenus *Sycomorus*, section *Sycocarpus*), is a functionally dioecious small- to medium-sized freestanding tree 3-5 m high (Hill, 1967, Corlett, 2006). This species displays asynchronous phenology at the population level, with a continuous presence of receptive figs, and of figs releasing pollinating wasps year-round (Patel, 1996, Corlett, 2006, Kuaraksa et al., 2012), although fig production peaks 4-5 times during the year (Yang et al., 2002, Kuaraksa et al., 2012). *Ficus hispida* is common in secondary habitats, at the edge of swamps, and along rivers (Berg and Corner, 2005).

As many other wide-ranging *Ficus* species, *F. hispida* is associated with several pollinating wasp species, belonging to the *Ceratosolen solmsi* species complex (Wiebes, 1994). The species complex is currently under revision (Jean-Yves Rasplus, unpublished data).

Study sites

Receptive fig odours were sampled between November 2017 and June 2019, in two sites in continental south China, one site in Hainan island, one site in south-western China, and 5 sites in south Thailand (Table1). In south Thailand, because only few trees were sampled per site, and because of the limited distance between sites, in the following, all the samples are grouped into a single location, Thailand. Collections in China were made in July, when average temperatures were about 28°C, in order to minimize temperature difference with Southern Thailand, and its potential proximate influence on odour emissions. In our sampling locations, two different *Ceratosolen* species have been collected from *F. hispida*, either by us or by other collectors (vouchers available at the fig wasp collection at CBGP, Montferrier-sur-Lez, France), *C. marchali* Mayr (SCBG, XTBG and Hainan) and *Ceratosolen nsp74* (Thailand and Hainan) (Jean-Yves Rasplus, pers. com.).

2.1 Volatile compounds collection

We collected and analysed odours released by receptive figs (15 to 70 figs/tree) from naturally growing trees. To sample the volatile organic compounds (VOCs), the Chromatoprobe head-space technique was used, following the same methods as in Souto-Vilarós et al., 2018. Receptive figs from individual trees were enclosed within a polyethylene terephthalate bag (Nalophan®, Kalle Nalo GmbH, Wursthüllen, Germany) for 30 min and the emitted volatiles were trapped in a chromatoprobe filter (filled with adsorbent mix: Carbotrap 20-40 and Tenax 60-80, Sigma Aldrich, Munich, Germany, 1.5 mg of each) by pumping air out of the bag at a rate of 200ml/min for 5 min. The adsorbent was blocked within the filters using glass wool. An identical Nalophan bag, without figs, but with a filter, was used to control for VOCs present in the ambient air in the location during the volatile collection. Odour collection was performed *in situ*, on sunny days, between 10 am and 5 pm. One microliter of a solution of internal standards (nonane and dodecane, 110 ng.µl-1 of each) was added to each filter, before scent VOC collection, to allow quantification. The samples were

then stored at -18 °C until chemical analysis and all collected samples were analysed within two months.

2.1.1 Chemical analyses

The samples were analyzed at the “Platform for Chemical Analyses in Ecology” (PACE, Montpellier) 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 (TDU) 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 210 to 250°C at 10°C/min, and finally held for 2 min at 250°C. 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 was 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 (alcane standard solution C8-C20, 04070, Sigma Aldrich®) were used to convert retention times into retention indexes.

The GC-MS data were processed using MZmine 2.18 (Pluskal et al., 2010) adapted to GC data processing (customized software), using the same automated protocol ensuring the consistency of peak integration. Following normalization of the dataset, peaks were detected

by local minima chromatogram deconvolution and aligned across samples based on mass spectra and retention times. Controls samples were used to substract contaminant compounds from the figs samples. VOCs were identified on the GC-MS samples based on their retention index and mass spectra, which were compared to those recorded in databases (NIST 2007 MS library, Wiley 9th edition), and when possible by comparison with reference compounds.

All statistical analyses of floral volatile data were performed in R (version 3.5.2; R Development Core Team, www.R-project.org). The composition of receptive floral scent (relative proportions of each compound) was compared among localities using the Vegan package (Oksanen et al., 2013). Data were standardized (range 0-1). Divergence in chemical profiles across locations was estimated for the standardized data with non-metric multidimensional scaling (NMDS) in two dimensions, based on a Bray-Curtis similarity matrix. The stress value was used to evaluate how well a particular configuration represents the observed dissimilarity matrix. The smaller the stress value, the better the fit of the reduced dissimilarity matrix to the observed dissimilarity matrix. Permutational multivariate analysis of variance (PERMANOVA) and Pairwise comparisons using permutation MANOVAs on the distance matrices based on 9999 permutations were used to test the difference in the scent profiles between the study localities. We used the false discovery rate method to evaluate the statistical significance of multiple comparisons. When the localities were significantly different or when there was a trend, the similarity percentage (SIMPER), was used to identify the compounds that accounted for most of the dissimilarity between localities.

We performed Mantel tests to investigate whether differences in floral scent composition correlated with the geographic distance between locations. We computed chemical Bray-Curtis dissimilarity indices. Geographic distances were calculated using our global positioning system (GPS) coordinates. Mantel tests (with 99 999 random iterations) were performed for the entire data set and for data subsets.

2.2 Insect behavioural tests

Behavioural assays were carried out in the laboratory in XTBG and SCBG between October and December 2018 using receptive male figs. We used a Y-tube olfactometer to test the attraction of wasps from the South China Botanical Garden (SCBG) and from the Xishuangbanna Tropical Garden (XTBG) to local receptive figs and to receptive figs from the other botanical garden. At the two locations, *F. hispida* is pollinated by *C. marchali* (JY Rasplus pers. com.). The two locations are separated by 1200 km. The bioassays were conducted under laboratory conditions using receptive male figs collected from plants in these two locations (SCBG and XTBG). We obtained non-local wasps by collecting figs a few days before wasp emergence, when the fig walls had begun to turn yellow or soft, and transferring them by express delivery from one location to another. Local and non-local wasp individuals were tested by using local receptive figs at both sites. We used the same size Y-tube olfactometer (stem 8 cm; arms 9 cm; internal diameter 1.5 cm) that has previously been used for attraction tests of the pollinating wasps of *F. hispida* (Proffit et al., 2009). Receptive figs were used as odour source, while an empty vial was connected as a control. Two receptive figs of *F. hispida* were used in the tests. To ensure continuous odour production, the receptive figs were renewed every two hours. Humidified and purified air was blown through two source bottles that connected to the arms of the Y-tube at a rate of 200 ml/min. One wasp was introduced into the central arm of the Y-tube and its movements were recorded for at most 15 minutes. To avoid a potential directional bias in the setup, the directions of control and odour source were reversed after each trial. The Y-tube and the source bottles were cleaned with pure acetone before each trial to eliminate scent contamination. For the bioassays, the same modalities were applied as for previous tests for fig wasps (Chen et al., 2009, Wang et al., 2013, Proffit et al., 2009). The wasp was considered to have made no choice when it stayed

motionless for 3 min in the departure section and/or the central arm. All the adult female fig wasps used in the experiments were active, newly emerged from mature male figs.

Binomial tests were used to determine whether individuals of pollinating wasps from SCBG and XTBG (N>40 per tested odour) were attracted to VOCs emitted by receptive figs originating from either SCBG or XTBG.

3. Results

Geographical variation in the chemical message emitted by receptive figs

The compounds detected at each site are listed in Table S1. They are ordered into chemical classes, which to some degree reflect their biosynthetic origin (Knudsen et al., 2006). A total of 39 compounds were identified in the odours of the receptive figs, and 4 compounds could not be identified (Table S1). The identified VOCs belong to three main chemical categories: 5 fatty acid derivatives, 12 monoterpenes, 18 sesquiterpenes, and 4 unknown compounds. The floral odours emitted by *F. hispida* were mainly composed of terpenes. In China, the most abundant chemical classes for all the locations were sesquiterpenes, while the odour was dominated by monoterpenes in Thailand (50.1%). The receptive odour of *F. hispida* from each site were dominated by 4-6 compounds, contributing each more than 5% of the total amount. Figure 1 represents the occurrence and relative contributions of these compounds in the different locations.

The NMDS (stress = 0.190) on the relative proportions of all VOCs in the odours emitted by each sample for the global dataset separates the different locations (PERMANOVA, $F_{(4,39)}$=3.86, P = 0.001). The NMDS plot clustered samples into 3 groups, South China, South-Western China (XTBG), and southern Thailand (Fig. 2). The pairwise comparisons on distance matrices shows that the difference between South-China and South-Western China is significant (permutation MANOVA, $p<0.05$ for each of the three pairwise comparisons, Table

2), while the south China locations (SCBG, Nan and Ding) were not significantly different in pairwise comparisons.

The SIMPER analysis conducted to identify the compounds responsible for dissimilarities between locations shows that both major and minor compounds are responsible for the differences between locations (Table 3). Variation in the relative proportions of 4-6 compounds explained 30% of the pairwise dissimilarities. Among these compounds, all the compounds present in at least one location at a relative abundance above 1.5% were detected in the three groups, while only 6 compounds, present at relative abundances below 1.5 % in the location where most abundant, were not observed in the three groups. Hence, most of the difference between groups of locations are quantitative and not qualitative.

Correlation between floral scents and geography

We found a significant correlation between chemical distance and geographic distance including all the samples (Mantel test, $r=0.3745$, $p<0.001$). A second test performed for all locations likely to be only pollinated by *C. marchali* (removing locations Thailand and Ding) was also significant (Mantel test, $r= 0.4812$, $p<0.001$). Finally, a third test performed for all locations in south China, including SCBG, Nan, and Ding, was not significant (Mantel test, $r=0.004309$, $p=0.458$, a result consistent with the lack of significant difference between these locations in the PERMANOVA results. Hence, chemical distance correlated with geographic distance at intermediate and long distances.

Response of fig wasps to receptive odours from local and nonlocal localities

In the behavioural experiments, a high proportion of fig wasps entered the olfactometer and chose either the scented or the control arm (Fig. 3). In olfactometer choices between a fig scent and the control, responses of wasps differed according to whether figs were from the same region as the wasps or from a different region. When wasps from SCBG and XTBG

were separately offered figs from corresponding locations, they were significantly attracted to figs from the same region (binomial test: $P<0.001$, n = 51, and $P<0.005$, n = 42; respectively). When the wasps from XTBG and SCBG were exposed to scents from figs from a different region, they were not attracted by the receptive figs and presented a slight trend to prefer the control (binomial test: $P=0.174$; n = 44, and binomial test: $P=0.072$, n = 45; respectively). In this experiment, wasps were only attracted by their local receptive figs.

4. Discussion

We show that receptive fig odours of *F. hispida* vary geographically. Receptive fig odours from locations in China were dominated by sesquiterpenes while in Thailand they were dominated by monoterpenes. Further, in the comparison between SCBG and XTBG, the odour variation is biologically meaningful for the wasps as a same pollinator species was attracted by local receptive figs and not by non-local ones. While biologically meaningful, the odour differences among locations correspond mainly to variation in the relative abundance of compounds shared among locations. Most compounds contributing to the difference among groups of locations were detected in all of them, and those that were not detected in some regions were never found in large quantities in any location. This suggests that all compounds were shared among locations.

Our results indicate that, in this widely distributed fig-pollinating wasp association, if a regional population of pollinators goes extinct, migrant wasps may fail to recolonise due to their inability to locate receptive figs. Therefore, we predict that the association between *Ficus hispida* and its pollinators may be sensitive to climatic accidents and to rapid global change, despite a huge range extending from Australia to the Himalayan foothills.

We document geographic quantitative variation in the general composition of odours emitted by receptive figs. Up to what point this variation is meaningful in terms of pollinator attraction can be questioned. Indeed, the seasonal variation in receptive fig odours reported in one species, *Ficus carica*, involves compounds that are not detected by the wasps (Proffit et al., 2020). In other species, growing in less seasonal climates, seasonal variation is limited (Rodriguez et al., 2017). Few studies on figs and fig wasps have determined which compounds are responsible for wasp attraction. Some try to establish which compounds in the floral odour are detected by the insects' antennal receptors by using coupled gas chromatography-electroantennographic detection (GC-EAD) (Proffit et al., 2020, Zhang et al., 2020, Wang et al., 2021). In some studies, the response of the insects in Y tube olfactometers to mixtures of synthetic compounds has been used to validate inferences about which compounds are active (Chen et al., 2009, Proffit et al., 2020, Vanderplanck et al., 2021, Wang et al., 2021). These studies show that at least 1-6 compounds of the receptive fig odours are detected by the wasps, or elicit wasp attraction (Proffit et al., 2020, Zhang et al., 2020, Wang et al., 2021). In *Ficus carica* and *F. semicordata*, compounds that have been shown to be involved in wasp attraction constitute a major fraction of the odour emitted by receptive figs (Proffit et al., 2020, Zhang et al., 2020), and in *Ficus auriculata*, the most abundant compounds are detected by the wasps (Zhang et al., 2020). This suggests that in the few cases where only minor compounds have been shown to elicit wasp antennal and/or behavioral response, further studies may reveal that more abundant compounds are also involved. The picture that a large fraction of the volatiles emitted at fig receptivity is involved in wasp attraction is supported by the two studies of floral odours in *Ficus* species locally sharing pollinators. In south Yunnan, *Ficus auriculata* and *F. oligodon* share two pollinating wasp species, and their receptive fig odours do not differ (Wang et al., 2016). Similarly, in South Africa, *Ficus natalensis* and *F. burkei* share pollinators, and their receptive fig odours do not

differ (Cornille et al., 2012). Such similitude of receptive fig odours has never been observed in any other interspecific comparison of receptive fig odours, suggesting convergent evolution due to pollinator sharing (Hossaert-McKey et al., 2010). Further, *Ficus natalensis* and *F. burkei* are not sister species (Cruaud et al., 2012). In that case, similar receptive fig odours were not inherited by descent. Hence, results show that the whole receptive fig odour mix, as analysed in our study, converges when plants share pollinators. This is most likely the product of selection to maximise wasp attraction. Convergence of receptive inflorescence odours is also documented in other brood site pollination mutualisms when plants share pollinators such as in some Cycadales (Suinyuy et al., 2012, Suinyuy and Johnson, 2018) and for Araceae in the *Schismatoglottis calyptrata* species complex that is pollinated by *Colocasiomyia* flies (Drosophilidae) (Hoe et al., 2018).

Within this general context, the case of linalool at XTBG is confusing and deserves further investigation. In some studies, large quantities of linalool have been observed in receptive *Ficus hispida* odours (Song et al., 2001, Proffit et al., 2008, Proffit et al., 2009) and the wasps have been shown in Y tube experiments to respond to synthetic linalool (Song et al., 2001). In another study, only minor compounds were found among the compounds detected by the wasps in total receptive fig odour extracts (Zhang et al., 2020) and in the present study we found little linalool despite the fact that wasps of XTBG were significantly attracted by the odour of receptive figs of this locality. All the receptive fig odours analysed in the current study were collected early in receptivity. These observations suggest that the emission of linalool increases during receptivity, and advancement in receptivity of analysed figs may have affected results. There is almost no data available on the qualitative variation of odours during anthesis (Delle-Vedove et al., 2017), and in *Ficus* we only have data on the variation after pollination or after wasp oviposition (Proffit et al., 2018).

Geographic variation of receptive fig odours has also been document in China for *Ficus hirta*, a small shrub ranging from the Himalayan foothills to Java and pollinated by a complex of parapatric wasp species (Deng et al., 2021). On the other hand, geographic variation of receptive fig odours at that scale was absent in the much larger fig-tree *Ficus racemosa* (Soler et al., 2011). Lack of odour variation at that scale correlates with gene flow, as in China-Thailand *Ficus racemosa* is pollinated by a single wasp species and neither plant nor insects present spatial genetic structure (Kobmoo et al., 2010, Bain et al., 2016) while *Ficus hirta* presents spatial genetic structure (Yu et al., 2019). Hence, we may suggest that low dispersal fig-fig wasp associations are potentially highly sensitive to local wasp extinctions while more dispersive ones could be more resilient. It has been suggested that local coevolution is one avenue by which mutualists can mitigate the effects of global change (Vidal et al., 2021). Our results suggest that local (co)evolution of plant signalling and wasp perception is an impediment to system resilience.

While the XTBG wasps are not attracted by receptive fig odours from SCBG and reciprocally, this does not mean that, given sufficient time, they would not evolve the capacity to recognise these odours. Indeed, the compounds mainly responsible for odour differences between locations and between regions are present in locations throughout the regions. Hence, the wasps' olfactive receptors are probably capable of detecting odours emitted by receptive figs in any location. What needs to evolve is an adjustment of the behavioural response to a particular mix of odours and maybe increased sensitivity to some compounds, both traits that can readily evolve in insects (Zhao and McBride, 2020).

Data from *Ficus septica* confirm that over evolutionary times fig wasps can colonise large geographic areas despite variation in receptive fig odours. Across the Philippines and Taiwan, *Ficus septica* presents geographic variation in receptive fig odours among islands and is pollinated by a set of different pollinating wasp species (Rodriguez et al., 2017). However,

one pollinating wasp species, *Ceratosolen jucundus*, has expanded throughout the region (Rodriguez et al., 2017). Hence, over evolutionary times, capacity to bridge populational differences in receptive fig odours are probably not a limiting factor for the wasps. Further, dated phylogenies show that *Ficus* is an old lineage that gradually accumulated species and exhibits very low extinction rates. This is even true in the dioecious lineages that include mainly shrubs and small trees and whose wasps do not disperse by drifting in the wind (Bruun-Lund et al., 2018). Low extinction rates over millions of years suggest strong resilience to the slow but large past climatic oscillations. Hence, the fig-pollinating wasp association could adjust to the speed of past climatic changes. Whether it will be capable of responding to the speed on ongoing climate changes is an open question.

Geographic variation of receptive inflorescence odours has also been documented in another obligate brood site mutualism. In the African cycads *Encephalartos villosus* (Suinyuy et al., 2015) and in *Encephalartos ghellinckii* (Suinyuy and Johnson, 2018), receptive cone odours varied geographically, and in both cases some pollinator species were shared among locations. In *E. villosus*, reciprocal choice experiments showed that pollinators were attracted by the local receptive cone odours, but not by non-local ones (Suinyuy et al., 2015). Hence, geographic structuring of receptive inflorescence odours with pollinators only responding to the local odours may be widespread in obligate brood site pollination mutualisms. In the facultative association between *Lithophragma* spp. and *Greya* moths, the geographic variation in floral odours at anthesis follows a different pattern from *Ficus hispida* with, in some species, qualitative variation of the emitted compounds (Friberg et al., 2017). As in other brood site mutualism cases, pollinators are only attracted by local flowers at anthesis (Friberg et al., 2014). However, the moths are capable of detecting a large array of compounds found in different populations, and even compounds absent from their native location (Schiestl et al., 2021), suggesting a strong potential for rapid evolutionary adjustment to local conditions.

Conversely, fig pollinating wasps in general (Wang et al., 2021), and pollinators of *Ficus hispida* in particular (Wang et al., 2014), are known to have a limited array of odorant-binding protein genes, suggesting a limited olfactory repertoire. Data on more reciprocal mutualisms will tell if variation in olfaction genes co-evolves with variation in the components of the floral odours of their host plants.

5. Conclusion

Geographic variation in the odours responsible for pollinator attraction is frequent in brood site pollination mutualisms. When odours vary, pollinators are only attracted by the odours emitted by receptive plants of their natal population. When such systems are exposed to extreme climatic episodes leading to local extinctions of pollinators, migrant pollinators may fail to recognise their host plants. Understanding whether micro-evolutionary processes will allow rapid response to such challenges in spatially structured mutualistic interactions is an open challenge. In a rapidly changing world, the answer may be crucial.

5. ACKNOWLEDGEMENTS

This research was supported by 'Franco-Thai Mobility Programme (PHC SIAM, France) 2018–2019' (project number 40533RA) and by LIA MOST.

Xiaoxia Deng's PhD grant was supported by China Scholarship Council [No. (2017)3109].

6. LITERATURE CITED

Bain A, Borges RM, Chevallier MH, Vignes H, Kobmoo N, Peng YQ, Cruaud A, Rasplus JY, Kjellberg F, Hossaert-Mckey M. 2016. Geographic structuring into vicariant species-pairs in a wide-ranging, high-dispersal plant–insect mutualism: the case of *Ficus racemosa* and its pollinating wasps. *Evolutionary Ecology* **30**: 663-684.

Berg CC, Corner EJH. 2005. Moraceae: Ficeae. *Flora Malesiana, Series I* **17**: 1-70.

Borges RM. 2016. On the air: broadcasting and reception of volatile messages in brood-site pollination mutualisms. *In Deciphering chemical language of plant communication*: 227-255.

Bruun-Lund S, Verstraete B, Kjellberg F, Rønsted N. 2018. Rush hour at the Museum-Diversification patterns provide new clues for the success of figs (*Ficus* L., Moraceae). *Acta Oecologica* **90** 4-11.

Chen C, Song Q, Proffit M, Bessière J-M, Li Z, Hossaert-McKey M. 2009. Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*. *Functional Ecology* **23**: 941-950.

Chiang Y-P, Bain A, Wu W-J, Chou L-S. 2018. Adaptive phenology of *Ficus subpisocarpa* and *Ficus caulocarpa* in Taipei, Taiwan. *Acta Oecologica* **90**: 35-45.

Christenhusz MJM, Byng JW. 2016. The number of known plants species in the world and its annual increase. *Phytotaxa* **261**: 201.

Cole KL, Ironside K, Eischeid J, Garfin G, Duffy PB, Toney C. 2011. Past and ongoing shifts in Joshua tree distribution support future modeled range contraction. *Ecological Applications* **21**: 137-149.

Cooper L, Bunnefeld L, Hearn J, Cook JM, Lohse K, Stone GN. 2020. Low-coverage genomic data resolve the population divergence and gene flow history of an Australian rain forest fig wasp. *Molecular Ecology* **29**: 3649-3666.

Corlett RT. 2006. Figs (*Ficus*, Moraceae) in Urban Hong Kong, South China *Biotropica: The Journal of Biology and Conservation* **38**: 116-121.

Cornille A, Underhill JG, Cruaud A, Hossaert-McKey M, Johnson SD, Tolley KA, Kjellberg F, van Noort S, Proffit M. 2012. Floral volatiles, pollinator sharing and diversification in the fig-wasp mutualism: insights from *Ficus natalensis*, and its two wasp pollinators (South Africa). *Proceedings of the Royal Society B: Biological Sciences* **279**: 1731-9.

Cruaud A, Rønsted N, Chantarasuwan B, Chou LS, *et al*. 2012. An extreme case of plant–insect codiversification: figs and fig-pollinating wasps. *Systematic Biology* **61**: 1029-1047.

Delle-Vedove R, Schatz B, Dufay M. 2017. Understanding intraspecific variation of floral scent in light of evolutionary ecology. *Annals of botany* **120**: 1-20.

DeLucia EH, Nabity PD, Zavala JA, Berenbaum MR. 2012. Climate change: resetting plant-insect interactions. *Plant Physiology* **160**: 1677-85.

Deng X, Bruno B, Yan-Qiong P, Yu H, Yufen C, Finn K, Magali P. 2021. Plants are the drivers of geographic variation of floral scents in a highly specialized pollination mutualism: a study of *Ficus hirta* in China. doi.org/10.21203/rs.3.rs-192226/v1

Deng W, Wei G, Zhao J-x, Zeng T. 2019. Anthropogenic effects on tropical oceanic climate change and variability: An insight from the South China Sea over the past 2000 years. *Quaternary Science Reviews* **206**: 56-64.

Forrest JRK. 2015. Plant-pollinator interactions and phenological change: what can we learn about climate impacts from experiments and observations? *Oikos* **124**: 4-13.

Friberg M, Schwind C, Guimarães Jr PR, Raguso RA, Thompson JN. 2019. Extreme diversification of floral volatiles within and among species of *Lithophragma* (Saxifragaceae). *Proceedings of the National Academy of Sciences* **116**: 4406-4415.

Friberg M, Schwind C, Roark LC, Raguso RA, Thompson JN. 2014. Floral scent contributes to interaction specificity in coevolving plants and their insect pollinators. *Journal of Chemical Ecology* **40**: 955-965.

Friberg M, Waters MT, Thompson JN. 2017. Nutrient availability affects floral scent much less than other floral and vegetative traits in *Lithophragma bolanderi*. *Annals of botany* **120**: 471-478.

García-Robledo C, Kuprewicz EK, Staines CL, Erwin TL, Kress WJ. 2016. Limited tolerance by insects to high temperatures across tropical elevational gradients and the implications of global warming for extinction. *Proceedings of the National Academy of Sciences* **113**: 680-685.

Gerard M, Vanderplanck M, Wood T, Michez D. 2020. Global warming and plant-pollinator mismatches. *Emerging topics in life sciences* **4**: 77-86.

Gilman RT, Fabina NS, Abbott KC, Rafferty NE. 2012. Evolution of plant-pollinator mutualisms in response to climate change. *Evolutionary Applications* **5**: 2-16.

Harrison RD. 2000. Repercussions of El Nino: drought causes extinction and the breakdown of mutualism in Borneo. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **267**: 911-915.

Harrison RD, Ronsted N, Xu L, Rasplus JY, Cruaud A. 2012. Evolution of fruit traits in *Ficus* subgenus *Sycomorus* (Moraceae): to what extent do frugivores determine seed dispersal mode? *PLoS One* **7**: e38432.

Harrower J, Gilbert GS. 2018. Context-dependent mutualisms in the Joshua tree-yucca moth system shift along a climate gradient. *Ecosphere* **9**: e02439.

Hegland SJ, Nielsen A, Lazaro A, Bjerknes AL, Totland O. 2009. How does climate warming affect plant-pollinator interactions? *Ecology letters* **12**: 184-95.

Hernández-Hernández T, Wiens JJ. 2020. Why are there so many flowering plants? A multiscale analysis of plant diversification. *The American Naturalist* **195**: 948-963.

Hill DS. 1967. Figs *(Ficus* spp*.) of Hong Kong*: Oxford University Press, Hong Kong & London.

Hoe YC, Gibernau M, Wong SY. 2018. Diversity of pollination ecology in the *Schismatoglottis calyptrata* complex clade (Araceae). *Plant Biology* **20**: 563-578.

Hossaert-McKey M, Soler C, Schatz B, Proffit M. 2010. Floral scents: their roles in nursery pollination mutualisms. *Chemoecology* **20**: 75-88.

IPCC 2005. Guidance Notes for Lead Authors of the IPCC Fourth Assessment Report on Addressing Uncertainties 5 (2005); available at http://www.ipcc.ch/pdf/supporting-material/uncertainty-guidance-note.pdf.

IPCC 2013 Climate Change 2013 – The physical science basis; Intergovernmental Panel on Climate Change**; https://www.ipcc.ch/report/ar5/wg1.**

Jaworski T, Hilszczański J. 2013. The effect of temperature and humidity changes on insects development their impact on forest ecosystems in the expected climate change. *Forest Research Papers* **74**: 345-355.

Jevanandam N, Goh AG, Corlett RT. 2013. Climate warming and the potential extinction of fig wasps, the obligate pollinators of figs. *Biology letters* **9**: 20130041.

Kawakita A, Kato M. 2009. Repeated independent evolution of obligate pollination mutualism in the Phyllantheae-*Epicephala* association. *Proceedings of the Royal Society B: Biological Sciences* **276**: 417-26.

Kharouba HM, Vellend M, Sarfraz RM, Myers JH. 2015. The effects of experimental warming on the timing of a plant-insect herbivore interaction. *Journal of Animal Ecology* **84**: 785-796.

Knudsen JT, Eriksson R, Gershenzon J, Stahl B. 2006. Diversity and distribution of floral scent. *Botanical Review* **72**: 1-120.

Kobmoo N, Hossaert-McKey M, Rasplus JY, Kjellberg F. 2010. *Ficus racemosa* is pollinated by a single population of a single agaonid wasp species in continental South-East Asia. *Molecular Ecology* **19**: 2700-2712.

Koh LP, Dunn RR, Sodhi NS, Colwell RK, Proctor HC, Smith VS. 2004. Species coextinctions and the biodiversity crisis. *Science* **305**: 1632-4.

Krishnan A, Pramanik GK, Revadi SV, Venkateswaran V, Borges RM. 2014. High temperatures result in smaller nurseries which lower reproduction of pollinators and parasites in a brood site pollination mutualism. *PLoS One* **9**: e115118.

Kuaraksa C, Elliott S, Hossaert-Mckey M. 2012. The phenology of dioecious *Ficus* spp. tree species and its importance for forest restoration projects. *Forest Ecology and Management* **265**: 82-93.

Leebens-Mack J. 2004. Patterns of genetic structure among populations of an oligophagous pollinating Yucca moth (*Tegeticula yuccasella*). *Journal of Heredity* **95**: 127-135.

Li J, Zhang G, Li XD, Qi SH, Liu GQ, Peng XZ. 2006. Source seasonality of polycyclic aromatic hydrocarbons (PAHs) in a subtropical city, Guangzhou, South China. *Science of the Total Environment* **355**: 145-55.

Morgan EJ, Sutton TL, Darwell CT, Cook JM. 2018. Restructuring of a mutualism following introduction of Australian fig trees and pollinating wasps to Europe and the USA. *Biological Invasions* **20**: 3037-3045.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, Simpson GL. 2013. Package 'vegan'. *Community ecology package, version* **2**: 1-295.

Ollerton J, Winfree R, Tarrant S. 2011. How many flowering plants are pollinated by animals? *Oikos* **120**: 321-326.

Parmesan C. 2007. Influences of species, latitudes and methodologies on estimates of phenological response to global warming. *Global Change Biology* **13**: 1860-1872.

Patel A. 1996. Variation in a mutualism: phenology and the maintenance of gynodioecy in two Indian fig species. *Journal of Ecology* 667-680.

Pellmyr O. 2003. Yuccas, yucca moths, and coevolution: a review. *Annals of the Missouri Botanical Garden*: 35-55.

Pluskal T, Castillo S, Villar-Briones A, Orešič M. 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* **11**: 1-11.

Powell JA. 2001. Longest insect dormancy: Yucca moth larvae (Lepidoptera: Prodoxidae) metamorphose after 20, 25, and 30 years in diapause. *Annals of the Entomological Society of America* **94**: 677-680.

Proffit M, Bessière J-M, Schatz B, Hossaert-McKey M. 2018. Can fine-scale post-pollination variation of fig volatile compounds explain some steps of the temporal succession of fig wasps associated with *Ficus racemosa* ? *Acta Oecologica* **90**: 81-90.

Proffit M, Chen C, Soler C, Bessière JM, 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. *Entomologia Experimentalis et Applicata* **131**: 46-57.

Proffit M, Lapeyre B, Buatois B, Deng X, Arnal P, Gouzerh F, Carrasco D, Hossaert-McKey M. 2020. Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction. *Scientific reports* **10**: 1-11.

Proffit M, Schatz B, Bessière 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**: 1-11.

Rafferty NE, CaraDonna PJ, Bronstein JL. 2015. Phenological shifts and the fate of mutualisms. *Oikos* **124**: 14-21.

Rodriguez LJ, Bain A, Chou L-S, Conchou L, Cruaud A, Gonzales R, Hossaert-McKey M, Rasplus J-Y, Tzeng H-Y, Kjellberg F. 2017. Diversification and spatial structuring in the mutualism between *Ficus septica* and its pollinating wasps in insular South East Asia. *BMC evolutionary biology* **17**: 207.

Schiestl FP, Wallin EA, Beck JJ, Friberg M, Thompson JN. 2021. Generalized olfactory detection of floral volatiles in the highly specialized *Greya-Lithophragma* nursery pollination system. *Arthropod-Plant Interactions* **15**: 209-221.

Settele J, Bishop J, Potts SG. 2016. Climate change impacts on pollination. *Nature Plants,* **2**: 16092.

Soler C, Hossaert-McKey M, Buatois B, Bessiere JM, Schatz B, Proffit M. 2011. Geographic variation of floral scent in a highly specialized pollination mutualism. *Phytochemistry* **72**: 74-81.

Song Q, Darong Yang, Zhang G, Yang C. 2001. Volatiles from *Ficus hispida* and their attractiveness to fig wasps. *Journal of Chemical Ecology* **27**: 1929-1942.

Souto-Vilarós D, Proffit M, Buatois B, *et al*. 2018. Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. *Journal of Ecology* **106**: 2256-2273.

Suinyuy TN, Donaldson JS, Johnson SD. 2012. Geographical variation in cone volatile composition among populations of the African cycad *Encephalartos villosus*. *Biological Journal of the Linnean Society* **106**: 514-527.

Suinyuy TN, Donaldson JS, Johnson SD. 2015. Geographical matching of volatile signals and pollinator olfactory responses in a cycad brood-site mutualism. *Proceedings of the Royal Society B: Biological Sciences* **282**: 20152053.

Suinyuy TN, Johnson SD. 2018. Geographic variation in cone volatiles and pollinators in the thermogenic African cycad *Encephalartos ghellinckii* Lem. *Plant Biology* **20**: 579-590.

Svensson GP, Michael O. Hickman J, Bartram S, Boland W, Pellmyr O, Raguso RA. 2005. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). *American Journal of Botany* **92**: 1624-1631.

Trisurat Y, Shrestha RP, Kjelgren R. 2011. Plant species vulnerability to climate change in Peninsular Thailand. *Applied Geography* **31**: 1106-1114.

Vanderplanck M, Lapeyre B, Brondani M, Opsommer M, Dufay M, Hossaert-McKey M, Proffit M. 2021. Ozone pollution alters olfaction and behavior of pollinators. *Antioxidants* **10**: 636.

Vidal MC, Anneberg TJ, Cure AE, Althoff DM, Segraves KA. 2021. The variable effects of global change on insect mutualisms. *Current Opinion in Insect Science* **47**: 46-52.

Wang G, Cannon CH, Chen J. 2016. Pollinator sharing and gene flow among closely related sympatric dioecious fig taxa. *Proceedings of the Royal Society B: Biological Sciences* **283**: 20152963.

Wang G, Compton SG, Chen J. 2013. The mechanism of pollinator specificity between two sympatric fig varieties: a combination of olfactory signals and contact cues. *Annals of botany* **111**: 173-81.

Wang N, Wang NX, Niu LM, Bian SN, Xiao JH, Huang DW. 2014. Odorant-binding protein (OBP) genes affect host specificity in a fig-pollinator mutualistic system. *Insect molecular biology* **23**: 621-631.

Wang R, Yang Y, Jing Y, *et al*. 2021. Molecular mechanisms of mutualistic and antagonistic interactions in a plant–pollinator association. *Nature Ecology & Evolution*.1-13.

Wiebes JT. 1994. The Indo-Australian agaoninae (pollinators of figs). *Koninklijke Nederlandse Akademie van Wetenschappen Verhandelingen Afdeling Natuurkunde, Tweede Reeks* **92**: 1-208.

Yang DR, Peng Y, Song Q, Zhang G. 2002. Pollination biology of *Ficus hispida* in the tropical rainforests of Xishuangbanna, China. *Acta Botanical Sinica* **44**: 519-526.

Yu H, Tian E, Zheng L, Deng X, Cheng Y, Chen L, Wu W, Tanming W, Zhang D, Compton SG, Kjellberg F. 2019. Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation. *Molecular Ecology* **28**: 2391-2405.

Zhang X, Wang G, Zhang S, *et al*. 2020. Genomes of the banyan tree and pollinator wasp provide insights into fig-wasp coevolution. *Cell* **183**: 875-889 e17.

Zhao J, Zhang Y, Song F, Xu Z, Xiao L. 2013. Phenological response of tropical plants to regional climate change in Xishuangbanna, south-western China. *Journal of Tropical Ecology 29*: 161-172.

Zhao Z, McBride CS. 2020. Evolution of olfactory circuits in insects. *Journal of Comparative Physiology A* **206**: 353-367.

Table1 Location of the study sites and dates of collection of odour samples. Sites are arranged in an east-west, north-south orientation.[1] Li et al. 2006, [2] Deng et al. 2019, [3] Zhao et al. 2013, [4]Trisurat et al., 2011.

region	location	GPS coordinates	Climate	Mean yearly temperature	dates of collection	# samples
South China	SCBG	113.35E, 23.18N	subtropical monsoon climate	20-22°C[1]	11/2017 11/2018	10
South China	Nan	108.39E, 22.79N	subtropical monsoon climate	20-22°C[2]	07/2019	3
South China	Ding	110.36E, 19.55N	tropical oceanic monsoon climate	24°C[2]	07/2019	5
South-western China	XTBG	101.27E, 21.93N	continental and oceanic climate	22°C[3]	06/2018 11/2018 12/2018	9
South Thailand	T5	100.52E, 13.73N	tropical monsoon climate	26.6 °C[4]	05/2018 03/2019	3
South Thailand	T4	99.9E, 13N	tropical monsoon climate		03/2019	2
South Thailand	T3	99.43E, 11.27N	tropical monsoon climate		05/2018 03/2019	2
South Thailand	T2	98.6E, 9.83N	tropical monsoon climate		05/2018 03/2019	2
South Thailand	T1	98.35E, 8.83N	tropical monsoon climate		05/2018 03/2019	4

Table 2 Significance of differences in pairwise comparisons using permutation MANOVAs on the chemical distance matrix between locations. We used the relative proportions of the different VOCs of each sample. Significant differences ($p < 0.05$) are indicated in bold.

	SCBG	Nan	Ding	XTBG	Thailand
SCBG					
Nan	0.208				
Ding	0.06	0.0978			
XTBG	**0.0117**	**0.0443**	**0.0117**		
Thailand	**0.0025**	**0.0025**	**0.0025**	**0.0025**	

Table 3. Main compounds responsible for dissimilarities between locations according to SIMPER analysis. The sum of the contributions of the compounds to the difference between location is indicated. Both major and minor compounds are involved.

	Average of similarity(%)									
	SCBG vs NN	SCBG vs Ding	SCBG vs XTBG	SCBG vs thailand	Nan vs Ding	Nan vs XTBG	Nan vs thailand	Ding vs XTBG	Ding vs thailand	XTBG vs thailand
Limonene	7.43	-	-	-	9.90	7.45	7.71	-	-	-
(Z)-3-Hexenyl acetate	7.00	5.82	5.51	6.30	-	-	6.53	-	5.55	6.15
(E)- β-Caryophyllene	5.94	4.89	-	-	5.33	8.32	8.49	6.72	7.72	-
α-Humulene	5.20	-	4.36	5.26	-	-	7.36	5.30	6.75	-
β-Elemene	4.53	-	6.82	-	-	15.42	-	6.60	-	8.37
α -Muurolene	-	5.83	-	-	7.45	-	-	5.66	6.01	-
γ-Muurolene	-	4.57	3.90	4.14	-	-	-	-	-	-
α-Copaene	-	4.31	-	-	5.34	-	-	5.89	6.23	-
γ-Elemene	-	4.95	-	-	5.69	-	-	-	-	-
Unknown a	-	-	3.82	-	-	-	-	-	-	4.49
1-8 Cineole	-	-	3.94	-	-	-	-	-	-	-
α-Copaene	-	-	-	4.12	-	-	-	-	-	-
(E)-β-Ocimene	-	-	-	5.63	-	-	-	-	-	6.83
(Z)-β-Ocimene	-	-	-	5.12	-	-	-	-	-	6.11
cumulative contributions	30.08	30.37	32.03	30.56	33.71	29.50	30.08	30.18	32.27	31.95

Table S1 Relative proportions (mean ± SD) of volatile organic compounds in the odours emitted by receptive *Ficus hispida* figs for each study location. O = number of individuals in which a particular compound was observed. RI = retention index.

RI	Compounds	SCBG		Nan		Ding		XTBG		Thailand	
	number of samples	10		3		5		9		12	
			O		O		O		O		O
	Fatty acid derivatives										
1005	(Z)-3-Hexenyl acetate	20.32±19.83	8		0	0.74±0.83	3	7.84±8.71	9	20.45±16.54	9
1012	Hexyl acetate	0.99±1.68	6	1.12±1.22	3		0	0.82±1.4	8		0
1014	(E)-2-Hexenyl acetate	2.76±4.94	5	1.04±0.97	3		0	0.9±1.39	5	1.51±1.81	6
1101	Nonanal	0.07±0.12	3		0		0	0.65±1.89	2	0.12±0.32	2
1203	Decanal	0.16±0.5	1		0		0		0	0.13±0.26	4
Mean of total percent		23.30		2.16		0.74		10.21		22.21	
	Monoterpenes										
935	α-Pinene	0.44±0.68	5	0.05±0.1	1		0	0.1±0.2	3	0.13±0.46	1
974	Sabinene	1.08±1.32	8	0.55±0.72	2	1.61±1.6	4	0.67±1.54	5	1.85±2.88	8
977	β-Pinene	0.1±0.2	4		0	0±0	0	0.05±0.12	2	0.04±0.13	1
990	β-Myrcene	4.88±4.23	9		0	5.66±3.5	4	2.03±1.75	9	7.03±10.55	9
1030	Limonene	0.93±1.06	7	6.3±4.85	3		0	1.03±1.03	6	0.42±1.45	1
1030	1,8-Cineole	1.96±2.42	8	0.23±0.4	1		0	0.65±1.96	1	1.2±1.84	6
1038	(Z)-β-Ocimene	0.13±0.18	5		0	0.09±0.21	1		0	0.83±0.89	8
1048	(E)-β-Ocimene	4.1±4.01	10	7±4.67	3	5.37±3.82	5	1.57±1.68	8	31.74±28.14	12
1090	(Z)- Linalool oxide (furanoid)	0.37±0.49	8	0.36±0.62	1	1.93±2.91	2	0.19±0.52	4	0.04±0.1	2
1100	Linalool	4.32±10.96	5	0.96±0.86	2	0.67±1.01	2	0.07±0.23	1	3.79±4.33	6
1117	perillene	0.16±0.28	4		0		0	0.08±0.17	2		0
1024	p-Cymene	2.24±3.86	9		0	1.67±2.05	4	1.11±1.3	8	2.11±1.23	11
1131	E,E-Cosmene	0.3±0.47	4	0.49±0.86	1	1.31±2.93	1	0.01±0.03	1	0.9±1.32	8
Mean of total percent		20.85		15.94		18.31		7.49		50.08	
	Sesquiterpenes										
1319	Unknown a	0.01±0.05	1		0		0	1.44±2.22	3		0
1335	Unknown b		0		0		0	1.03±1.82	3		0
1341	δ-Elemene	0.13±0.27	3		0	2.88±4.25	3	0.97±2.03	5	1.13±1.4	8
1347	α-Cubebene	0.15±0.3	5	0.17±0.19	2	0.4±0.4	3	0.31±0.79	2		0
1375	α-Ylangene	0.03±0.09	2		0	0.24±0.54	1	0.1±0.27	2		0
1374	Cyclosativene	0.55±0.82	5		0		0		0		0
1377	α-Copaene	7.12±4.68	10	9.57±6.43	3	14.1±7.56	5	2.82±3.25	6	2.18±2.33	9

1390	Unknown c	4.32±7.19	6	0.43±0.76	1	0.09±0.19	1	5.37±9.16	8	0.09±0.22	2
1395	β-Elemene	8.36±15.9	8	22.21±24.64	3	3.86±1.63	5	38.63±31.07	9	4.65±5.65	8
1423	cis-Thujopsene	0.01±0.02	1		0	0.15±0.34	1	0.3±0.58	3		0
1429	β-Caryophyllene	14.79±8.06	10	31.5±17.12	3	31.26±10.17	5	7.99±7.43	9	4.15±4.14	9
1435	β-Copaene	0.22±0.22	7	0.04±0.06	1	0.07±0.1	2	0.53±1.31	2	0.02±0.06	1
1439	γ-Elemene	1.65±2.23	7	1.78±1.57	2	2.86±2.41	4	0.6±1.19	5	0.21±0.46	4
1451	Geranyl acetone	0.6±1.2	7		0	0.35±0.49	2	0.1±0.18	5	0.2±0.25	6
1444	(E)- β-Farnesene	3.21±5.17	10	2.44±4.23	1	3.81±4.3	4	15.26±21.19	9	9.79±9.48	10
1460	α-Humulene	4.58±3.31	10	6.22±3.47	3	6.38±0.96	5	2.37±2.12	9	0.96±0.97	8
1484	γ-Muurolene	2.95±3.43	10	2.5±0.61	3	3.97±1.41	5	1.06±1.29	9	0.7±0.86	6
1490	Germacrene D	1.98±1.78	10	1.65±1.34	3	2.81±2.15	4	1.3±3.17	3	0.88±2.86	3
1506	Bicyclogermacrene	1.51±3.36	5	0±0	0	3.51±6.2	2	0.18±0.45	3	1.89±1.98	10
1505	α-Muurolene	0.6±0.67	7	0.89±0.53	3	1.57±1.57	3	0.8±0.94	5	0.29±0.59	3
1530	δ-Cadinene	1.92±1.91	10	2.5±1.8	3	2.63±1.12	5	1.07±1.37	8	0.59±0.47	9
Mean of total percent		54.69		81.9		80.94		82.23		27.73	

Main fragments for unknown compounds	
Unknown a	91;105/90;133/80;148/55;119/50;161/50
Unknown b	105;91/95;161/80;189/75;133/65;204/60;147/60
Unknown c	161;105/50;91/40;79/40;119/35

1

Figure legends

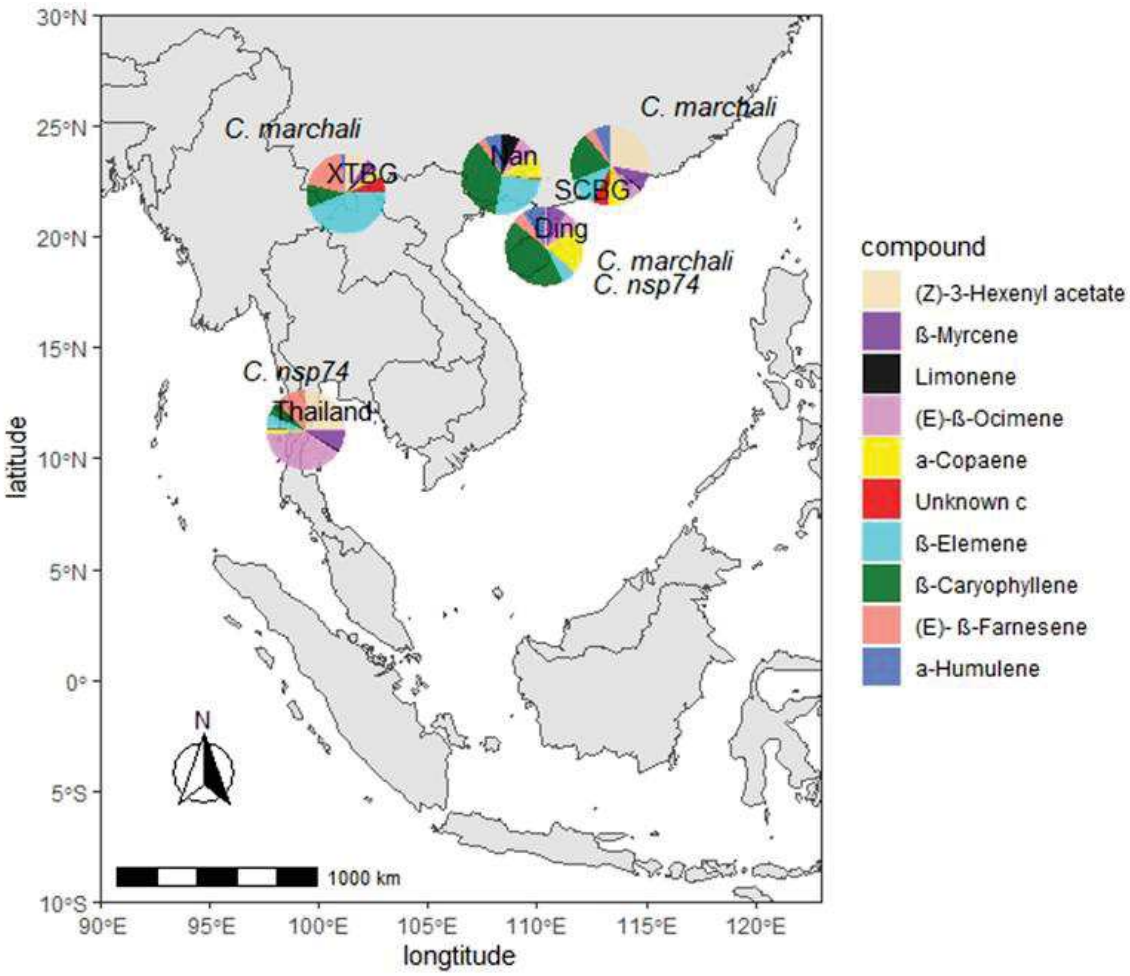


Fig. 1. Geographic variation in the mean composition of receptive fig odour. The pie charts represent the mean proportion per site of compounds present in a proportion higher than 5% in at least one location. In the CBGP fig wasp collection, wasps originating from SCBG and XTBG belong to *Ceratosolen marchali*, while wasps originating from Hainan island and South Thailand belong to *Ceratosolen n*sp 74. Due to limited wasp sampling, the presence of more wasp species at the different location is possible.

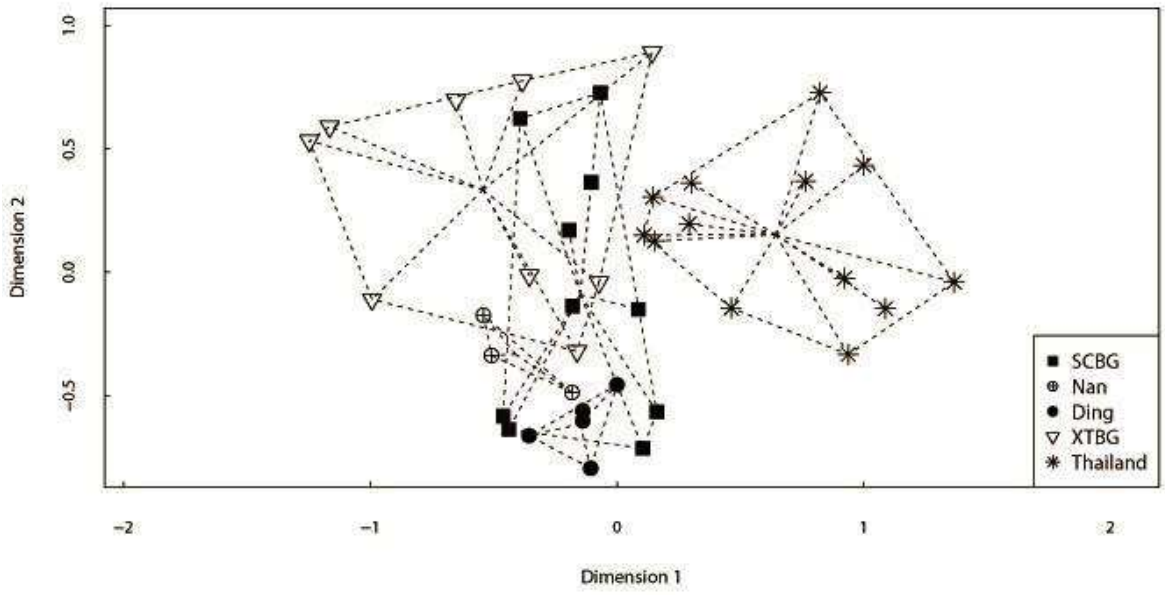


Fig. 2. Non-metric multi-dimensional scaling of the relative proportions of VOCs in the global bouquets emitted by receptive figs of *F. hispida* in the different localities based on Bray-Curtis dissimilarity Index (stress=0.190). Different localities are represented by different point shapes. The envelope of the odours for each locality is drawn (dashed lines) and all the samples are connected to the centroid of the group. Three groups are apparent: the south China locations, XTBG, Thailand

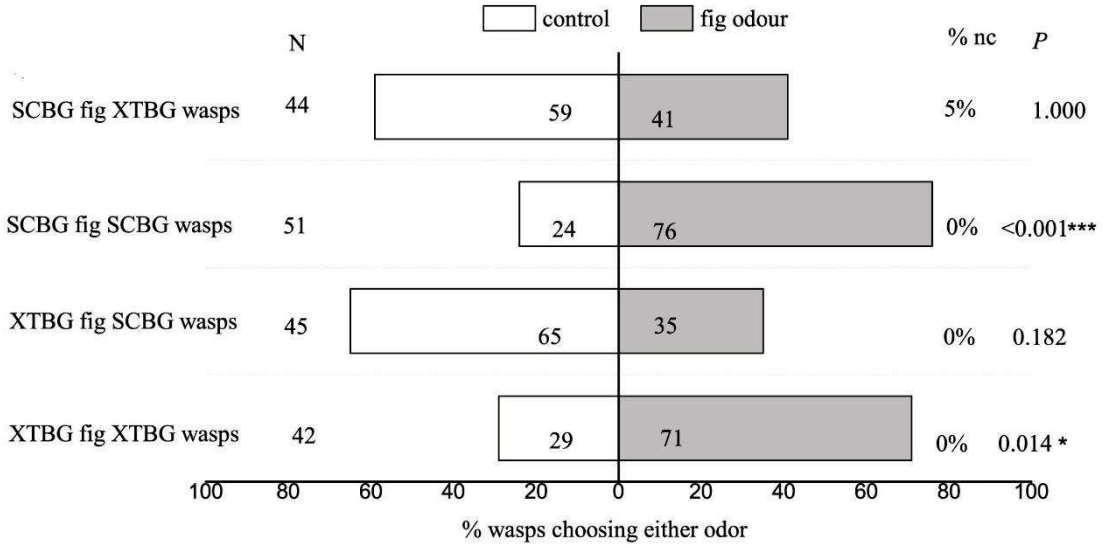


Fig. 3. Behavioural responses of female *Ceratosolen marchali* fig wasps when confronted with odours of local versus non-local receptive figs localities in Y-tube experiments. Fresh receptive male figs were used as odour sources. Behaviour choice to scent combinations in Y-tube test are as follows: nonlocal host versus air and local host versus air (binomial test). control: purified air; N: Numbers of tested fig wasps; NC: percentage of unresponding fig wasps (*P<0.05; ***P<0.001)

Limited interspecific divergence in olfactive signalling coupled with geographic variation may result in localised pollinator sharing between closely related *Ficus* species

DENG Xiaoxia, CHENG Yufen, PENG Yan-Qiong, YU Hui, PROFFIT Magali, KJELLBERG Finn

Keywords: speciation, *Ficus hirta*, *Ficus triloba*, *Valisia javana*, *Valisia esquirolianae*, co-speciation, mutualism, chemical signalling

Abstract

Background and Aims

In brood site pollination mutualisms, pollinators are attracted by odours produced at anthesis. In *Ficus*, odours of receptive figs differ among species and pollinators generally only enter figs of their host species ensuring a pre-zygotic barrier to plant interspecific hybridisation. Field observations recorded that, in Guangdong province, pollinators of *F. hirta* entered the figs of the closely related *F. triloba* on a regular basis. We propose that closely related *Ficus* species produce similar receptive fig odours. Under particular contexts of odours locally present, the receptive fig odours of non-host figs of a *Ficus* species may become attractive to pollinators of closely related *Ficus* species.

Methods

We used the headspace technique to collect *in situ* receptive fig odours of *F. triloba* in a series of locations in China. Under controlled conditions, we tested the attraction of fig pollinating wasps from *F. hirta* and *F. triloba* to host figs and non-host figs in Y tube experiments.

Key Results

Receptive fig odours of *F. triloba* though different from those of *F. hirta*, were mainly composed of a same set of volatile organic compounds. When given the choice between receptive fig odours and air, the pollinating wasps were only attracted by their host's odours. However, when given a choice between host and non-host figs the pollinators of *F. hirta* were equally attracted by the two odours while the pollinators of *F. triloba* tended to be more attracted by their host's fig odours.

Conclusions

Receptive fig odours vary geographically within species and the differentiation of receptive fig odours between closely related *Ficus* species is often incomplete. This allows localised or occasional pollinator sharing following different modalities. Cross stimulation when wasps are exposed simultaneously to odours of host and non-host species may be important. While occasional pollinator sharing may play a marginal role when wasp populations are robust, it may ensure the provisioning of new pollinators from the closest relative of a *Ficus* species if its pollinators go extinct.

Introduction

Successful speciation involves establishing barriers to gene flow between incipient sister-species. While allopatric speciation is frequent, the distribution of sister species often strongly overlap (Hernandez-Hernandez et al. 2021). Therefore, reinforcement processes reducing introgression play a central role in speciation (Servedio and Noor 2003). Sister species with overlapping ranges often occupy different ecological niches (Hernandez-Hernandez et al. 2021). Models show that species coexist more easily if barrier reinforcement relies on habitat preferences rather than on species recognition (Kyogoku and Kokko 2020). In plants, the pre-

zygotic barrier often involves change in pollinators (Hernandez-Hernandez et al. 2021), and pollinators may be habitat specialists (Lami et al. 2021).

Within this general context, systems in which plants associate with pollinators that breed in floral structures, *i.e.* brood pollination mutualisms, may ensure efficient pre-zygotic isolation among plant species. Indeed, the pollinators are often host specialists (Brookes et al. 2015). Plants typically attract their pollinating insect by releasing particular odours at anthesis that constitute species signatures (Hossaert-McKey et al. 2010). Among such systems, figs and fig pollinating wasps provide an extreme case of specialised brood site pollination mutualism in which parallel cladogenesis between plants and insects has been the main form of diversification over the last 70 Ma (Cruaud et al. 2012). They also provide a system where the range of plant species strongly overlap. Sympatry is generalised among *Ficus* species (Harrison 2005). Do brood site pollination mutualisms and *Ficus* in particular, follow the general rules associated with pre-zygotic barriers among related species, or do the particularities of these systems allow different diversification processes?

Fig pollinating wasps breed in the enclosed inflorescences (the figs) that characterise genus *Ficus*. The wasps are the sole pollinators of figs. Generally, a wasp species is associated with a single *Ficus* species, while a *Ficus* species is pollinated by a species or a species complex (Yu et al. 2019) and related *Ficus* species have related pollinator species (Cruaud et al. 2012). The wasps are attracted to figs by a species-specific odour released when flowers are ready to be pollinated and receive wasp oviposition (Proffit et al. 2020). Most *Ficus* species produce distinctive receptive fig odours (Hossaert-McKey et al. 2010), and wasps are sensitive to the ratio of different volatile organic compounds (VOCs) in the odour (Proffit et al. 2020). This allows high host-specificity.

The biology of the association suggests a simple, automatically enforced, reproductive isolation mechanism between incipient *Ficus* species. When the distribution of a *Ficus* species

becomes fragmented (*e.g.* in glacial refugia during climatic oscillations), allopatric differentiation of pollinator and *Ficus* host may occur. If there is a local particularity in plant insect communication, *i.e.* in the odour emitted by receptive figs and in how it is interpreted by the wasps, this may result in a pre-zygotic barrier with respect to other populations surviving in other refugia (Cook and Segar 2010). In some *Ficus* species, receptive fig odours vary geographically (Rodriguez et al. 2017, Deng et al. 2021), pollinating wasp species vary geographically (Yu et al. 2019) and some wasps are attracted by the local odours of their host plant, and not by non-local odours (Deng et al. 2021)). Hence, biological data suggests that a scenario of allopatric speciation in climatic refugia with geographic receptive fig odour differentiation instantly enforcing pre-zygotic isolation is plausible: in case of secondary contact between populations expanding from different refugia, the incipient species will remain distinct.

Receptive fig odours differ markedly between non-sister *Ficus* species and pollinators are not attracted by the odours of non-sister *Ficus* species (*e.g.* Proffit et al. 2009, Okamoto and Su 2021). On the other hand, sister *Ficus* species may present similar odours, because of shared ancestry, and some pollinators are attracted by receptive figs of their host's sister-species in experimental setups and/or in the field (Yokoyama 2003, Wang et al. 2013, Souto-Vilarós et al. 2018). With receptive fig odours varying geographically within species and closely related species presenting similar receptive fig odours, we may expect a geographic patchwork of receptive fig odours, where receptive fig odours sometimes locally overlap sufficiently between closely related *Ficus* species to affect the specificity of wasp attraction.

Ficus hirta and *Ficus triloba* provide a model system to investigate such a situation. *Ficus hirta* presents clinal genetic variation across its range (Yu et al. 2019) and receptive fig odours diverge with increasing distance (Deng et al. 2021). Throughout its range, it is pollinated by a set of parapatric wasp species of the *Valisia javana* species group. Its closest

relative, *Ficus triloba*, occurs throughout most of that range and is pollinated by *Valisia esquirolianae* (Berg and Corner 2005, Hu et al. 2020, Yu et al. 2021), a wasp that is closely related to the *Valisia javana* species groups (Yu et al. 2019) but is morphologically distinct (Chen and Chou 1997). In Guangdong, South China, figs of *Ficus triloba* are visited in some places by both *Valisia esquirolianae* and *Valisia javana hilli* , the local species of the *V. javana* complex (Yu et al. 2021). In samples collected throughout the range of *F. hirta*, *V. esquirolianae* was not found in the figs (Yu et al. 2019), though in a more recent survey it was obtained from *F. hirta* figs in one location (Yu unpublished).

Here we test the hypothesis that 1) *F. hirta* and *F. triloba* have similar though different receptive fig odours and that 2) in Guangdong, receptive fig odours of *F. triloba* are attractive to *Valisia javana hilli,* while *V. esquirolianae* is not attracted by figs of *F. hirta*.

Material and Methods

Study system and collection sites

Ficus triloba Buch.-Ham. ex Voigt (= *Ficus esquiroliana* Léveillé) (subg. *Ficus*, sect. *Eriosycea*, subsect. *Eriosycea*) is a dioecious tree up to 15 m tall while *Ficus hirta* is a small shrub (Berg 2007, Kuaraksa et al. 2012, Lu et al. 2016). *Ficus triloba* male trees produce a single main crop releasing pollen loaded wasps in July in time to pollinate the main crop on female trees that ripens in September-October (Kuaraksa et al. 2012). *Ficus hirta*, its closest relative (Berg and Corner 2005, Hu et al. 2020), produces figs year-round, with seasonal peaks, in June-July, and in October-November (Yu et al. 2006, Yu et al. 2008) thus overlapping with *F. triloba* phenology. *Ficus triloba* has large figs, about 30 mm in diameter at receptivity (Kuaraksa et al. 2012), while those of *F. hirta* are about 10-15mm (Yu et al. 2018). Receptive figs of *F. triloba* emit a strong floral scent while the smell of receptive figs of *F. hirta* is hardly detectable by the human nose (Hu et al. 2020). *Ficus hirta* is pollinated

by a set of 9 different wasps throughout its distribution (Yu et al. 2019), while a same pollinator (*Valisia esquirolianae*) has been collected on *F. triloba* in Taiwan, in continental China, and in Thailand (Chen and Chou 1997, Jiang et al. 2006). The two species are sympatric across most of their distributions that extends from northeast India and subtropical China to the Malay Peninsula (Berg 2007). While their habitats differ, the two species may grow side by side in secondary habitats, for instance in abandoned tree plantations or close to each other as in our study sites in Dinghu Mountain (DHS, a National Nature Reserve, established in 1956) and in Shimen (a forest park established in1995) in Guangdong province, China. In these two sites, *V. javana hilli* was observed to develop in figs of *F. triloba* (Yu et al. 2021).

Between November 2017 and June 2019, in rainy and dry season, to explore the diversity of receptive fig odours, we collected receptive fig odours from *Ficus triloba* at DHS, Shimen and at the Xishuangbanna Tropical Botanical Garden (XTBG) in Yunnan. We collected 15 samples in the region of Dinghu mountain (DHS, 112.54 E, 23.16 N), 3 samples from a single tree in South China Botanical Garden (SCBG, 113.35 E, 23.17 N), 13 samples in Shimen National Forest Park (Shimen, 113.45 E, 23.27 N), and 4 samples in the Xishuangbanna Tropical Botanical Garden (XTBG,101.15 E, 21.55 N).

Volatile collections

The chromatoprobe head-space method, which was initially used in *Silene*, was adopted to collect fig odours *in situ* (Dötterl et al. 2005, Soler et al. 2012, Deng et al. 2021). Odour collection was performed outdoors in the shade between 10:00 am and 5:00 pm on sunny days, corresponding to the insects' period of peak activity during our field season. Five-15 receptive figs were enclosed together 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) through a 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. In parallel, at 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 of each) was added to each filter before scent extraction, so that we could control for VOC loss during storage and transport. The samples were stored at -20 °C until VOC analysis.

VOC analysis

The samples were analysed using gas chromatography coupled with mass spectrometry and the compounds were identified as detailed in Deng et al. 2021. We obtained a global dataset, where the composition of the odour extracted from each sample is expressed by the relative proportions of each VOC in the emitted odour (semi-quantitative data). This dataset was complemented by previous data obtained from *Ficus hirta* (Deng et al. 2021) to compare the odours of the two species, and from *F. hispida* (subgenus *Sycomorus*) (Deng et al. submitted) to provide an outgroup.

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 R-package vegan (Oksanen et al. 2013).Two-dimensional plots were constructed using the "metaMDS" function algorithm after data standardization with "decostand" function in R (v. 3.5.1). 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 variation in chemical composition between locations was significant, we carried out permutational multivariate analysis of variance tests (PERMANOVA) based on the Bray-Curtis distance matrix. A significant difference may imply either different dispersion of the data or a difference in volatile compound composition. SIMPER (similarity percentage) was used to identify the compounds responsible for dissimilarities between groups. The chemical distance matrices were calculated with the function "vegdist", and PERMANOVA were performed using the function "adonis" in the vegan package (Oksanen et al. 2013).

Insect behavioural tests

In Dinghu mountain (DHS), *Valisia javana hilli* was observed to develop in figs of *F. triloba* along with *V. esquirolianane*. On the contrary, *V. esquirolianae* was not observed to develop in the figs of *F. hirta* at DHS (Yu et al. 2021).

In order to test if the local populations of *Valisia esquirolianae* and *V. javana hilli* are attracted by the odours released by receptive figs of *F. triloba* and *F. hirta*, wasp attraction was tested using Y-tube olfactometers in DHS. Bioassays were conducted outside, on a sunny day between 9 am and 12 am. We tested the response of the wasps when given the choice between odours emitted by *F. triloba* or by *F. hirta* and filtered air, and their response to a choice between the odours of the two species. Three different series of tests were used: receptive figs of host versus control, receptive figs of non-host versus control and receptive figs of host versus receptive figs of non-host. We used the same size Y-tube olfactometer (stem 8 cm; arms 9 cm; diameter 1.5 cm) as Proffit et al. (2009) to test the attraction of the pollinating wasps of *F. hispida*. Humidified air was purified with activated charcoal and blown into a glass vial connected to each lateral arm (200 ml/min per arm). Air was extracted

from the central arm (380 ml/min). The vial connected to one arm contained receptive figs stemming from several trees, and in the other, the vial was either empty or it contained receptive figs of the other species. For tests involving *Ficus triloba*, 2 receptive figs were put into the vial, while for *F. hirta*, 4 receptive figs were put into the vial. When comparing the attraction by receptive figs of the two species, due to the large difference in size, an equal weight of fresh figs was used. To ensure continuous odour production, we changed the odour source every two hours. Wasps were introduced individually into the central arm of the Y-tube and their movements were recorded for 10 minutes. To avoid a potential directional bias, the directions of control and odour source were reversed after each trial. To eliminate scent contamination, the Y-tubes were cleaned with pure acetone before each trial, as was the entire network of connecting plastic tubes after each five trials. The observer noted the behavioural choice made by each individually tested fig wasp for 10 min among three modalities: choice for odour, choice for control, or no choice. We considered that wasps made no choice when they stayed motionless for 3 min in the departure section and/or the central arm before the bifurcation of the olfactometer. All the adult female fig wasps were newly emerged from male figs. For each experiment, we used binomial tests to compare the number of choices for odour versus choices for no odour or other odour (excluding the no-choice response).

Results

Variation in scent profiles

The chemical composition of the odours emitted by receptive *F. triloba* figs is summarised per location in Table 1. GC-MS analysis revealed 46 compounds, with 20 compounds shared by all the locations. Based on their biosynthetic origin (Knudsen 2006), the detected compounds fell into three distinct chemical classes: fatty acid derivatives, monoterpenes, and

sesquiterpenes. The odours comprised 3 fatty acid derivatives, 8 monoterpenes, and 28 sesquiterpenes, and 7 compounds could not be identified. Ten compounds represented more than 5% of the odours in at least one location, namely α-cubebene, cyclosativene, α-copaene, β-cubebene, cis-thujopsene, E- β-farnesene, β-caryophyllene, α-muurolene, germacrene D, δ-cadinene and unknown 6. All these compounds were also found at least once in *F. hirta* receptive fig odours (Deng et al. 2021).

Table 1 Volatile organic compounds emitted by receptive figs of *F. triloba* (mean percentages) in four study sites (mean ± SD per site). RI = Retention Index; Occ = occurrence of each VOC, total and by population; N = number of sample

	RI	Shimen		SCBG		DHS		XTBG	
		Occ	N=13	Occ	N=3	Occ	N=15	Occ	N=4
Fatty acid derivatives									
1005	(E)-3-hexenyl acetate	9	0.77±1.84	2	0.24±0.24	5	1.47±3.92	4	1.26±1.2
1102	nonanal	6	0.38±0.57	0		1	0±0.01	0	
1203	decanal	3	0.04±0.1	0		1	0±0.02	0	
	sum		1.19		0.24		1.47		1.26
Monoterpenes									
934	α-pinene	3	0.02±0.05	0		1	0±0.02	1	0.01±0.01
973	sabinene	2	0±0.01	0		1	0±0.02	0	
979	β-pinene	0		0		1	0±0	0	
984	6-methyl-5-hepten-2-one	7	0.16±0.2	2	0.13±0.11	7	0.17±0.31	2	0.39±0.74
991	β- myrcene	3	0.01±0.03	0		5	0.18±0.31	0	
1030	limonene	7	0.73±1.2	2	4.56±6.9	6	0.18±0.42	2	0.47±0.84
1048	cis-β-ocimene	2	0.03±0.07	0		0		0	
1101	linalool	4	0.03±0.09	0		2	0.7±2.61	0	
	sum		0.98		4.69		1.23		0.87
Sesquiterpenes									
1343	δ-elemene	10	0.92±1.46	1	0.12±0.21	15	1.78±1.61	4	1.25±0.06
1355	α-cubebene	10	1.16±1.7	3	7.41±2.11	8	0.92±2.4	4	5.4±0.91
1365	α-ylangene	9	1.11±1.06	0		8	0.66±1.3	1	0.01±0.02
1375	cyclosativene	11	1.49±1.99	3	5.05±1.83	6	1.21±3.08	4	0.37±0.1
1382	Isoledene	9	0.38±0.54	0		7	2.79±8.58	0	
1384	α-copaene	11	9.83±14.8	3	41.25±6.86	15	7.11±16.88	4	54.21±7.5
1392	β-bourbonene	11	1±0.85	3	0.6±0.3	9	0.36±0.49	4	0.05±0.01
1387	β-cubebene	8	0.92±1.12	3	10.65±1.45	5	0.68±1.52	4	2.63±0.93
1389	β-elemene	6	0.52±0.8	1	0.04±0.06	11	1.5±1.67	3	1.34±2.63
1410	α-cis-bergamotene	3	0.3±0.71	0		1	0.1±0.38	0	
1425	α-cedrene	11	0.13±0.13	0		6	0.18±0.32	0	
1427	α-gurjunene	4	0.1±0.2	0		7	0.25±0.91	3	0.03±0.02

1429	cis-thujopsene	10	18.32±16.46	0		5	15.45±25.21	0	
1430	β-caryophyllene	13	21.47±8.68	3	12.92±2.07	11	27.16±21.18	4	14.15±4.05
1435	β-copaene	11	1.21±1.31	2	0.47±0.47	11	2.97±2.27	4	2.21±0.84
1440	α-trans-bergamotene	2	0.02±0.04	3	0.31±0.15	2	0.01±0.02	0	
1446	Z-β-farnesene	0		0		4	0.33±0.7	0	
1454	alloaromadendrene	5	0.3±0.43	0		9	0.91±0.82	3	0.51±0.41
1457	E-β-farnesene	13	6.74±3.65	2	0.76±0.66	13	4.87±4.57	4	1.26±1.35
1463	humulene	8	2.73±2.99	1	0.35±0.6	11	4.71±3.62	3	1.48±1.02
1482	γ-muurolene	13	1.55±0.56	3	1.75±0.25	12	3.79±5.64	0	
1488	germacrene D	11	6.55±6.42	2	2.68±3.01	12	8.92±8.18	4	5.33±2.12
1494	α-selinene	5	0.11±0.19	0		1	0.28±1.09	1	0.09±0.18
1503	α-bulnesene	6	0.15±0.26	0		10	1.23±1.83	0	
1505	α-muurolene	13	5.16±4.5	3	2.75±0.62	10	2.67±2.64	4	1.99±0.44
1510	β-bisabolene	4	0.72±1.66	2	0.77±0.69	2	0.11±0.39	0	
1520	γ-cadinene	10	0.54±0.72	2	0.34±0.32	15	1.49±2.14	4	0.03±0.02
1528	δ-cadinene	10	3.19±3.82	3	5.55±0.73	11	1.99±1.47	3	2.48±1.78
	sum		86.62		93.77		94.43		94.82
unknown compound									
1318	Unknown1	6	0.48±0.98	0		2	0.04±0.11	0	
1359	Unknown2	4	0.1±0.29	0		0	0±0	0	
1378	Unknown3	5	0.36±0.9	0		4	0.1±0.19	0	
1395	Unknown4	10	0.83±0.68	0		9	0.45±0.87	1	0.01±0.02
1451	Unknown5	10	1.59±1.36	0		2	0.31±1.17	0	
1465	Unknown6	13	7.11±3.52	3	1.31±0.25	13	1.86±1.49	4	3.04±0.23
1480	Unknown7	10	0.73±1.13	0		7	0.11±0.2	2	0.01±0.01
	sum		11.2		1.31		2.87		3.06

The NMDS (stress = 0.123) on the *Ficus triloba* dataset (i.e., relative proportions of each VOCs in the odour emitted by each studied sample) showed that, while many point overlapped among locations, the odours of receptive figs differed among locations (Fig. 1; PERMANOVA: $F_{(3;34)} = 4.08$, $P=0.001$). Pairwise comparisons using permutation MANOVAs on a distance matrix by locality showed differences between all the localities ($P<0.05$). The results within location at Shimen and even more at DHS presented a large variance. The three samples of SCBG collected from the same tree suggests small odour variation within trees.

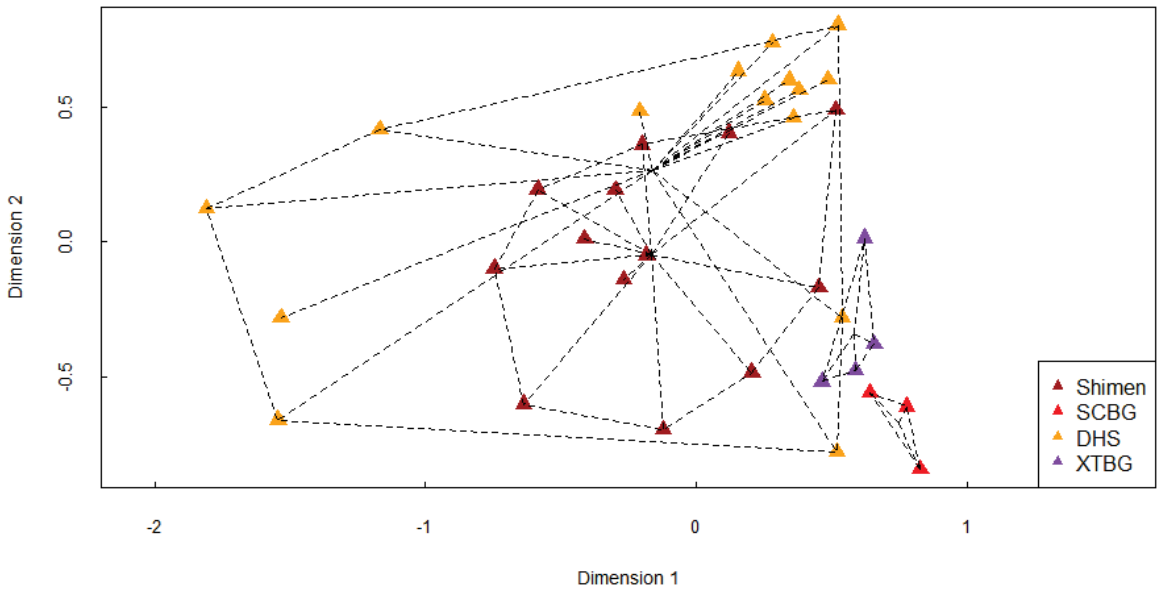


Fig. 1 Non-metric multi-dimensional scaling representation of the relative proportions of VOCs in the odours emitted by individual plants of *Ficus triloba* based on Bray-Curtis dissimilarity Index (stress=0.123).

Inter-specific variation in the chemical message emitted by receptive figs

All the compounds representing more than 5% of the odour of *F. triloba* in at least one location were also detected in the odours produced by at least one individual of *F. hirta*. Reciprocally all the compounds representing more than 5% of the odour of *F. hirta* in at least one location were also detected in at least one individual of *F. triloba* (Table 2). In contrast, out of these 17 compounds, 12 were not detected in *F. hispida* odours, while 5 compounds representing over 5% of receptive fig odours of *F. hispida* in at least one location where not detected in *F. triloba* and *F. hirta* odours. In agreement, in the NMDS plot there was a large overlap between *F. triloba* and *F. hirta* odours, while *F. hispida* was separated (Fig. 2a).

Nevertheless, receptive fig odour differed between *F. hirta* and *F. triloba* (PERMANOVA, $F_{(1,79)}$ =9.65, P =0.001, Fig. 2b) despite 28 shared compounds (Table 1; Deng et al. 2021).

Table 2. Volatile Organic Compounds representing more than 5% of the odours in a least one location in at least one species. Note the strong difference between *F. triloba-hirta* and *F. hispida*

species	locality	limonene	linalool	α-cubebene	cyclosativene	α-copaene	β-cubebene	β-elemene	α-cedrene	cis-Thujopsene	β-caryophyllene	E-β-farnesene	humulene	Unknown6	Unknown5	germacrene D	α-muurolene	δ-cadinene	(Z)-3-Hexenyl acetate	β-Myrcene	E)-β-Ocimene	α-Copaene	Unknown c
F.hirta	Ning	0.1	0.0	3.1	1.7	23.3	1.5	1.7	0.1	2.9	29.1	1.2	4.9	2.7	3.6	5.9	1.7	2.6	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	Sha	0.1	0.0	0.0	0.2	1.7	0.0	3.5	0.0	0.8	44.6	0.6	7.2	0.9	9.2	5.8	1.6	1.1	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	Sui	2.5	0.0	0.8	1.7	5.6	0.4	2.2	0.0	0.0	34.0	1.3	4.6	1.7	7.2	12.4	2.8	1.4	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	SCBG	0.0	0.0	0.6	1.4	10.4	0.0	4.0	3.3	0.0	56.0	2.2	8.9	0.5	0.0	1.0	1.7	1.0	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	DHS	0.0	0.0	0.8	0.4	8.9	0.0	2.7	13.3	0.5	46.4	2.7	7.1	1.1	0.1	2.2	2.8	1.3	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	Nan	1.4	0.1	1.1	2.0	12.0	0.4	2.5	3.0	0.0	57.3	0.2	6.2	0.9	0.3	3.8	1.6	1.2	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	Ding	0.4	0.0	4.8	0.4	27.5	1.0	2.5	2.1	0.0	29.8	0.4	4.8	3.3	0.2	5.0	3.6	4.1	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	Wan	9.5	5.2	0.7	1.2	10.6	0.1	1.9	5.2	0.0	40.7	2.7	6.1	1.8	0.3	2.2	1.8	2.6	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.hirta	XTBG	2.4	8.9	0.2	0.8	3.0	0.0	18.3	0.0	0.0	22.9	1.2	0.3	1.2	0.0	9.1	5.5	1.5	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
F.triloba	shimen	1.3	0.0	0.7	1.3	6.3	0.7	0.5	0.2	21.0	19.7	6.6	2.4	8.2	0.6	7.0	5.6	4.9	**0.0**	**0.0**	**0.0**	**0.0**	
F.triloba	SCBG	4.6	0.0	7.4	5.0	41.2	10.7	0.0	0.0	0.0	12.9	0.8	0.3	1.3	0.0	2.7	2.7	5.5	**0.0**	**0.0**	**0.0**	**0.0**	
F.triloba	DHS	0.3	0.0	0.1	3.2	2.2	0.3	2.2	0.2	0.0	40.3	2.8	7.2	2.1	0.2	16.8	3.5	2.7	**0.0**	**0.0**	**0.0**	**0.0**	
F.triloba	XTBG	0.5	0.0	5.4	0.4	54.2	2.6	1.3	0.0	0.0	14.2	1.3	1.5	3.0	0.0	5.3	2.0	2.5	**0.0**	**0.0**	**0.0**	**0.0**	
F.hispida	SCBG	0.9	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	8.4	**0.0**	**0.0**	14.8	3.2	4.6	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	20.3	4.9	4.1	7.1	4.3
F.hispida	Nan	6.3	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	22.2	**0.0**	**0.0**	31.5	2.4	6.2	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	0.0	0.0	7.0	9.6	0.4
F.hispida	Ding	0.0	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	3.9	**0.0**	**0.0**	31.3	3.8	6.4	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	0.7	5.7	5.4	14.1	0.1
F.hispida	XTBG	1.0	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	38.6	**0.0**	**0.0**	8.0	15.3	2.4	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	7.8	2.0	1.6	2.8	5.4
F.hispida	Thailand	0.4	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	4.7	**0.0**	**0.0**	4.2	9.8	1.0	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	20.5	7.0	31.7	2.2	0.1

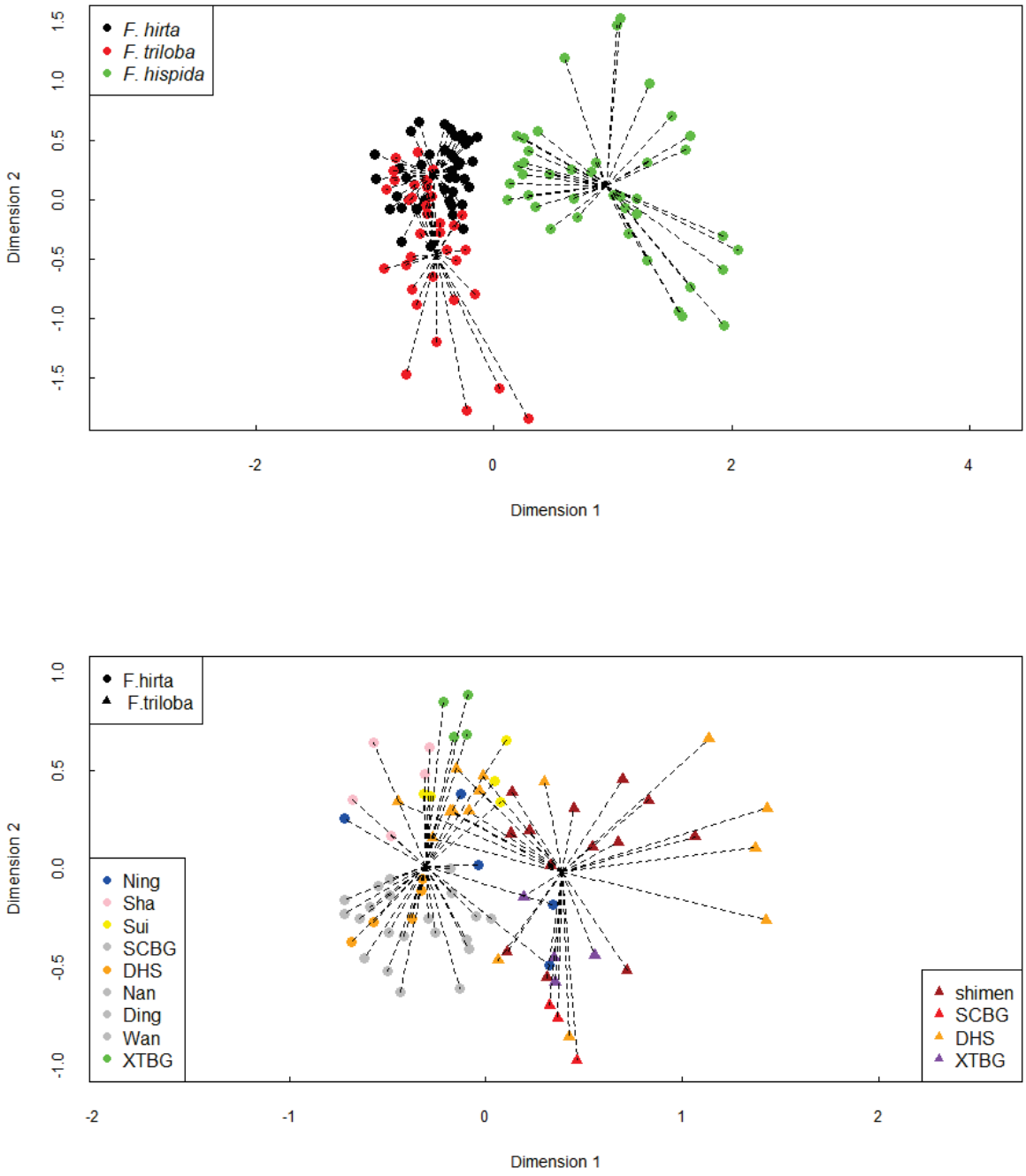


Fig. 2 Comparison of receptive fig odours among species. 2a: comparison between *Ficus triloba*, *F. hirta* and *F. hispida*. 2b comparison between *F. triloba* and *F. hirta*. Non-metric multi-dimentional scaling representation of the relative proportions of VOCs in the odors emitted by individual plants based on Bray-Curtis dissimilarity Index (stress=0.18 for 2a and stress= 0.20 for 2b).

The geographic variation in receptive fig odours of *F. triloba* and *F. hirta* is illustrated in Fig. 3. There is no obvious correlation between variation in the two species.

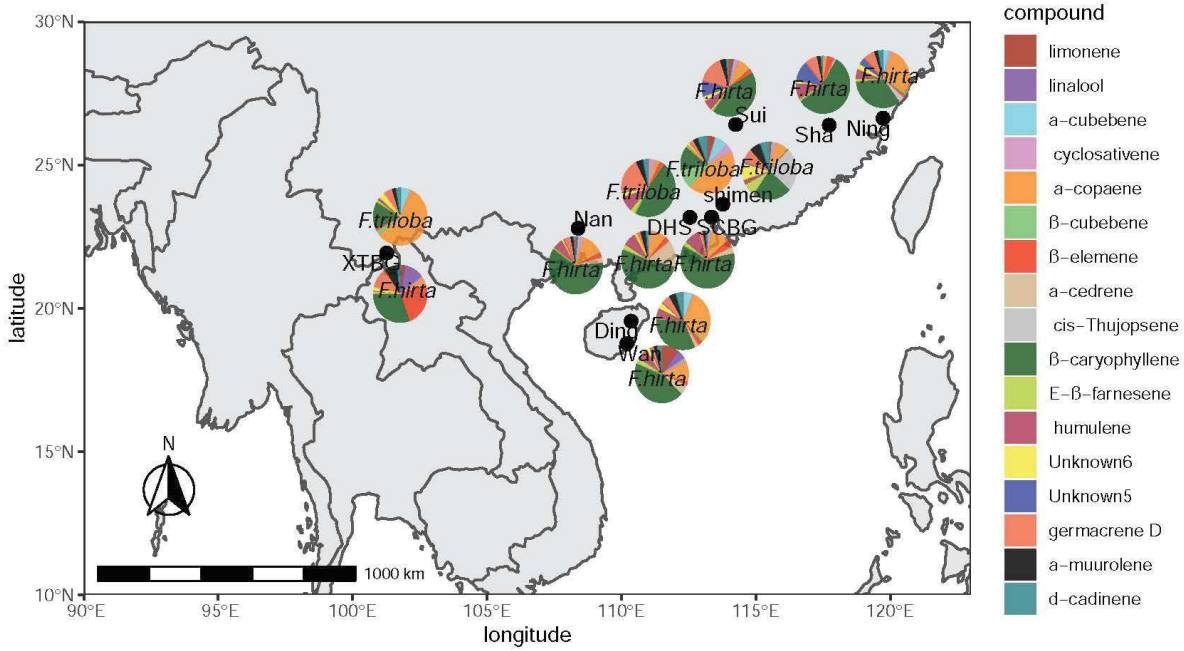


Fig. 3 Geographic variation in receptive fig odour fig odour composition for *Ficus hirta* and *Ficus triloba*.

Insect behavioral tests

Results of Y-tube olfactometer tests are presented in Fig 4. When given the choice between the odour of receptive figs against cleaned air, both *V. javana hilli* and *V. esquirolianae* were attracted by odours emitted by the receptive fig odour of their host species (binomial test, $P<0.005$, $n = 36$ and $P<0.001$, $n = 41$, respectively) and they were not attracted by the receptive fig odours of the other species (binomial test, $P=0.253$; $n = 49$, and $P=0.323$, $n = 37$, respectively).

However, when *Valisia javana hilli* was given the choice between the odours emitted by receptive figs of its host plant *F. hirta*, and those emitted by *F. triloba*, it was equally attracted by the odours emitted by receptive figs of the two species (binomial test, $P=1$; $n = 42$), while *Valisia esquirolianae* tended to be more attracted by the odours of *F. triloba* (two-tailed

binomial test, P=0.066; n = 43).

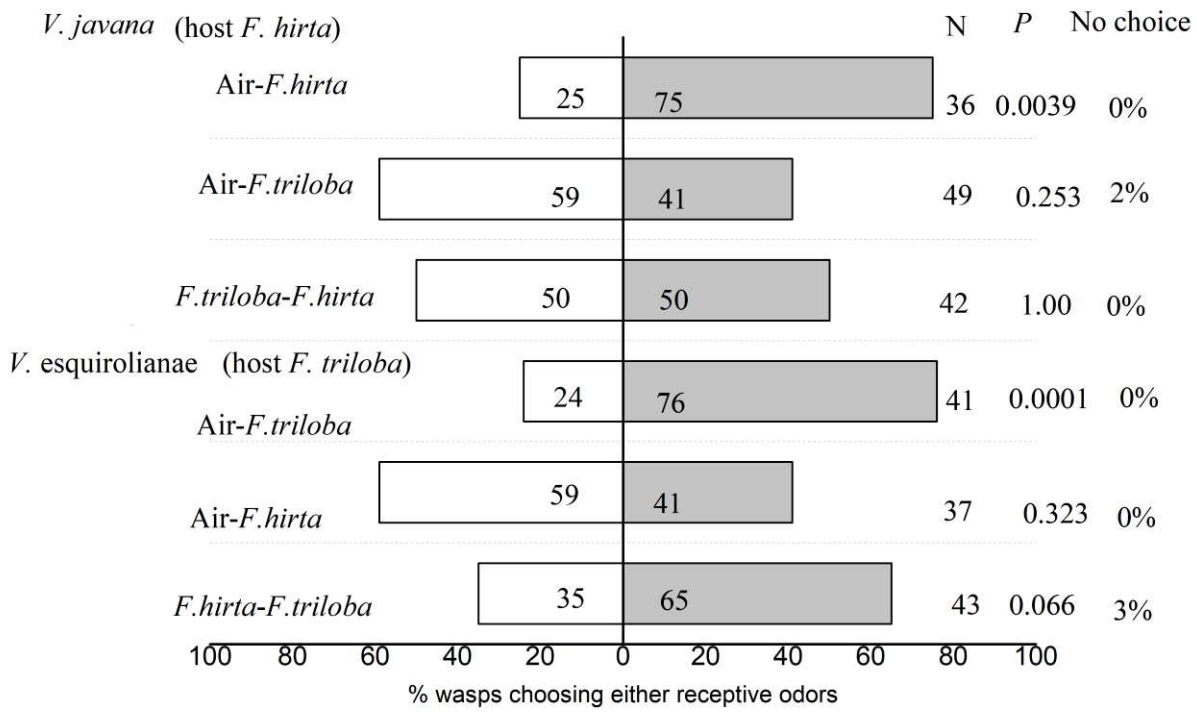


Fig 4. Choices made by *Valisia javana hilli* and *V. esquirolianae* when confronted with different odour sources (receptive figs) in a Y-tube olfactometer. We used binomial tests for statistical comparisons between the number of choices for odour versus clean air or choice between odours of the two *Ficus* species. N: number of tested wasps. P: probability, two tailed.

Discussion

Despite significant differences in receptive fig odours between *F. hirta* and *F. triloba*, there was a large overlap in the VOCs constituting these odours. All the compounds present at a concentration above 5% in at least one location in one species were also detected in the other species. This overlap was much more marked than with the VOCs constituting the receptive fig odours of *F. hispida* suggesting an effect of phylogenetic distance. A similar situation was observed for the species group of *Ficus itoana* (subgenus *Sycomorus*) in Papua New-Guinea, with overlap of receptive figs odours of the species group on an NMDS plot and separation

from other species of subgenus *Sycomorus* belonging to other sections (Souto-Vilarós et al. 2018).

The similarity in VOCs constituting the odours suggests that the pollinating wasps of both *F. hirta* and *F. triloba* are capable of detecting at least some of the VOCs composing the receptive fig odour of the other *Ficus* species. On the other hand fig-pollinating wasps have a limited repertoire of olfactory genes (Wang et al. 2021). The wasps may not have the olfactory receptors allowing them to detect the VOCs constituting receptive fig odours of *F. hispida*. However, detection of the VOCs emitted by receptive figs is different attraction by these figs. Indeed, the relative proportions of the VOCs determine whether wasps are attracted (Proffit et al. 2020). The difference between receptive fig odours in *F. hirta* increased with geographic distance (Deng et al. 2021) while the difference between *F. hirta* and *F. triloba* receptive fig odours was independent of geographic distance. This suggests lack of interference between the two species in the local evolution of their receptive fig odours. We suggest that independent odour variation in the two species leads to occasional situations of local overlap of the part of the chemical message detected by one or the other species of wasp.

In the Y tube experiment, when wasps were given the choice between air and receptive fig odours, they were attracted by their host species' figs and were not attracted by non-host figs. However, when given a choice between receptive fig odours from the two species, *Valisia javana hilli* was attracted by figs of both species, while the response of *V. esquiroliana* was intermediate with a trend towards preferring their host's figs. In these experiments, during their passage through the main arm of the olfactometer, the wasps were exposed to a mix of receptive fig odours of the two species. We propose that during this exposition, *F. hirta* odours stimulate *Valisia javana hilli* so that it subsequently responds to the previously non-attractive odour of *F. triloba*.

The attraction of host specialist pollinating wasps by receptive figs of closely related *Ficus*

species has previously been investigated in Y tube olfactometer experiments for three situations. *Ficus boninsimae* and *F. nishimurae* are two very closely related species co-occurring in the Ogasawara islands, Japan. *Ficus boninsimae* is an open habitat species while *F. nishimurae* is an understory tree. In Y tube experiments, pollinators of *F. boninsimae* were equally attracted by figs of *F. boninsimae* and *F. nishimurae*, while the pollinators of *F. nishimurae* were more attracted by *F. nishimurae* fig odours (Yokoyama 2003). In Papua New Guinea, the closely related *F. microdyctia*, *F. sp.* and *F. itoana* replace each other along an altitudinal gradient. Their receptive fig odours overlap in an NMDS plot. In Y tube test against air, the pollinator of *Ficus sp.* was attracted by fig odours of *F. sp* and of *F. microdyctia*, but not those of *F. itoana*. The pollinator of *F. itonana* was attracted by receptive fig odours of *F. itoana*, but not those of the two other species. Finally, the pollinator of *F. microdyctia* was avoiding the odours of receptive figs of *F. sp.* and *F. itoana* (Souto-Vilarós et al. 2018). *Ficus semicordata semicordata* and *F. s. montana* co-occur from Nepal to Laos through South-China but have distinct habitats (Wang et al. 2013). Receptive fig odours of *Ficus s. semicordata* is mainly constituted by a highly unusual compound, p-methylanisole (Chen et al. 2009), and this compound was also found in receptive fig odours of *F. s. montana* inviduals. Pollinators of *F. s. semicordata* were preferentially attracted by their host species when given a choice, but when given no choice, they were attracted by non-host figs. Pollinators of *F. s. montana* were equally attracted by receptive figs of the two varieties. Finally, the ranges of *Ficus auriculata*, *F. oligodon* and *F. hainanensis* which form a species complex, overlap throughout continental Asia but they occupy distinct habitats (Wang et al. 2016). They share pollinators throughout their regions of co-occurrence and the receptive fig odours of *F. auriculata* and *F. oligodon* were not distinguishable (Wang et al. 2016).

Hence, in all investigated sister *Ficus* species that occur in sympatry, the species occupy different habitat and their receptive fig odours present similarities. Generally, they do not

share pollinators, but their pollinators may be attracted by non-host receptive figs in Y tube experiments, following variable modalities and directionality. There is no evidence supporting selection for divergence in olfactive signalling between these closely related *Ficus* species and there is no evidence supporting selection on the wasps to use several hosts. All the investigated cases involve dioecious *Ficus* species, in which pollinator dispersal is limited (Harrison and Rasplus 2006). Hence, for dioecious *Ficus* species, habitat differentiation between closely related species may constitute the main barrier to gene flow between species. Pollinator specificity is a complementary force, but it is leaky. As such, *Ficus* follow the general pattern of separation between closely related species in the tree of life (Hernandez-Hernandez et al. 2021).

On islands, small population sizes may lead to local extinctions of pollinators. In such situations, because of the limited barriers to wasps detecting receptive figs of close relatives of their usual host species, recolonization of a *Ficus* species by pollinators of a close relative is expected. This is the case in Taiwan where *Blastophaga nipponica* pollinates *Ficus erecta* as elsewhere, but distinct host-races of *B. nipponica* pollinate the more localised *F. formosana* and *F. tannoensis*. (Wachi et al. 2016). In an artificial situation in Hawaii, *Ficus rubiginosa* was introduced with its pollinator *Pleistodontes imperialis*. *Ficus watkinsiana*, a close relative of *F. rubiginosa* was also introduced. It is now beginning to be pollinated by *P. imperialis*, while in their native range the two *Ficus* species co-occur and are pollinated by different wasp species (Bernard et al. 2020). Hence, the barrier to colonisation of closely related host species by a same wasp species could often be competition with the established populations of pollinating wasp. Reciprocally, when a *Ficus* species is introduced into a part of the world where no closely related species sustains a population of wasps, it will remain unpollinated as long as its pollinator is not introduced (Compton et al. 2019). Within this perspective, specialised pollination in *Ficus* may limit their invasiveness when introduced into new parts

of the world as long as pollinators from their continent of origin are not introduced.

Acknowledgments

Xiaoxia Deng was supported by a grant from the China Scholarship Council No. (2017)3109.

References

Berg, C. C. 2007. Precursory taxonomic studies on *Ficus* (Moraceae) for the Flora of Thailand. Thai Forest Bulletin (Botany) **35**:4-28.

Berg, C. C., and E. J. H. Corner. 2005. Moraceae: Ficeae. Flora Malesiana, Series I **17**:1-70.

Bernard, J., K. C. Brock, V. Tonnell, S. K. Walsh, J. P. Wenger, D. Wolkis, and G. D. Weiblen. 2020. New species assemblages disrupt obligatory mutualisms between figs and their pollinators. Frontiers in Ecology and Evolution **8**.

Brookes, D. R., J. P. Hereward, L. I. Terry, and G. H. Walter. 2015. Evolutionary dynamics of a cycad obligate pollination mutualism - Pattern and process in extant *Macrozamia* cycads and their specialist thrips pollinators. Molecular Phylogenetics and Evolution **93**:83-93.

Chen, C.-H., and L.-Y. Chou. 1997. The Blastophagini of Taiwan (Hymenoptera: Agaonidae: Agaoninae). Journal-Taiwan Museum **50**:113-154.

Chen, C., Q. Song, M. Proffit, J.-M. Bessière, Z. Li, and M. Hossaert-McKey. 2009. Private channel: a single unusual compound assures specific pollinator attraction in *Ficus semicordata*. Functional Ecology **23**:941-950.

Compton, S. G., M. Stavrinides, C. Kaponas, and P. J. Thomas. 2019. No escape: most insect colonisers of an introduced fig tree in Cyprus come from the plant's native range. Biological Invasions **22**:211-216.

Cook, J. M., and S. T. Segar. 2010. Speciation in fig wasps. Ecological Entomology **35**: 54-66.

Cruaud, A., N. Rønsted, B. Chantarasuwan, L. S. Chou, W. L. Clement, A. Couloux, B. Cousins, G. Genson, R. D. Harrison, P. E. Hanson, M. Hossaert-mckey, R. Jabbour-zahab, E. Jousselin, C. Kerdelhué, F. Kjellberg, C. Lopez-vaamonde, J. Peebles, Y.-q. Peng, R. A. S. Pereira, T. Schramm, R. Ubaidillah, S. V. Noort, G. D. Weiblen, D.-r. Yang, A. Yodpinyanee, R. Libeskind-hadas, J. M. Cook, J.-y. Rasplus, and V. Savolainen. 2012. An extreme case of plant-insect codiversification: figs and fig-pollinating wasps. Systematic Biology **61**:1029-1047.

Deng, X., B. Bruno, P. Yan-Qiong, H. Yu, C. Yufen, K. Finn, and P. Magali. 2021. Plants are the drivers of geographic variation of floral scents in a highly specialized pollination mutualism: a study of *Ficus hirta* in China.

Dötterl, S., L. M. Wolfe, and A. Jürgens. 2005. Qualitative and quantitative analyses of flower scent in Silene latifolia. Phytochemistry **66**:203-213.

Harrison, R. D. 2005. Figs and the diversity of tropical rainforests. AIBS Bulletin **55**:1053-1064.

Harrison, R. D., and J.-Y. Rasplus. 2006. Dispersal of fig pollinators in Asian tropical rain forests. Journal of Tropical Ecology **22**:631-639.

Hernandez-Hernandez, T., E. C. Miller, C. Roman-Palacios, and J. J. Wiens. 2021. Speciation across the Tree of Life. Biol Rev Camb Philos Soc.

Hossaert-McKey, M., C. Soler, B. Schatz, and M. Proffit. 2010. Floral scents: their roles in nursery pollination mutualisms. Chemoecology **20**:75-88.

Hu, R., P. Sun, H. Yu, Y. Cheng, R. Wang, X. Chen, and F. Kjellberg. 2020. Similitudes and differences between two closely related *Ficus* species in the synthesis by the ostiole of odors attracting their host-specific pollinators: A transcriptomic based investigation. Acta Oecologica **105**:103554.

Jiang, Z. F., D. W. Huang, C. D. Zhu, and W. Q. Zhen. 2006. New insights into the phylogeny of fig pollinators using Bayesian analyses. Molecular Phylogenetics and Evolution **38**:306-315.

Knudsen, J. T. 2006. Diversity and distribution of floral scent. The botanical review **72**:1-120.

Kuaraksa, C., S. Elliott, and M. Hossaert-Mckey. 2012. The phenology of dioecious *Ficus* spp. tree species and its importance for forest restoration projects. Forest Ecology and Management **265**:82-93.

Kyogoku, D., and H. Kokko. 2020. Species coexist more easily if reinforcement is based on habitat preferences than on species recognition. Journal of Animal Ecology **89**:2605-2616.

Lami, F., I. Bartomeus, D. Nardi, T. Beduschi, F. Boscutti, P. Pantini, G. Santoiemma, C. Scherber, T. Tscharntke, and L. Marini. 2021. Species-habitat networks elucidate landscape effects on habitat specialisation of natural enemies and pollinators Ecology Letters **24**:288-297.

Lu, J., P. Gui, H.-q. Li, Z.-L. Lu, L.-f. Zhang, H.-z. Tian, and M. G. Gilbert. 2016. Phylogenetic analysis and taxonomic delimitation of the “hairy-fig” complex of Ficus sect. *Eriosycea* (Moraceae) in China. Phytotaxa **261**:121-136.

Okamoto, T., and Z.-H. Su. 2021. Chemical analysis of floral scents in sympatric *Ficus* species: highlighting different compositions of floral scents in morphologically and phylogenetically close species. Plant Systematics and Evolution **307**.

Oksanen, J., F. G. Blanchet, M. Friendly, R. Kindt, P. Legendre, D. McGlinn, P. R. Minchin, R. B. O'Hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, E. Szoecs, and H. Wagne. 2013. Package ‘vegan’.Community Ecology Package. Acta Oecologica **2**:1-295.

Proffit, M., C. Chen, C. Soler, J. M. Bessière, B. Schatz, and M. Hossaert-McKey. 2009. Can chemical signals, responsible for mutualistic partner encounter, promote the specific exploitation of nursery pollination mutualisms?–The case of figs and fig wasps. Entomologia Experimentalis et Applicata **131**: 46-57.

Proffit, M., B. Lapeyre, B. Buatois, X. Deng, P. Arnal, F. Gouzerh, D. Carrasco, and M. Hossaert-McKey. 2020. Chemical signal is in the blend: bases of plant-pollinator encounter in a highly specialized interaction. Scientific reports **10**:1-11.

Rodriguez, L. J., A. Bain, L.-S. Chou, L. Conchou, A. Cruaud, R. Gonzales, M. Hossaert-McKey, J.-Y. Rasplus, H.-Y. Tzeng, and F. Kjellberg. 2017. Diversification and spatial structuring in the mutualism between *Ficus septica* and its pollinating wasps in insular South East Asia. BMC Evolutionary Biology **17**:207.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: theory and data. Annual Review of Ecology, Evolution, and Systematics **34**:339-364.

Soler, C. C., M. Proffit, J. M. Bessiere, M. Hossaert-McKey, and B. Schatz. 2012. Evidence for intersexual chemical mimicry in a dioecious plant. Ecol Lett **15**:978-985.

Souto-Vilarós, D., M. Proffit, B. Buatois, M. Rindos, M. Sisol, T. Kuyaiva, B. Isua, J. Michalek, C. T. Darwell, M. Hossaert-McKey, G. D. Weiblen, V. Novotny, and S. T. Segar. 2018. Pollination along an elevational gradient mediated both by floral scent and pollinator compatibility in the fig and fig-wasp mutualism. Journal of Ecology **106**:2256-2273.

Wachi, N., J. Kusumi, H. Y. Tzeng, and Z. H. Su. 2016. Genome-wide sequence data suggest the possibility of pollinator sharing by host shift in dioecious figs (Moraceae, *Ficus*). Molecular Ecology **25**:5732-5746.

Wang, G., C. H. Cannon, and J. Chen. 2016. Pollinator sharing and gene flow among closely related sympatric dioecious fig taxa. Proceedings of the Royal Society B: Biological Sciences **283**:20152963.

Wang, G., S. G. Compton, and J. Chen. 2013. The mechanism of pollinator specificity between two sympatric fig varieties: a combination of olfactory signals and contact cues. Annals of Botany **111**:173-181.

Wang, R., Y. Yang, Y. Jing, S. T. Segar, Y. Zhang, G. Wang, J. Chen, Q.-F. Liu, S. Chen, Y. Chen, A. Cruaud, Y.-Y. Ding, D. W. Dunn, Q. Gao, P. M. Gilmartin, K. Jiang, F. Kjellberg, H.-Q. Li, Y.-Y. Li, J.-Q. Liu, M. Liu, C. A. Machado, R. Ming, J.-Y. Rasplus, X. Tong, P. Wen, H.-M. Yang, J.-J. Yang, Y. Yin, X.-T. Zhang, Y.-Y. Zhang, H. Yu, Z. Yue, S. G. Compton, and X.-Y. Chen. 2021. Molecular mechanisms of mutualistic and antagonistic interactions in a plant–pollinator association. Nature Ecology & Evolution 1-13.

Yokoyama, J. 2003. Cospeciation of figs and fig-wasps: a case study of endemic species pairs in the Ogasawara Islands. Population ecology **45**: 249-256.

Yu, H., D. Liang, E. Tian, L. Zheng, and F. Kjellberg. 2018. Plant geographic phenotypic variation drives diversification in its associated community of a phytophagous insect and its parasitoids. BMC Evolutionary Biology **18**:134.

Yu, H., Y. Liao, Y. Cheng, K. Fushi, J. Yongxia, and S. Compton. 2021. The extent of pollinator sharing among fig trees in southern China.

Yu, H., E. Tian, L. Zheng, X. Deng, Y. Cheng, L. Chen, W. Wu, W. Tanming, D. Zhang, S. G. Compton, and F. Kjellberg. 2019. Multiple parapatric pollinators have radiated across a continental fig tree displaying clinal genetic variation. Molecular Ecology **28**:2391-2405.

Yu, Hui, Nan-Xian Zhao, Yi-Zhu Chen, Yuan Deng, Jin-Yan Yao, and Hua-Gu Ye. 2006. Phenology and reproductive strategy of a common fig in Guangzhou. Botanical studies **47**:435-441.

Yu, H., N. Zhao, Y. Chen, and E. A. Herre. 2008. Male and female reproductive success in the dioecious fig, *Ficus hirta* Vahl. in Guangdong Province, China: Implications for the relative stability of dioecy and monoecy. Symbiosis **45**:121.

Zhou, Z.K. & Gilbert, M.G. 2003. Moraceae: *Ficus*. In: Wu, C.Y., Raven, P.H. & Hong, D.Y. (eds.) Flora of China. Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, pp. 37-71.