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Research Article

Population genetic structure in three sympatric *Ficus* species associated with copollinator

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Abstract Understanding the factors contributing to genetic structure among closely related sympatric species is crucial for grasping adaptive divergence and speciation initiation. We focused on three dioecious fig trees (*Ficus hispida*, *Ficus heterostyla*, and *Ficus squamosa*) that constitute a clade of closely related species pollinated by closely related *Ceratosolen* wasps. Analyzing microsatellite data (64 sampling locations) and chemical volatiles for fig trees and inferring the phylogenetic relationships of their pollinating wasps, we show that despite sharing of a large proportion of volatile compounds and a few exchanges of pollinators, all species maintain genetic and morphological integrity. Admixture of *F. heterostyla* and *F. hispida* in *F. squamosa* is detected at its distribution margin. Two genetically distinct clusters of *F. heterostyla*, possibly indicating cryptic fig species pollinated by distinct pollinators, are highlighted. *Ficus hispida* is genetically homogeneous over its studied range but associated with at least three pollinator species. Life history traits of each *Ficus* species (fruiting mode, population density, flowering pattern, habitat preference) and seed dispersal mode (hydrochorous, zoochorous) are discussed together with elements on the morphology and biology of their pollinators to explain observed results. This study contributes to our understanding of how species in the fig–wasp mutualism diversify and coexist.

Key words: *Ficus*, life history trait, pollinating wasp, population structure, volatile compound.

1 Introduction

Comprehending the genetic structure of populations and the underlying factors that influence it is essential for understanding the processes of species evolution, adaptation, and speciation, and is of paramount importance in formulating effective conservation strategies (Mayr, 1942; Avise, 2000; Frankham et al., 2002). In particular, gaining insights into the factors, such as life history traits, that contribute to the population genetic structure of closely related sympatric species is highly advantageous, as it enables the isolation of trait variation effects without the confounding influences of ecological traits or evolutionary histories (Williams et al., 2001). This understanding is also key to better understanding how species differentiate, coexist, and adapt, which are all fundamental questions in evolutionary biology (Coyne & Orr, 2004; Weber & Strauss, 2016).

Plant–pollinator interactions are essential to terrestrial ecosystems. They can boost the reproductive success of plants while reducing interspecific competition and foraging costs for pollinators (Chittka & Thomson, 1997; Waser & Ollerton, 2006). Coevolution between plants and insect pollinators is generally considered as a driver for the diversification of both groups (Labandeira et al., 1994; Ramos & Schiestl, 2019; van der Kooi & Ollerton, 2020). More precisely, reciprocal adaptation is expected to strengthen specialization between interacting partners and reciprocal specialization is supposed to have driven their divergence and speciation (Schemske & Bradshaw, 1999; Kay & Sargent, 2009). Specialist pollinated plants, with well-defined pollinators, allow for better control of variables and isolation of specific effects during investigations of population structure. Given this advantage, obligate pollination mutualisms are particularly relevant systems to investigate how genetic diversity is distributed among populations and its underlying determinants.

Pantropical *Ficus* (Moraceae), with over 800 described species, is one of the most diverse woody plant genera. It is distinguished by extraordinarily high species diversity both globally and locally, showcasing a broad spectrum of growth forms (trees, hemiepiphytes, shrubs, climbers) and significant ecological importance (Shanahan et al., 2001; Berg

& Corner, 2005; Harrison, 2005; Harrison & Shanahan, 2005; Gardner et al., 2023). Fig trees depend on tiny wasps (Agaonidae) for pollination, that, in turn, reproduce exclusively in the fig pistillate flowers (Cruaud et al., 2012). This intimate relationship has resulted in coadaptation of both partners regarding chemical attraction (Hossaert-Mckey et al., 2010, 2016), reproductive phenology (Patel, 1996; Chen et al., 2018) or partner identification, and anatomical compatibility (Verkerke, 1989; van Noort & Compton, 1996). These coadaptations reduce interspecific pollen transfer and contribute to the maintenance of high local species diversity in *Ficus*.

However, molecular evidence has revealed growing cases of breakdown in one-to-one matching between fig tree and wasp species (e.g., Molbo et al., 2003; Sun et al., 2011; Chen et al., 2012; Wang et al., 2016; Souto-Vilarós et al., 2019; Yu et al., 2019, 2021; Su et al., 2022), with an estimated proportion of approximately 30% of *Ficus* species that have multiple pollinators (copollinator) (Yang et al., 2015). This is mainly due to pollinator duplication (i.e., the wasps speciate, while the host fig does not), likely because *Ficus* generation time is longer than wasp generation time (Cook & Segar, 2010), and to a lesser extent, due to host shift (i.e., a wasp forms a host race on a new host, ultimately resulting in wasp speciation). The concept of prevalent hybridization in genus *Ficus* caused by frequent host shifts and heterospecific visitation (Machado et al., 2005; Wang et al., 2021a) raises the question of how closely related sympatric fig species maintain their species integrity, especially since documented heterospecific visitation is more common among them. A broad geographic spectrum study is essential to enable definitive conclusions on whether or not this is a general trend in the fig wasp mutualism. Cases of heterospecific visitation that can lead to interspecific pollination are primarily recorded in areas where closely related species of fig trees co-occur or where species complexes that are undergoing speciation are encountered (e.g., Rasplus, 1996; Kerdelhué et al., 1999; Moe et al., 2011; Wang et al., 2016; Souto-Vilarós et al., 2019; Yu et al., 2021, 2022; Su et al., 2022; Huang et al., 2023a, 2023b). Therefore, investigating the patterns of population structure and pollinator sharing among closely related sympatric species of fig trees seems relevant for gaining insight into how species diversify and coexist in this textbook example of plant–pollinator mutualism.

Here we focused on the dioecious *Ficus hispida* L.f., *Ficus heterostyla* Merr., and *Ficus squamosa* Roxb. (subgenus *Sycomorus* section *Sycocarpus*) that constitute a clade of closely related species pollinated by closely related wasps in the genus *Ceratosolen* (Cruaud et al., 2012; Fungjanthuek et al., 2022; Gardner et al., 2023). These fig trees coexist in the Oriental region and exhibit noticeable disparity in growth forms, fig placement, habitat preferences (Fig. 1), population density, and seed dispersers. Over its distribution range, *F. hispida* is historically known to be pollinated by at least two pollinating wasps (considered as subspecies) (Wiebes, 1963). However, during field sampling in the last 10 years, multiple morphospecies of wasps with distinct colors have been observed and molecular analyses confirmed this observation (Cruaud et al., 2017; Wong et al., 2023). Whether some of these morphospecies could be also associated with either *F. squamosa* or *F. heterostyla* seems possible but is not yet known. Heterospecific colonization of *F. squamosa* figs by the wasp species that usually pollinates *F. heterostyla* was documented in Xishuangbanna (southwest China) and experimental hybrid seeds were produced (Liu et al., 2015; Huang et al., 2023b). Therefore, these three fig species may represent a species complex with at least partial exchange of pollinating wasps on which studies exploring mechanisms of genetic diversity distribution among populations seem relevant.

In this study, by analyzing microsatellite data, attractant chemical volatiles, and life history traits from the fig species and by inferring the phylogenetic relationships of their pollinating wasps, we aimed to: (1) reveal the patterns of genetic and chemical divergence among and possibly within the three focal fig species; (2) investigate patterns of copollinators; and (3) explore factors that influenced the population structure in this complex.

2 Material and Methods

2.1 Fig tree species

Ficus hispida is a small tree (up to 15 m) that ranges from India to Australia. The species is found in various habitats, including evergreen and mixed deciduous forests, dry forests, forests along streams or river edges, and also appears frequently in secondary growth areas. Figs are predominantly cauliflorous or flagelliflorous on short branchlets arising from the main branches or the trunk (Berg & Corner, 2005) (Figs. 1A, 1B). *Ficus heterostyla* is morphologically related to *F. hispida* and was considered as a distinct species by Berg & Chantarasuwan (2007) mostly because of striking differences in the position of figs and the presence of brown indumentum on leaves and stems. It grows in the understorey of evergreen forests or sometimes in secondary growth areas, ranging from southwest China to southern Vietnam. Figs are located on elongated stolons running on or under the soil (Berg & Chantarasuwan, 2007) (Figs. 1C, 1D). *Ficus squamosa* is a compact rheophytic shrub up to 2–3 m tall with a creeping stem. The species ranges from northern India to southern Thailand and thrives abundantly in forests along riverbanks or near streams. It exhibits unusually long persistent styles featuring short, stiff retrorse hairs that facilitate seed attachment to the substrate (Berg & Corner, 2005). The figs originate on branches near the water level or even below (Pothasin et al., 2016) (Fig. 1E). *Ficus heterostyla* and *F. squamosa* co-occur from southern Thailand to southwest China. They are distributed within the ranges of *F. hispida* (Fig. 1).

2.2 Fig tree population sampling, microsatellite genotyping, and processing

2.2.1 Fig tree population sampling

Leaves were sampled from 311 individuals belonging to 30 locations of *F. hispida*, 250 individuals in 21 locations of *F. heterostyla* and 143 individuals in 13 locations of *F. squamosa* (Fig. 1; Table S1). Our sampling encompasses most of continental southeast Asia from southern China and Myanmar to Thailand, Laos, Vietnam, and Cambodia.

2.2.2 Microsatellite genotyping

We developed a set of 19 nuclear microsatellite loci for the three targeted species of fig trees and 14 of them (3-N173, 4-101, 5-N108, 6-N104, 7-N245, 19-N530, 20-N291,

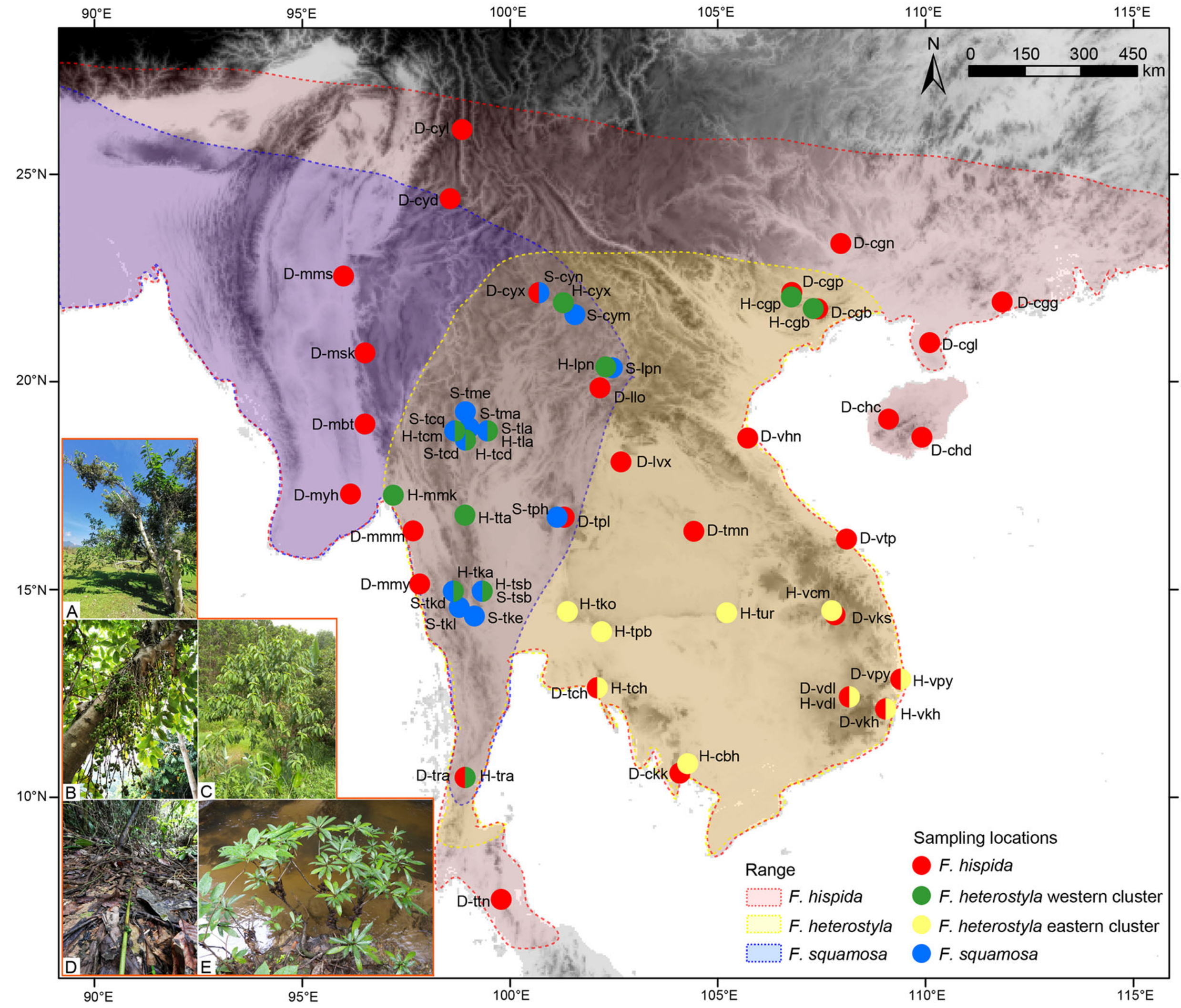

Fig. 1. Species range map (colored areas) and sampling locations (colored dots) of the three studied fig species. **A,** Habitat and growth form of *Ficus hispida*. **B,** Fruiting mode of *F. hispida*. **C,** Habitat and growth form of *Ficus heterostyla*. **D,** Fruiting mode of *F. heterostyla*. **E,** Habitat, growth form and fruiting mode of *Ficus squamosa*.

21-N197, 23-N724, 26-N180, 28-N247, 29-N105, 30-N457, and 32-N125) were selected for specimen genotyping according to the amplification protocols described by Li et al. (2020). Post-polymerase chain reaction products were analyzed by capillary electrophoresis on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA) with the GeneScan 500 ROX Size Standard. Microsatellite fragment sizes were determined using GeneMapper version 3.2.

2.2.3 Population genetic structure

Two approaches, Bayesian clustering analysis and principal coordinates analysis (PCoA) were used to evaluate the genetic structure. Four microsatellite genotype datasets were created: three datasets that were composed of pairs of species (*F. hispida* + *F. heterostyla*, *F. heterostyla* + *F. squamosa*, *F. hispida* + *F. squamosa*), and one dataset that included all three fig species.

Bayesian genetic clustering of individual genotypes was carried out in STRUCTURE version 2.3.4 (Pritchard et al., 2000). Ten independent runs were conducted for each value of *K* (*K* = 1–10) using 1 000 000 Markov chain Monte Carlo iterations, after a burn-in of 100 000 iterations. We used the admixture model with correlated allele frequencies. The optimal value for *K* was determined with the STRUCTURE HARVESTER online program (Earl & Vonholdt, 2012) using Evanno's delta *K* method (Evanno et al., 2005). CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) was used to summarize the estimated membership coefficients into clusters, and the outputs were visualized in DISTRUCT 1.1 (Rosenberg, 2004). The PCoA was undertaken with GeneAIEx 6.5 (Peakall & Smouse, 2012) based on Euclidean distance to estimate the genetic affinity of sampling locations and individuals. Scatterplots were visualized in 2D space for the first and second principal coordinates.

We estimated genetic differentiation between sampling locations using F_{ST} values calculated with Arlequin 3.5 (Excofffer & Lischer, 2010). To illustrate the pairwise F_{ST} matrix, we used the "pheatmap" function in R to derive a heatmap. Genetic differentiation between species and between the two clusters of *F. heterostyla* (see Section 3.1) highlighted during our study was calculated using the same program. Additionally, we compared the patterns of isolation by distance (IBD) among the three fig species and the two *F. heterostyla* clusters to examine the possible effect of geographic distance on their genetic structures. A Mantel test was conducted with the R package Vegan version 2.6-4 (Oksanen et al., 2022) involving 9999 permutations to evaluate the IBD patterns, using $F_{ST}/(1-F_{ST})$ and logarithmic geographic distance as measures.

2.3 Wasp sampling, ultraconserved element sequencing, and phylogenetics

To investigate the pattern of pollinator duplication and heterospecific visitation, we randomly sampled a male-phase fig from a host tree and then collected a female agaonid wasp from that fig. A total of 29 wasps from the three fig species were sampled, including 16 wasps from *F. hispida*, eight from *F. heterostyla*, and five from *F. squamosa*, representing the different sampling areas of fig trees. These individual wasps, together with three outgroups (Table S2) were used to build a phylogenetic tree.

DNA extraction (Qiagen [Valencia, CA, USA] DNeasyBlood and Tissue kit) and library preparation followed Cruaud et al. (2019). Ultraconserved elements (UCEs) were captured using the 2749 RNA probes designed by Faircloth et al. (2015) (myBaits UCE Hymenoptera 1.5Kv1 kit; Arbor Biosciences [Ann Arbor, Michigan, USA]). Assembly of UCEs followed Cruaud et al. (2019). Briefly, quality filtering and adapter trimming were performed with Trimmomatic-0.36 (Bolger et al., 2014). Overlapping reads were merged using FLASH-1.2.11 (Magoc & Salzberg, 2011) and demultiplexed with a bash custom script (Cruaud et al., 2019). Assembly of cleaned reads was performed with CAP3 (Huang & Madan, 1999). Contigs were aligned to the set of 1432 reference UCEs that resulted from the assembly of probes using LASTZ release 1.02.00 (Harris, 2007). Contigs that aligned with more than one reference UCE were removed and different contigs that aligned with the same reference UCE were filtered out using Geneious R11.1.4 4 (https://www.geneious.com).

Only UCEs present in at least 50% of the samples were retained for phylogenetic analysis ($N = 919$). Alignment of each UCE was performed with MAFFT (linsi option (Katoh & St&ley, 2013). Alignment cleaning was conducted with SeqTools (Mirarab et al., 2014). Positions with more than 50% gaps and sequences with more than 25% gaps were removed. Individual UCE trees were built with IQ-TREE 2.2.2.6 (Minh et al., 2020) with best fit models selected by ModelFinder (BIC criterion) (Kalyaanamoorthy et al., 2017). Only common substitution models were tested. Two rounds of Treeshrink (Mai & Mirarab, 2018) were performed on these individual UCE trees to remove abnormally long branches ($b = 20$ and realignment of loci between rounds of Treeshrink with MAFFT).

Phylogenetic inference was undertaken on the concatenated cleaned UCEs using IQ-TREE either with or without partitioning. For the partitioned analysis, each UCE was first split into one core and two flanking regions using the Sliding-Window Site Characteristics (SWSC) method (Tagliacollo et al., 2018) and the best partitioning scheme inferred by PartitionFinder 2.1.1 (Lanfear et al., 2017) that joins core and flanking regions that share similar characteristics into subsets was used for phylogenetic inference (model selection = AICc; algorithm = rclusterf; branch lengths linked).

The best fit model for the unpartitioned dataset was selected by ModelFinder and FreeRate models with up to 10 categories of rates included in tests. The best fit models for each data subset of the partitioned dataset were also selected by ModelFinder but only common substitution models were tested. The candidate tree set for all tree searches using IQ-TREE was composed of 98 parsimony trees + 1 BIONJ tree and only the 20 best initial trees were retained for NNI search. Statistical support of nodes was assessed with ultrafast bootstrap (UFBoot) with a minimum correlation coefficient set to 0.99 and 1000 replicates of SH-aLRT tests (Guindon et al., 2010).

2.4 Volatile samples and data analyses

Floral volatiles released during the receptive phase of the fig development are key components for the attraction of pollinating wasps (Hossaert-McKey et al., 2010, 2016). From November 2020 to November 2021, volatile samples were collected by Chen et al. (2023) during the receptive phase from seven female and 12 male trees of *F. hispida*, two female and 10 male trees of *F. heterostyla*, and six female and 10 male trees of *F. squamosa* (Table S3) in Xishuangbanna Tropical Botanical Garden. We used these data to generate a dissimilarity matrix using the Bray–Curtis index. Permutational multivariate ANOVA (PERMANOVA) (permutations = 999) in Vegan version 2.6-4 was used to test the significant differences in total quantity of volatiles. Two-dimensional nonmetric multidimensional scaling (NMDS) ordination was used to explore the similarities among samples by calculating the best dimensional representation of the distance matrix between samples. All analyses were performed in R version 3.6.1. In addition, one-way ANOVA followed by Tukey's post-hoc test was used to test the dissimilarity level between species based on the dissimilarity matrix.

3 Results

3.1 Population genetic structure in fig trees

Barplots of the genetic composition of each individual at optimal K values for all analyses conducted with STRUCTURE are illustrated in Fig. 2. STRUCTURE HARVESTER identified $K = 2$ as the most likely number of genetic clusters for the datasets that comprised *Ficus hispida* + *Ficus heterostyla* (Figs. 2A, S1A) on one side and *F. hispida* + *Ficus squamosa* on the other (Figs. 2B, S1B), in agreement with species limits. An optimal K value of 3 was obtained when the species pair composed of *F. heterostyla* + *F. squamosa* was analyzed (Figs. 2C, S1C) with two intraspecific geographic clusters revealed within *F. heterostyla* (west/east; Fig. 1). When all three species were included in a single analysis, delta K reached its peak at four genetic clusters (Figs. 2D, S1D) and the two intraspecific clusters of *F. heterostyla* were well visible. Five locations of the 13 sampled for *F. squamosa* (38%) comprised individuals with a blend of genetic characteristics, suggesting potential hybridization with either *F. hispida* or *F. heterostyla* (Fig. 2D). Thus, a few individuals from locations S-tme (25%) and S-tph (33.3%) of *F. squamosa* exhibited high levels of genetic admixture with *F. hispida*, while all individuals sampled in

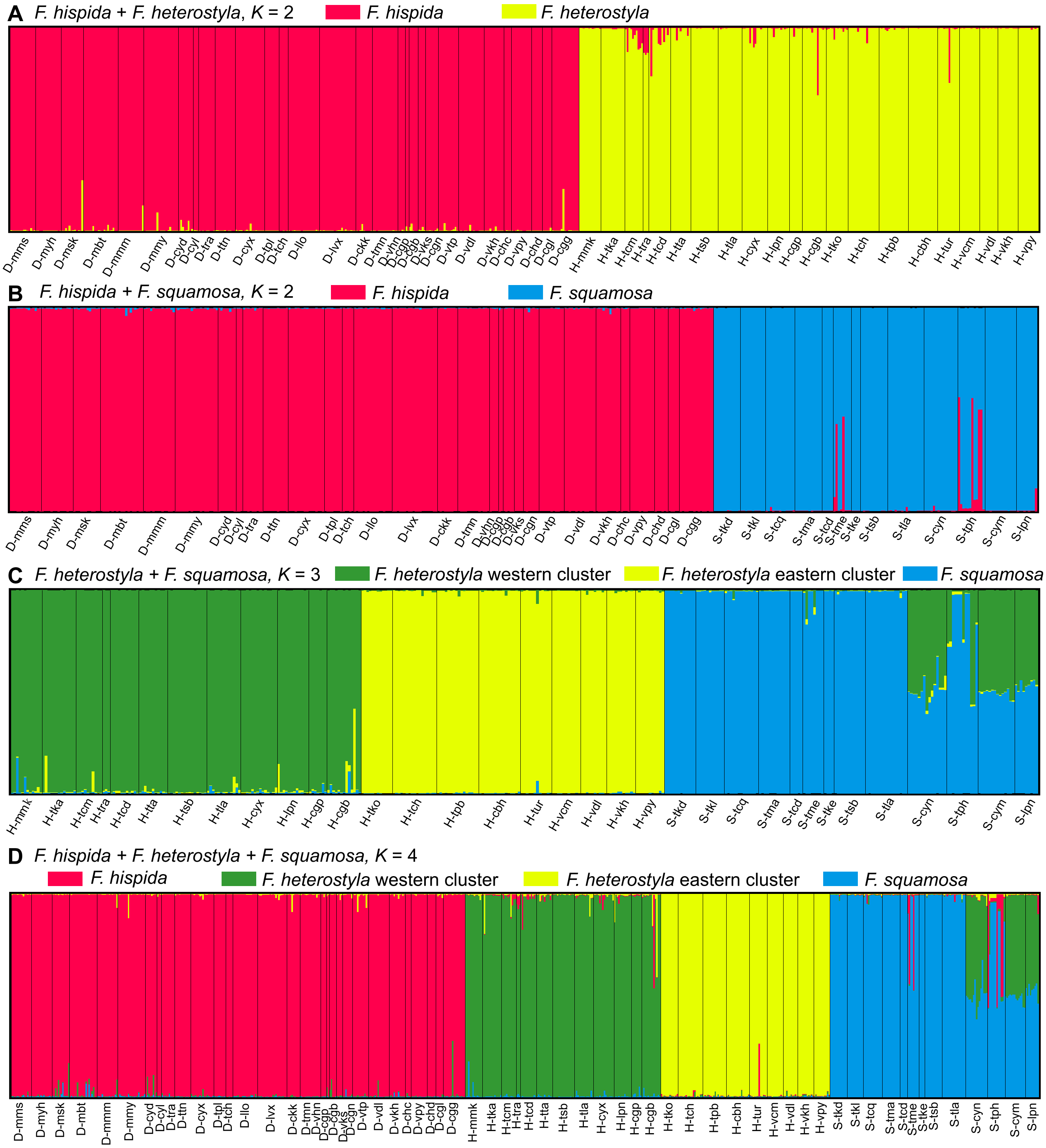

Fig. 2. Results of the Bayesian clustering analysis for the three studied fig species. For each species or genetic cluster, locations are listed from the westernmost location (left) to the easternmost location (right) (see Fig. 1). Colored barplots indicate estimated genetic composition of each individual when *K* clusters are considered. **A,** *K* = 2 for the dataset of *Ficus hispida* + *Ficus heterostyla*. **B,** *K* = 2 for the dataset of *F. hispida* + *Ficus squamosa*. **C,** *K* = 3 for the dataset of *F. heterostyla* + *F. squamosa*. **D,** *K* = 4 for the dataset of *F. hispida* + *F. heterostyla* + *F. squamosa*.

locations S-cym, S-cyn, and S-lpn of *F. squamosa* and one sampled in S-tph (8.3%) showed a high level of admixture with the western cluster of *F. heterostyla*. In contrast, admixture between *F. hispida* and any of the two clusters of *F. heterostyla* was low. The easternmost location sampled for the western cluster of *F. heterostyla* (H-cgb) comprised two individuals (15.4%) with a significant level of admixture with either *F. hispida* or the eastern cluster of *F. heterostyla*. Membership proportion of three fig plants at *K* = 3 is also shown in Fig. S2, further supporting the admixture among fig species.

The PCoA scatterplots of the four datasets at both location and individual levels are shown in Fig. S3. As compared to the dispersed organization observed among samples of *F. heterostyla* and *F. squamosa*, the locations and individuals of *F. hispida* showed a more pronounced clustering pattern, indicating stronger gene flow. As observed in the STRUCTURE analysis, a clear boundary was detected between *F. hispida* and *F. heterostyla* (Figs. S3A, S3B). The two intraspecific clusters observed within *F. heterostyla*, and admixture between *F. squamosa* and the western cluster of *F. heterostyla* (Figs. S3E, S3F), were also supported.

Genetic differentiation (F_{ST}) varied from 0 to 0.286 among sampling locations of *F. hispida* (mean ± SD: 0.134 ± 0.051), from 0.030 to 0.465 among locations of *F. heterostyla* (mean ± SD: 0.250 ± 0.105), and from 0.012 to 0.561 among locations of *F. squamosa* (mean ± SD: 0.325 ± 0.120). Notably, the estimated genetic differentiation between the two geographic clusters within *F. heterostyla* reached 0.167. *Ficus hispida* locations formed a tightly clustered group on the heatmap of F_{ST} values, whereas *F. squamosa* locations showed a varying range of genetic differentiation from low to high. Interestingly, *F. heterostyla* locations showed differentiation into three distinct genetic clusters (Fig. 3). The western cluster already highlighted in STRUCTURE analyses was recovered but two subgroups within the eastern cluster ($F_{ST} = 0.280$) were also revealed that comprised either the easternmost or the westernmost locations of the eastern cluster of *F. heterostyla* (Figs. 1, 3).

As for interspecific level analysis, the highest genetic differentiation was between *F. hispida* and *F. squamosa* ($F_{ST} = 0.290$), followed by *F. hispida* and *F. heterostyla* ($F_{ST} = 0.206$), while the lowest differentiation was observed between *F. heterostyla* and *F. squamosa* ($F_{ST} = 0.151$). When the two clusters of *F. heterostyla* are considered, F_{ST} values are as follows: *F. hispida*–western *F. heterostyla* cluster = 0.231; *F. hispida*–eastern *F. heterostyla* cluster = 0.292; *F. squamosa*–western *F. heterostyla* cluster = 0.145; *F. squamosa*–eastern *F. heterostyla* cluster = 0.256.

Within each species and the two *F. heterostyla* clusters, genetic differentiation was positively and significantly correlated to geographic distance (Fig. 4). The slope of the regression line for *F. squamosa* (slope = 0.428) was approximately two to three times higher than in *F. heterostyla* (slope = 0.194) and *F. hispida* (slope = 0.132). Notably, the regression line slope of the eastern *F. heterostyla* cluster (slope = 0.549) is the largest, while that of the western cluster is the smallest (slope = 0.067), with the eastern slope over eight-fold steeper.

3.2 Phylogenetic relationships of wasps

Phylogenetic relationships of *Ceratosolen* specimens associated with the three focal fig tree species are shown in Fig. 5 and Fig. S4. Trees obtained from the unpartitioned and partitioned datasets were identical. The 29 ingroup wasps grouped into five strongly supported lineages that might represent species. Lineages 1, 2, and 5 (identified as *Ceratosolen solmsi*) comprised wasps collected from *F. hispida* figs, and lineage 4 consisted of pollinators strictly associated with the eastern cluster of *F. heterostyla*. This last lineage occurring in southeastern Asian lowlands formed with the sundaic *C. solmsi* (see Wiebes, 1963), a strongly supported clade. Lineage 1 appeared strictly associated with *F. hispida*. Lineage 2 (i.e., *Ceratosolen marchali*) grouped wasps pollinating *F. hispida* and *F. squamosa*. Lineage 3 consisted of wasps sampled from *F. squamosa* and a few individuals from the *F. heterostyla* western cluster. These findings strongly suggest that both pollinator duplication and pollinator sharing occurred for the studied fig trees.

3.3 Volatile sharing among three fig species

A total of 60 volatile compounds were detected from receptive figs of the three studied species, with 44 from *F. hispida*, 37 from *F. heterostyla*, and 29 from *F. squamosa*. *Ficus hispida* and *F. heterostyla* shared 24 compounds, *F. heterostyla* and *F. squamosa* shared 25 compounds, and *F. hispida* and *F. squamosa* shared 22 compounds. Twenty-one volatile compounds were shared among the three species (Fig. 6; Table S3).

Despite a high level of shared volatile compounds between species, the statistics of volatile profiles differed significantly between species (PERMANOVA, *F. hispida* versus *F. heterostyla*, $F_{(1,\ 29)} = 20.515$, $P < 0.001$; *F. hispida* versus *F. squamosa*, $F_{(1,\ 33)} = 18.605$, $P < 0.001$; *F. heterostyla* versus *F. squamosa*, $F_{(1,\ 26)} = 7.963$, $P < 0.001$). The NMDS graph (Fig. S5) also demonstrated the separation between interspecific samples. The Bray–Curtis distance between *F. heterostyla* and *F. squamosa* (mean ± SD: 0.676 ± 0.134) was significantly lower than that between *F. hispida* and *F. heterostyla* (mean ± SD: 0.749 ± 0.095), and that between *F. hispida* and *F. squamosa* (mean ± SD: 0.756 ± 0.115) (Fig. S6), supporting that lower volatile divergence corresponded to lower genetic divergence between fig species (see Section 3.1).

4 Discussion

We expand upon the coevolutionary scenario of three closely related fig trees (*Ficus heterostyla*, *Ficus hispida*, and *Ficus squamosa*) and their complex of pollinating wasps, by analyzing population genetics data of plants, chemical volatiles emitted by figs, and phylogenetic relationships among wasps.

4.1 Fig tree population structure, life history traits, and copollinators

The three studied species exhibit contrasting genetic structure (Figs. 2, 3). Gene flow is higher between sampling locations of *F. hispida* (Fig. 3), which may be explained by life history traits of this species (Fig. 1). *Ficus hispida* can indeed colonize various habitats, including degraded land. It is even considered as a good candidate for forest restoration (Yang et al., 2002; Elliott et al., 2003; Kuaraksa et al., 2012). Local density can be high. The fruits of *F. hispida* are relatively large, visually inconspicuous (often pale yellow when mature), odorous, and typically grow along the main branches or trunk (cauliflorous), all of which are features of bat dispersal (Lomáscolo et al., 2010). Field observations also confirm that frugivorous bats are the primary consumers (Corlett, 2006). Bats are highly mobile and capable of long-distance seed dispersal (Shilton et al., 1999; Bernard & Fenton, 2003; Randhawa et al., 2020; Aziz et al., 2021). By defecating or spitting out seeds during flight (Thomas et al., 1988; Corlett, 1998, 2006), they increase the likelihood of seeds reaching disturbed and open areas beyond their

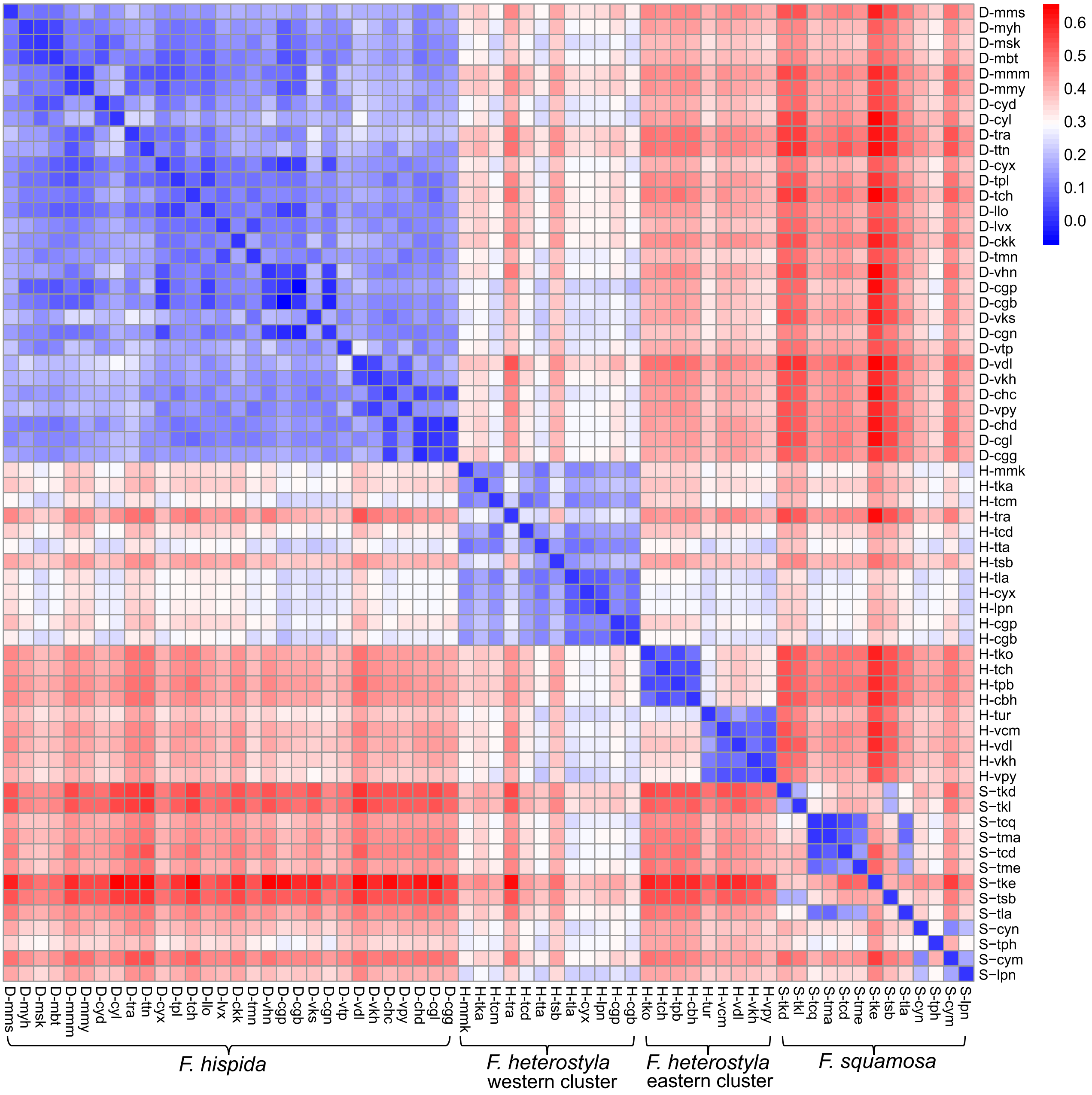

Fig. 3. Heatmap illustrating pairwise F_{ST} values among the 64 sampling locations. High F_{ST} values are shown in red and low F_{ST} values are shown in blue.

perching and nesting sites (Whittaker & Jones, 1994; Muscarella & Fleming, 2007). Hence, bat-mediated dispersion of pioneer plants plays a significant role in forest restoration and succession (Muscarella & Fleming, 2007; Aziz et al., 2021; Parolin et al., 2021). Bat-assisted dispersion of pioneer *F. hispida* could contribute to expand its ecological range while promoting habitat continuity, thereby enhancing seed flow. Additionally, *F. hispida* individuals bloom asynchronously (Patel, 1996; Kuaraksa et al., 2012), necessitating pollinators to move between them. However, the elevated placement of figs improves wind accessibility, thereby potentially facilitating wind-assisted dispersal by pollinators to receptive figs (Ware & Compton, 1994a, 1994b) and enhancing pollen flow. Three wasp species are hosted by *F. hispida* (Fig. 5); however, they can coexist within locations and even on a host individual (Miao et al. in prep.). Therefore, copollinators may not have exacerbated the population differentiation of *F. hispida*.

In contrast to *F. hispida*, *F. heterostyla* and *F. squamosa* fig trees occur in forest habitats, with *F. heterostyla* exhibiting fragmentation as it grows in localized groups within the forest understory, while *F. squamosa* is specifically restricted to riparian forests. Two strongly divergent genetic clusters (west/east) were identified within *F. heterostyla* (Figs. 2, 3; Huang et al., 2023a, 2023c), with two subclusters further distinguished within the eastern cluster (Fig. 3). The

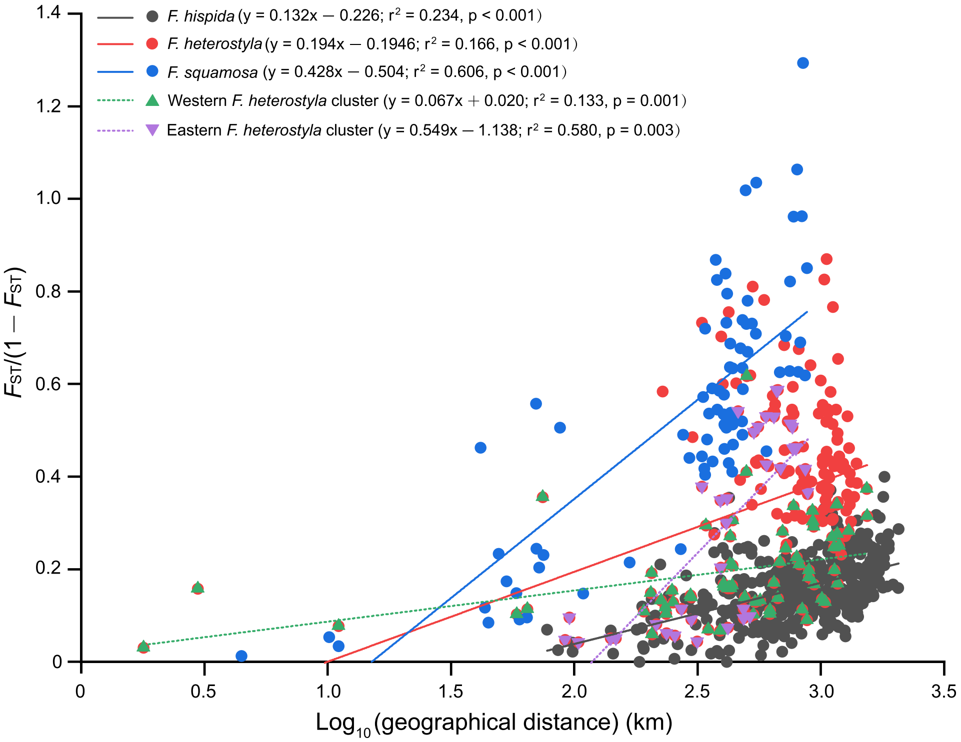

Fig. 4. Correlation between genetic differentiation (estimated as $F_{ST}/[1 - F_{ST}]$) and geographical distances (km, log scale) for *Ficus hispida*, *Ficus heterostyla*, *Ficus squamosa*, and western and eastern clusters of *F. heterostyla*.

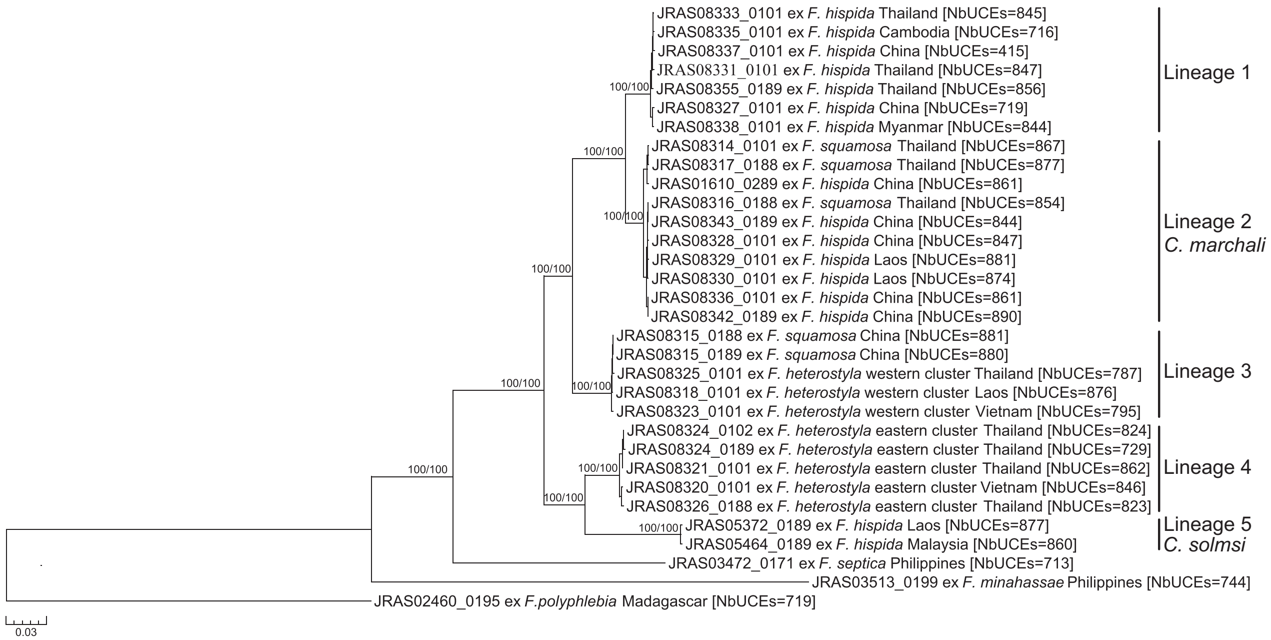

Fig. 5. Maximum likelihood tree of the pollinating wasps obtained from the concatenated ultraconserved element (UCE) dataset (919 UCEs, 560,965 bp, with partitioning). Statistical support (SHaLRT/UFBoot) is shown at main nodes. Tips are labeled with specimen voucher number and the *Ficus* species from which they were sampled. *C. marchali, Ceratosolen marchali*; *C. solmsi, Ceratosolen solmsi.*

clustering of *F. heterostyla* in fragmented patches in the wild seem to obstruct gene flow between patches but sustaining strong gene flow within patches, as suggested by the IBD pattern (Fig. 4). Ground-foraging animals like rodents and deers (Shanahan, 2000) could consume figs of *F. heterostyla*, although this has not been documented yet. Disturbed or open areas, with limited resources and increased predator exposure, are often undesirable habitats for these animal dispersers, hindering seed exchange among forest fragments inhabited by *F. heterostyla*. *Ficus heterostyla* fructifies close to

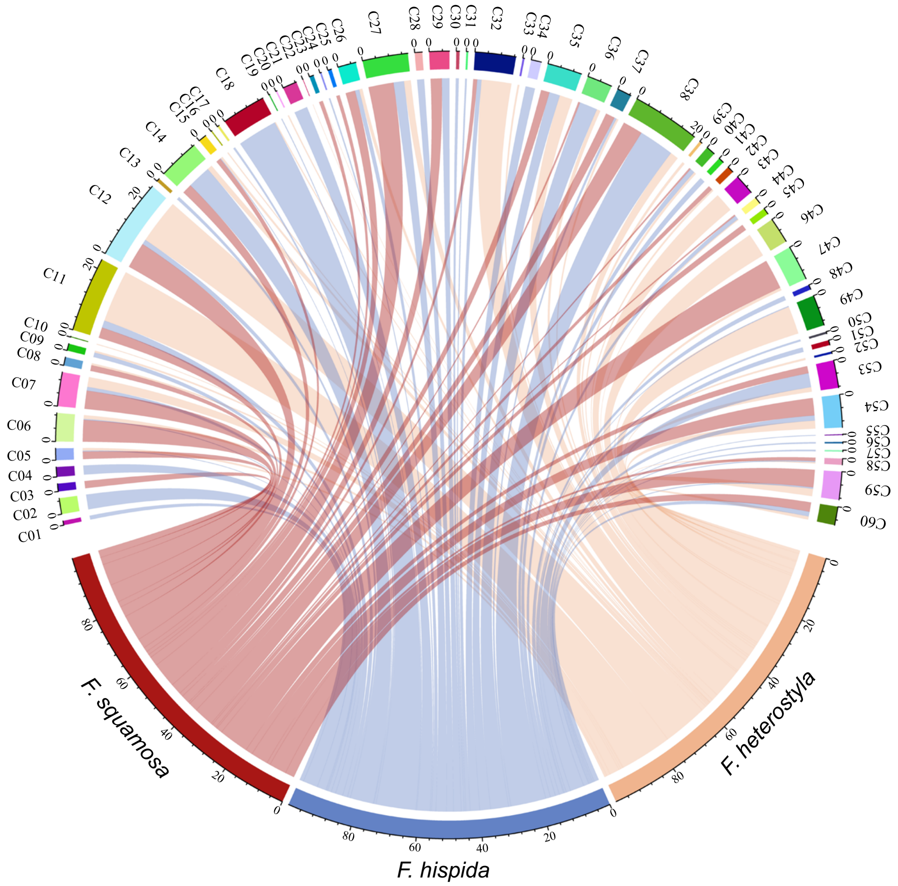

Fig. 6. Circular chart showing the relative percentage of volatile organic compounds in receptive *Ficus hispida*, *Ficus heterostyla*, and *Ficus squamosa* figs.

the ground and figs can be partially buried into the litter (Fig. 1), a factor that is considered a potential limitation on pollinator dispersal, as suggested by studies on *Ficus tikoua* Bureau (Chen et al., 2011). However, *F. heterostyla* produces a much larger crop of figs compared to *F. tikoua*, which may enhance its effectiveness in attracting pollinators. Consequently, a pattern with high divergence among patches and low genetic distance within patches emerges. Interestingly, each cluster is pollinated by a different species of wasp (Fig. 5), which clearly intensifies the genetic divergence between the eastern and western clusters.

Ficus squamosa has a restricted habitat along forest stream banks and exhibits one of the highest population densities among fig species (Pothasin et al., 2014, 2016). High population density enables wasps to complete their life cycle locally, nullifying the requirement for long-distance dispersal to search for receptive figs. Isolation by distance is more pronounced than in the two other species, which may be also explained by the fact that, based on seed morphology and seed dispersal of rheophytes (Berg et al., 2011; Pothasin et al., 2016), water is considered as the primary disperser for *F. squamosa* seeds. Forest streams, often narrow and characterized by shallow level of water, rich vegetation, obstacles (e.g., stones, gravel) and curved paths, likely hamper long distance dispersal of seeds. Animal involvement in dispersal of *F. squamosa* seems possible and would enable intercatchment and upstream dispersal (Pothasin et al., 2014). Nevertheless, the reliance of *F. squamosa* on watercourse habitats likely restricts seed dispersal and gene flow between locations. *Ficus squamosa* hosts two wasp species, one from China and one from Thailand (Fig. 5). Nevertheless, the genetic differentiation between the Chinese and Thai locations ($F_{ST} = 0.281$) is slightly lower than the average among Thai locations ($F_{ST} = 0.291$). Geographic isolation is more likely to be the key factor shaping the population structure of *F. squamosa*.

4.2 Pattern of genetic admixture between fig species, pollinator sharing, and chemical compounds emitted by the figs

Interestingly, all individuals in locations sampled at the northeastern edge of the distribution range of *F. squamosa* are characterized by a high level of admixture with the western cluster of *F. heterostyla* (Figs. 1, 2). We previously discussed the hybridization pattern between *F. heterostyla* and *F. squamosa* in detail (Huang et al., 2023a). Many individuals in the location sampled at the eastern edge of the distribution range of *F. squamosa* (S-tph; Fig. 1) and a few in another unremarkable location (S-tme) are characterized by a high level of admixture with *F. hispida* (Fig. 2). Therefore, it seems that heterospecific pollination is more frequent at the distribution range margin. Habitats at the range margin are vulnerable to climate change, human activities, and stochastic events, and populations are often sparse, fragmented, and prone to local extinctions (González-Megías et al., 2005; Kawecki, 2008; Fréjaville et al., 2020). The tiny (2–3 mm), short-lived (1–2 days) pollinating wasps are highly vulnerable to environmental changes. Rising temperature and decreased humidity, for instance, can reduce their vitality and lifespan (Dunn et al., 2008; Warren et al., 2010; Jevanandam et al., 2013; Sutton, 2016). Research has shown that fig trees and pollinating wasps are less fit in high latitude margins (Chen et al., 2018). Marginal wasp populations are more vulnerable to extinction or temporary absence, and/or a reduction of host availability, increasing the likelihood of pollinator-specificity breakdown and heterospecific visitation (Janzen, 1979). In addition, *F. squamosa* bears figs with high seasonal variation and can suffer from a seasonal pollinator decline (Liu et al., 2015; Pothasin et al., 2014, 2016), which could facilitate the colonization by pollinators of sympatric *F. hispida* and *F. heterostyla* that produce figs year-round (Patel, 1996; Kuaraksa et al., 2012; Liu et al., 2015). Pollinators of the riparian *F. squamosa* are expected to be hydrophilic, which can complicate access to *F. hispida* and *F. heterostyla* figs. *Ficus heterostyla* figs, clinging to the ground or often partially buried, further present challenges for pollinators of *F. hispida* and *F. squamosa* to locate them. In contrast, pollinators of *F. hispida* and *F. heterostyla* may more easily access habitats of *F. squamosa* figs. Volatiles emitted by *F. hispida* figs may disperse widely through air currents, but may not reach *F. heterostyla* and *F. squamosa* pollinators that are confined to forest and riparian habitats.

Wasp phylogeny (Fig. 5) confirms the presence of pollinator sharing among *F. squamosa* and *F. hispida* on one side and *F. squamosa* and *F. heterostyla*, as already reported (Liu et al., 2015), in the other. No sharing of pollinators was observed between *F. hispida* and *F. heterostyla*. As a consequence, approximately 38% of the locations sampled for *F. squamosa* are characterized by a high level of admixture with either *F. heterostyla* or *F. hispida* (Fig. 2).

Host recognition by wasps heavily depends on floral volatiles released during the receptive phase (Grison-Pigé et al., 2002; Hossaert-McKey et al., 2010, 2016) and may be mediated by only one or a few specific attractive compounds (Chen et al., 2009; Proffit et al., 2020; Wang et al., 2021b; Yang et al., 2023). The distinct difference in volatile profiles enable wasps to discriminate among the three co-occurring fig species. Even so, heterospecific visitation can still occur between closely related fig species partly due to the high similarity of their floral volatiles, such as between *Ficus microdictya* Diels and *Ficus umbrae* Ezedin & Weiblen (Souto-Vilarós et al., 2018; Ezedin & Weiblen, 2019), *Ficus hirta* Vahl and *Ficus triloba* Buch.-Ham. ex Voigt (Deng et al., 2022; Yu et al., 2022), or members of the *Ficus auriculata* Lour. complex (Wang et al., 2016). The volatiles emitted by *Ficus natalensis* Hochst. demonstrate notable similarity to those produced by the unrelated *Ficus burkei* Miq., and both are pollinated by the wasp *Elisabethiella stuckenbergi*, implying a case of evolutionary convergence in the chemical attractants for wasps (Cornille et al., 2011). The high proportion of volatile compounds shared among the studied fig trees (>20) suggests possible cross-attraction of pollinators (Fig. 6).

Despite the existence of pollinator sharing and significant sharing of volatile compounds, genetic admixture is limited and occurs in marginal populations. Life history traits and habitat differentiation among the three fig tree species are likely to reduce the frequency of heterospecific visitation, which contributes to maintaining their morphological identity, as observed in the *F. auriculata* complex (Wei et al., 2014). Although *F. auriculata* and *Ficus oligodon* are highly similar in floral volatile profiles (Wang et al., 2016), they rarely hybridize due to ecological differences and limited overlap in their reproductive seasons (Kuaraksa et al., 2012; Wei et al., 2014).

4.3 Allopatric species and duplication of pollinators

The five wasp lineages highlighted in our study (Fig. 5) likely correspond to different species as they can be morphologically distinguished by differences in the sculpture of the pronotum, axilla, and lateral panel of axilla, and several other characters (Rasplus et al., in prep.). A close look at the wasp phylogenetic tree shows that the first split separated southeastern lineages of wasps (sundaic) from wasps associated with the three focal fig trees in the Sino-Himalayan mountains. Therefore, the wasp species pollinating *F. heterostyla* and *F. squamosa* seemed to originate lineages that may have been initially associated with *F. hispida*. The asymmetry of transfer of genetic material from *F. heterostyla* western cluster to *F. squamosa* tend to support that lineage 3 was initially associated with *F. heterostyla*, although the opposite hypothesis cannot be definitively rejected. The three wasp species hosted by *F. hispida* (Fig. 5) are likely the result of speciation on their host, which is linked with much shorter generation times and faster accumulation of genetic differentiation of wasps compared to those of their host fig trees. This allows for quicker isolation and speciation of wasps in response to environmental heterogeneity or geographic barriers, while the host fig trees have not yet differentiated into new species (Cook & Segar, 2010; Souto-Vilarós et al., 2019).

4.4 Possible overlooked speciation in *F. heterostyla* and insights

The genetically distinct eastern and western clusters are highlighted within *F. heterostyla* (Figs. 2, 3). A close look at individuals from the two clusters revealed that they differ morphologically. Indeed, the eastern form has more elongate leaves that are basally subcordate and apically relatively more acuminate, while they are shorter, cuneate, and acute in the western form (Fig. S7). The leaves of the eastern form also appear more coriaceous than those of the western form that are chartaceous. Finally, treelets seem larger in the eastern form than in the western form, but this last character needs to be better assessed. Clearly, these two clusters deserve more thorough morphological and molecular investigation. They each possess distinct pollinators (Fig. 5), likely leading to increased genetic differentiation and potential evolution of allopatric sibling species. Without investigation of genetic structure within the host plant this observation would have led to the conclusion of another breakdown to the one species of pollinator to one species of *Ficus* "rule". Indeed, in published reports, species boundaries in the genus *Ficus* are rarely investigated with molecular data. They are mostly based on superficial morphological assessment without questioning the status or validity of infraspecific entities observed decades ago. Therefore, this result calls for studying the genetic structure of wasps but also to improve our circumscription of fig tree species, before drawing any definitive conclusion regarding the codiversification between fig trees and wasps.

5 Conclusions

Life history traits significantly shape how species reproduce, disperse, and adapt, impacting their population genetic structure and evolutionary adaptation. The three closely related fig species we are focusing on tend to allocate genetic diversity differently among their populations. Multiple factors, including growth form, fruit-bearing position, population density, flowering pattern, habitat preference, mode of seed dispersal, and volatile compounds, certainly contribute to the development of adaptive divergence and collectively determine the genetic structure of our focal species. This study holds specific implications for understanding the population structures and formation of biodiversity within the extremely diverse genus *Ficus*, particularly in light of the considerable variability in life history traits among fig trees. It also contributes to comprehending the mechanisms by which other organisms allocate genetic diversity within their populations.

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Author Contributions

J.F.H. and Y.Q.P. designed this study and collected the samples. J.F.H. and B.G.M. generated and processed the fig data. J.F.H. wrote the paper. A.C. and J.Y.R. generated and processed wasp data, contributed to conceptual development of the manuscript, and edited the paper. W.B. and M.B.C. generated the volatile data. P.P. and S.K. helped with sample collection. All authors have read and approved of the content.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability Statement

Nuclear microsatellite genotyping data needed to replicate this study are provided as a supplementary file.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.13178/suppinfo:

Fig. S1. Diagram of delta *K* and variance per *K* value for different datasets. **A,** Dataset of *Ficus hispida* + *Ficus heterostyla*. **B,** Dataset of *F. hispida* + *Ficus squamosa*. **C,** Dataset of *F. heterostyla* + *F. squamosa*. **D,** Dataset of *F. hispida* + *F. heterostyla* + *F. squamosa*. They are plotted by the HARVESTER online program, determining the optimal *K* value.

Fig. S2. Bayesian STRUCTURE clustering results of 64 sampling locations for *Ficus hispida*, *Ficus heterostyla*, and *Ficus squamosa* based on 14 nuclear microsatellite loci at $K = 3$.

Fig. S3. Two-dimensional scatter diagram based on principal coordinate analysis of genetic variation in datasets at location and individual levels, respectively. **A,** Diagram of the dataset *Ficus hispida* + *Ficus heterostyla* at the location level. **B,** Diagram of the dataset *F. hispida* + *F. heterostyla* at the individual level. **C,** Diagram of the dataset *F. hispida* + *Ficus squamosa* at the location level. **D,** Diagram of the dataset *F. hispida* + *F. squamosa* at the individual level. **E,** Diagram of the dataset *F. heterostyla* + *F. squamosa* at the location level. **F,** Diagram of the dataset *F. heterostyla* + *F. squamosa* at the individual level. **G,** Diagram of the dataset *F. hispida* + *F. heterostyla* + *F. squamosa* at the location level. **H,** Diagram of the dataset *F. hispida* + *F. heterostyla* + *F. squamosa* at the individual level.

Fig. S4. Phylogenetic trees for the pollinating wasps and statistical support (SHaLRT/UFBoot) are shown at nodes. **A,** Phylogenetic tree obtained from the unpartitioned ultraconserved element (UCE) dataset. **B,** Phylogenetic tree obtained from the partitioned UCE dataset.

Fig. S5. Nonmetric multidimensional scaling of the relative percentage of volatile organic compounds emitted by receptive figs of *Ficus hispida*, *Ficus heterostyla*, and *Ficus squamosa* based on Bray–Curtis distance.

Fig. S6. Average flora volatile dissimilarity (Bray–Curtis distance) level among species pairs. Data are shown as mean ± SD. One-way ANOVA with post-hoc Tukey test was used for multiple comparisons.

Fig. S7. Morphological traits and fruiting modes of *Ficus heterostyla* in different sampling areas. **A**–**C**, Eastern Thailand. **D,** Southern Vietnam. **E,** Cambodia. **F,** Southern Vietnam. **G,** Myanmar. **H, I**, Northern Laos. Samples from eastern Thailand, southern Vietnam, and Cambodia group in the eastern cluster of *F. heterostyla*, while those from Myanmar and northern Laos group in the western cluster.

Table S1. Detailed information of sampling locations of *Ficus hispida*, *Ficus heterostyla*, and *Ficus squamosa*.

Table S2. Detailed information of wasp samples included in this study.

Table S3. Volatile organic compounds emitted by figs of *Ficus hispida*, *Ficus heterostyla*, and *Ficus squamosa* at receptive phase.