

Data for both!

Contrasting genetic responses to population fragmentation in a coevolving fig and fig wasp across a mainland–island archipelago

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Abstract

Interacting species of pollinator–host systems, especially the obligate ones, are sensitive to habitat fragmentation, due to the nature of mutual dependence. Comparative studies of genetic structure can provide insights into how habitat fragmentation contributes to patterns of genetic divergence among populations of the interacting species. In this study, we used microsatellites to analyse genetic variation in Chinese populations of a typical mutualistic system – *Ficus pumila* and its obligate pollinator *Wiebesia* sp. 1 – in a naturally fragmented landscape. **The plants and wasps showed discordant patterns of genetic variation and geographical divergence.** There was no significant positive relationship in genetic diversity between the two species. Significant isolation-by-distance (IBD) patterns occurred across the populations of *F. pumila* and *Wiebesia* sp. 1 as whole, and IBD also occurred among island populations of the wasps, but not the plants. However, there was no significant positive relationship in genetic differentiation between them. The pollinator populations had significantly lower genetic variation in small habitat patches than in larger patches, and three island pollinator populations showed evidence of a recent bottleneck event. No effects of patch size or genetic bottlenecks were evident in the plant populations. Collectively, the results indicate that, in more fragmented habitats, the pollinators, but not the plants, have experienced reduced genetic variation. The contrasting patterns have multiple potential causes, including differences in longevity and hence number of generations experiencing fragmentation; different dispersal patterns, with the host's genes dispersed as seeds as well as a result of pollen dispersal via the pollinator; asymmetrical responses to fluctuations in partner populations; and co-existence of a rare second pollinating wasp on some islands. These results indicate that strongly interdependent species may respond in markedly different ways to habitat fragmentation.

Keywords: co-evolution, *Ficus*, gene flow, genetic variation, habitat fragmentation, *Wiebesia*

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Introduction

Habitat fragmentation alters the sizes, isolation and spatial patterns of remnant habitats and has been recognized as one of the most critical threats to genetic

diversity (Young *et al.* 1996; Chen 2000). Genetic variation within and among fragmented populations is the outcome of interplay between genetic drift, gene flow, selection and mating system. Because these factors need many generations to substantially alter the genetic composition of large populations, it may take thousands of years to detect significant genetic change in long-lived species. Consequently, some studies have reported little

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erosion of genetic variation in long-lived perennials growing in fragmented habitats, leading to the view that trees may be relatively resistant to habitat fragmentation (Jump & Peñuelas 2006; Kramer *et al.* 2008; Dubreuil *et al.* 2010).

Interspecific interactions can amplify the effects of habitat fragmentation via their influence on gene flow (animal dispersal of seeds or pollen grains) or patterns of selection (herbivory, parasitism or mutualism; Magalhaes *et al.* 2011). Habitat fragmentation can reduce the abundance and restrict the movement of pollinators, leading to reduced host plant population sizes and an increased risk of their local extinction. Empirical studies have confirmed that pollination processes are sensitive to fragmentation (Harris & Johnson 2004; Aguilar *et al.* 2006), and the more specific the relationship, the more sensitive are these interactions to disturbance through causes such as habitat fragmentation (Koh *et al.* 2004). Hence, there may be losses in genetic variation among both partners in plant–pollinator mutualisms in response to fragmentation of habitats.

Here, we focus on the obligate mutualism between fig trees (*Ficus* species, Moraceae) and their pollinator fig wasps (Agaonidae). Fig trees rely on small, delicate and short-lived adult female fig wasps for their pollen dispersal, and in turn, the pollinating wasps require the florets inside figs for their larval development (Janzen 1979; Weiblen 2002; Cook & Rasplus 2003). Earlier studies found that each fig tree species had one specific pollinating wasp species (the ‘one fig-one wasp rule’), but results in recent years using molecular markers have revealed increasing numbers of exceptions (e.g. Molbo *et al.* 2003; Lin *et al.* 2010). Fig wasps are slow, weak fliers (Compton *et al.* 2000), but can be transported by air currents, and ecological and molecular studies have revealed examples of extremely long-distance dispersal of more than 160 km (Ahmed *et al.* 2009).

The dioecious creeping fig *F. pumila* has a natural distribution that extends from the Ryukyu Islands to mainland China and Vietnam. The genetic structure of its populations in China indicates moderate levels of gene flow among populations (Chen *et al.* 2008), with effective dispersal estimated to be <1800 m (Wang *et al.* 2009). *Wiebesia pumilae* (Hymenoptera: Agaonidae) was described as *Blastophaga pumilae* by Hill (1967) from *F. pumila* growing in Hong Kong and until recently was assumed to be its only pollinator. Molecular markers have now revealed that there are three cryptic species of pollinators of *F. pumila* in southeastern China, referred to as *Wiebesia* spp. 1, 2 and 3 (Chen *et al.* 2012). *Wiebesia* sp. 2 is probably the true *W. pumilae* with a distribution in Hong Kong and southern China. In the northern part of the range of *F. pumila*, including Jiangxi, Anhui, Jiangsu and Zhejiang provinces, it is

replaced by *Wiebesia* sp. 1. The third species, *Wiebesia* sp. 3, has a more limited distribution restricted to eastern China, where it is mainly found on offshore islands of the Zhoushan Archipelago and Taiwan Island (Chen *et al.* 2012). Based on mtDNA *cytb* sequences, the pollinating wasp of *F. pumila* in the Ryukyu Islands is distinct from the mainland pollinators and is closer to *Wiebesia* sp. 3 than to *Wiebesia* spp. 1 and 2 (Chen *et al.* unpublished data).

Naturally fragmented landscapes generally have longer histories of fragmentation than anthropogenically altered habitats, providing an opportunity to explore the long-term genetic consequences of fragmentation on interacting mutualists. The Zhoushan Archipelago, southeast of Shanghai, formed about 7200–8100 year ago by rising sea levels after the last glacial maximum (Fig. S1, Supporting information; Xin *et al.* 2006). *Ficus pumila* has a wide natural distribution across the islands of the archipelago, where it currently supports two species of pollinators, although one species is rare and restricted to a small number of islands (Chen *et al.* 2012).

Ficus pumila and the pollinators are intimately interacting species. The tree’s pollinating fig wasps develop only inside its figs, and population sizes of the insects are therefore dependent on those of their hosts. We therefore hypothesize that there is a concordant pattern of genetic structure between *F. pumila* and its pollinating fig wasps. However, the pollinators have a much shorter generation time, and the survival of each generation is strictly dependent on the number of available figs, which fluctuates greatly between seasons, suggesting a second hypothesis that pollinating fig wasp is more sensitive to fragmentation than its hosts. To test these hypotheses, we used microsatellites to evaluate genetic variation among island populations of *F. pumila* and its more common pollinator (*Wiebesia* sp. 1) and then compared the results with adjacent mainland populations. We specifically addressed the following questions: 1) Do these two mutually dependent species show concordant genetic structures? 2) Have *F. pumila* and its pollinator been affected by habitat fragmentation? And if they have, 3) were similar differences between the mainland and island populations exhibited by both the plant and its pollinator, indicating similar susceptibility to habitat fragmentation?

Materials and methods

Locations and sample collection

The study region comprised the Zhoushan Archipelago and adjacent mainland areas of Zhejiang Province in Eastern China (Fig. 1). The Zhoushan Archipelago

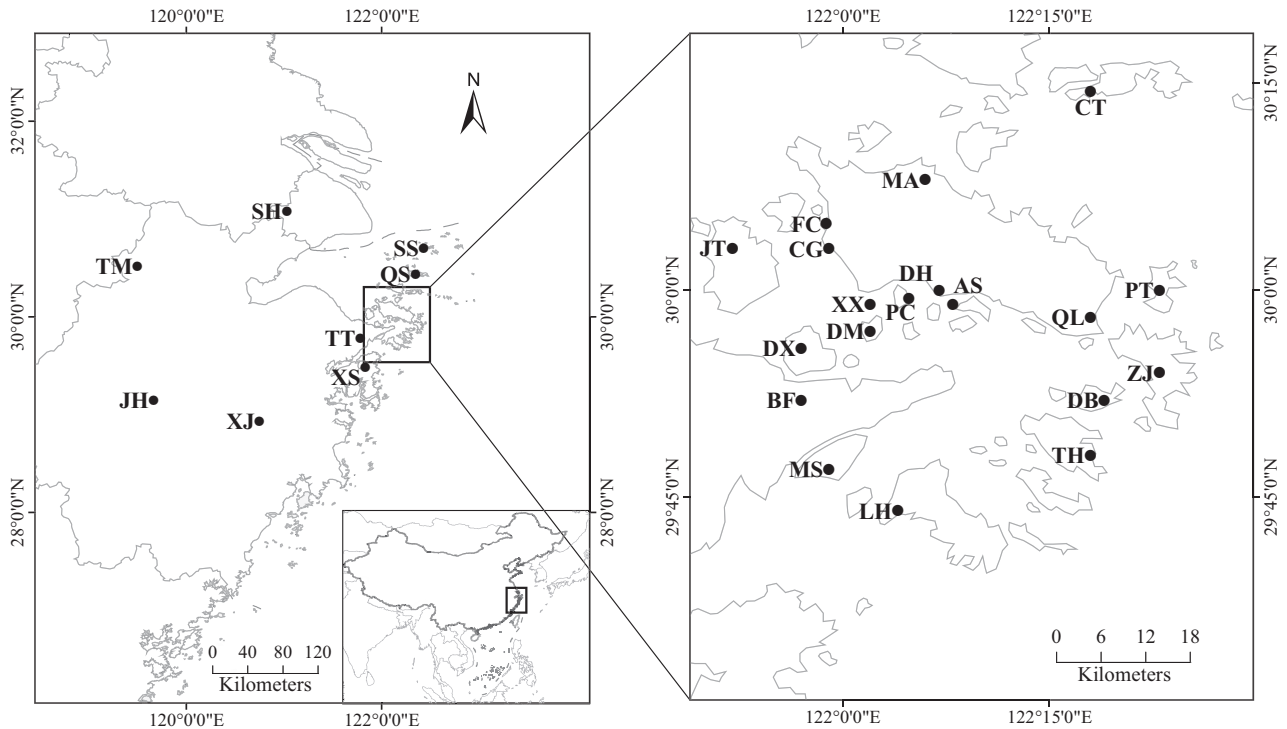


Fig. 1 Sample sites for *Ficus pumila* and its pollinator *Wiebesia* sp. 1 in the Zhoushan Archipelago and adjacent mainland China. Inset in lower right corner of the left panel shows position of the study area within China.

provides an ideal area to study interactions between fig trees and their pollinators in a fragmented landscape. It is the largest Chinese offshore archipelago, with over 1300 islands, of which Zhoushan Island, with an area of about 502 km², is the largest. During the last glacial maximum, the islands were joined to the mainland, but they became separated by rising sea levels about 7200–8100 years ago (Fig. S1, Supporting information; Xin *et al.* 2006).

Ficus pumila is commonly found on walls of abandoned houses, on rocks or climbing big trees. Human activities, such as agriculture and urbanization, may reduce the habitats of *F. pumila*, which would affect the genetic structure of *F. pumila* and the pollinators. However, it was easy to find *F. pumila* in rural areas, and sometimes in the urban areas, although there were relatively few mature fig trees. Furthermore, urbanization only began in recent decades, which is a very short time compared with the thousands of years since the islands became isolated. Comparisons between the genetic structures of the island and adjacent mainland populations can therefore provide evidence for genetic erosion that has resulted from long-term habitat fragmentation.

Figs of *F. pumila* are large, reaching over 70 mm in diameter, and contain thousands of flowers. As in other dioecious fig trees, male plants only support the development of pollen-carrying fig wasps, whereas female plants produce only seeds. Male trees growing in Hong

Kong have been reported to produce three overlapping crops (Hill 1967). Further north in China, just one or two crops are produced annually, maturing in the spring and sometimes additionally in the summer (Ma & Wu 1989; Luo *et al.* 2000). Female trees usually have a crop that is pollinated in spring and matures in autumn. Although some female trees have a small second crop in summer, those figs are generally aborted before they mature (Zhao *et al.* unpublished data). Female figs split open when mature, exposing the yellowish, pectin-rich perianth, which attracts birds and fruit bats. In Hong Kong, *F. pumila* was recorded to be dispersed by fruit bats (Corlett 2006). However, there are no fruit bats in Zhejiang Province, and seeds are most probably dispersed by birds (Shanahan *et al.* 2001b).

Three congeneric cryptic pollinating wasps pollinate *F. pumila* in mainland China (Chen *et al.* 2012). The dominant pollinator in SE China and the Zhoushan Archipelago is *Wiebesia* sp. 1, which has been recorded from most of the islands (Fig. 1). Another pollinator, *Wiebesia* sp. 3, is also present on some of the offshore islands in the SE of the Archipelago, but its frequency is much lower than that of *Wiebesia* sp. 1 (Chen *et al.* 2012). No nonpollinating fig wasps have been recorded from *F. pumila* in this area.

In the spring months (April to May) of 2005–2008, we sampled populations of *F. pumila* and its pollinating

Table 1 The location and genetic characteristics of *Ficus pumila* and *Wiebesia* sp. 1 populations

Population	Code	Location	<i>Ficus pumila</i>						<i>Wiebesia</i> sp. 1					
			<i>N</i>	<i>N</i> _A	<i>A</i> _R	<i>H</i> _O	<i>H</i> _E	<i>F</i> _{IS}	<i>N</i>	<i>N</i> _A	<i>A</i> _R	<i>H</i> _O	<i>H</i> _E	<i>F</i> _{IS}
Mainland														
Jinhua	JH	29°09'N, 119°40'E	36	9.0	5.893	0.451	0.689	0.348	29	7.1	4.634	0.621	0.712	0.130
Tianmu	TM	30°31'N, 119°30'E	10	4.3	4.250	0.588	0.616	0.048	29	6.0	4.613	0.683	0.743	0.082
Xianju	XJ	28°56'N, 120°45'E	20	3.1	2.841	0.444	0.406	−0.095	31	7.5	4.769	0.584	0.749	0.224
Shanghai	SH	31°05'N, 121°12'E	22	3.5	3.081	0.477	0.504	0.054	24	7.8	5.002	0.758	0.764	0.008
Xiangshan	XS	29°29'N, 121°50'E	20	4.6	4.028	0.561	0.562	0.001	29	8.4	5.335	0.717	0.778	0.079
Tiantong	TT	29°47'N, 121°47'E	25	4.0	3.410	0.465	0.475	0.021	30	8.3	5.307	0.647	0.789	0.183
Baifeng	BF	29°52'N, 121°57'E	25	4.8	3.867	0.468	0.565	0.175	26	7.8	5.144	0.623	0.756	0.179
Mean			22.5	4.7	3.910	0.493	0.545	0.079	28.3	7.6	4.972	0.662	0.756	0.126
Islands														
Liuheng	LH	29°44'N, 122°04'E	32	5.6	4.558	0.594	0.628	0.055	15	6.4	5.021	0.727	0.759	0.044
Meishan	MS	29°47'N, 121°59'E	29	4.3	3.407	0.476	0.455	−0.047	9	5.5	4.585	0.478	0.682	0.312
Daxie	DX	29°56'N, 121°57'E	13	4.5	4.199	0.587	0.599	0.022	19	6.4	4.688	0.690	0.743	0.074
Damao	DM	29°57'N, 122°02'E	28	5.8	4.655	0.513	0.622	0.177	19	5.7	4.258	0.526	0.731	0.286
Panzhi	PC	29°59'N, 122°05'E	33	5.5	4.298	0.581	0.588	0.012	25	6.9	4.801	0.624	0.722	0.138
Aoshan	AS	29°59'N, 122°08'E	10	3.9	3.875	0.550	0.566	0.080	6	4.3	4.300	0.767	0.727	−0.060
Xixiezhi	XX	29°59'N, 122°02'E	15	3.3	3.113	0.500	0.517	0.033	27	6.6	4.543	0.659	0.728	0.096
Dinghai	DH	30°00'N, 122°07'E	36	5.9	4.519	0.555	0.621	0.108	29	6.9	4.624	0.638	0.736	0.135
Cengang	CG	30°03'N, 121°59'E	13	4.1	3.905	0.567	0.590	0.039	25	7.0	4.850	0.664	0.757	0.125
Jintang	JT	30°03'N, 121°52'E	34	4.9	3.906	0.603	0.600	−0.004	15	5.6	4.532	0.773	0.730	−0.062
Fuchi	FC	30°05'N, 121°59'E	14	4.4	4.077	0.500	0.557	0.106	12	5.3	4.365	0.575	0.713	0.201
Mao	MA	30°08'N, 122°06'E	27	4.9	3.881	0.551	0.569	0.033	20	6.3	4.853	0.765	0.765	−0.001
Taohua	TH	29°48'N, 122°18'E	29	5.6	4.363	0.678	0.612	−0.111	6	4.6	4.600	0.717	0.694	−0.036
Dengbu	DB	29°52'N, 122°19'E	21	4.4	3.843	0.649	0.617	−0.054	12	5.0	4.247	0.667	0.733	0.094
Zhujiajian	ZJ	29°54'N, 122°23'E	30	6.0	4.726	0.625	0.673	0.072	16	3.7	3.123	0.406	0.571	0.296
Qionglong	QL	29°58'N, 122°18'E	18	4.4	3.833	0.542	0.548	0.011	22	5.7	4.009	0.614	0.688	0.110
Putuoshan	PT	30°00'N, 122°23'E	32	5.4	4.243	0.629	0.641	0.019	19	5.6	4.116	0.611	0.681	0.106
Dachangtu	CT	30°14'N, 122°18'E	29	5.5	3.985	0.509	0.548	0.074	25	6.2	4.319	0.672	0.725	0.074
Qushan	QS	30°26'N, 122°21'E	21	3.6	3.016	0.449	0.439	−0.024	21	4.8	3.816	0.605	0.696	0.133
Shengsi	SS	30°42'N, 122°26'E	32	3.9	3.107	0.422	0.466	0.095	18	5.0	3.834	0.528	0.643	0.184
Mean			24.8	4.8	3.975	0.554	0.573	0.035	18	5.7	4.374	0.635	0.711	0.112

N, sample size; N_A, mean number of alleles per locus; A_R, allelic richness per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; trees/figs.

Values in bold are significantly biased from 0.

wasps from 17 islands of varying sizes (from 0.82 to 502 km²) and from seven areas of the adjacent mainland (Fig. 1, Table 1). The islands were selected according to their accessibility. Between 10 and 33 host individuals were sampled in each population, with each sample separated from others by at least 30 m, to avoid repeat sampling of the same individual. About five healthy leaves were collected from each plant and dried using silica gel. Because few mature figs were produced in some populations, we collected 2–24 figs in each populations based on the numbers of mature figs, to prevent local extinctions of the pollinators. Mature figs of male plants were collected and stored in netting bags to let the fig wasps emerge naturally. The emerging wasps were placed into absolute ethanol and stored at 4 °C.

DNA extraction and microsatellite genotyping

Total genomic DNA of *F. pumila* was extracted from about 50 mg samples of silica gel-dried leaves using the Plant Genomic DNA Kit (Tiangen). Eleven nuclear microsatellite loci (FP9, FP38, FP102, FP134, FP213, FP327, FP328, FP435, FP540, FP556 and FP601) were genotyped using fluorescently labelled PCR primers as described by Zhang *et al.* (2011).

Genomic DNA of the pollinating wasps was isolated from the whole body of one female wasp using the modified method of Sambrook *et al.* (1989). Between one and three fig wasps per fig were used, with the remaining individuals retained in alcohol storage. The diameter of receptive figs of *F. pumila* is about 2–3 cm (Rui Zhao,

unpublished data), which is larger than that of most *Ficus* species and routinely allows more than ten foundress fig wasps to enter (Yan Chen, personal observations). Lower inbreeding rates than most fig wasps are therefore expected for the pollinators of *F. pumila*.

Genotyping was carried out using 10 microsatellite primers developed previously (Liu *et al.* 2009) with 5'-labelled with fluorescent dye on the forward primer. The PCR amplification was performed in 10 µL volumes. The amplification products were combined into three mixtures [mixture 1: WP447 (6-FAM), WP294 (ROX) and WP076 (6-FMA); mixture 2: WP403 (ROX), WP554 (TAMRA), WP399 (HEX) and WP231 (6-FAM); mixture 3: WP522 (6-FAM), WP439 (HEX) and WP004 (6-FAM)], and each mixture was scanned on an ABI 3730 Automated DNA Sequencer (Applied Biosystems). Allele sizes were scored using GENEMAPPER version 4.0 (Applied Biosystems) or PEAKSCANNER (Applied Biosystems). The species status of each wasp was identified based on the product sizes of loci WP294 and WP439, which show substantial differences between *Wiebesia* spp. 1 and 3 (Liu *et al.* unpublished data). Only *Wiebesia* sp. 1 was used in the subsequent analyses.

Hardy–Weinberg equilibrium, linkage disequilibrium and presence of null alleles

To address the effectiveness of the microsatellite loci, we conducted three analyses. First, population-specific tests for deviation from Hardy–Weinberg equilibrium (HWE) were performed with GENEPOP 4.0 (Rousset 2008). Second, linkage disequilibrium (LD) among loci per population was conducted using FSTAT version 2.9.3 (Goudet 1995). All multiple tests were adjusted by sequential Bonferroni correction (Rice 1989). Third, the frequency of null alleles was inspected by MICRO-CHECKER (Van Oosterhout *et al.* 2004).

Within-population genetic diversity and its relationship with patch size

Genetic diversity provides a means of evaluating the potential effects of habitat fragmentation. We characterized genetic diversity of *F. pumila* and its pollinating wasps using the following parameters: mean number of alleles per locus (N_A), allelic richness per locus (A_R , adjusting sample size of each population to the minimal sample size), observed (H_O) and unbiased expected heterozygosities (H_E). These analyses were performed using FSTAT and TFGA (Miller 1997). Inbreeding coefficients (F_{IS}) were calculated for each population by FSTAT.

We also analysed the relationships between genetic diversity and areas of available habitats to test whether populations in small habitat patches exhibited lower

genetic diversity than populations in the larger patches or mainland. We divided the island populations into three groups according to their areas (small islands: area <10 km²; moderate-sized islands: 10–100 km²; large islands: four populations on Zhoushan Island, the largest in the archipelago). Seven mainland populations constituted the 4th group. We tested the differences in genetic diversity (N_A , A_R , H_E and H_O) using one-way ANOVA analysis and post hoc Tukey's HSD.

Populations of host plants with higher genetic diversity are expected to support higher genetic diversity within their pollinator populations, because they may provide more diverse resources to support them. To test this hypothesis, we analysed the relationship between genetic diversity (N_A , A_R , H_E and H_O) of *F. pumila* and the pollinator, using linear regression analyses. All analyses were performed in R version 2.13.1 (R Development Core Team 2011).

Genetic differentiation and isolation by distance

If populations have been affected by habitat fragmentation, there would be high genetic differentiation among populations. To test this, we calculated population genetic differentiation at the overall and pairwise levels. We first estimated F_{ST} (0) (Weir & Cockerham 1984) using the software FSTAT. For overall F_{ST} , standard error was estimated by Jackknifing, and 1000 bootstraps were used to estimate the 95% upper and lower bounds, with significance levels ($P = 0.05$) of pairwise F_{ST} determined with 1000 permutations, applying sequential Bonferroni corrections (Rice 1989). However, F_{ST} is likely to underestimate genetic differentiation between populations for markers that show high levels of allelic variability. We therefore also calculated the standardized F_{ST} ($F'_{ST} = F_{ST}/F_{STmax}$) (Hedrick 2005). F_{STmax} was calculated after recoding the data using RECODEDATA (Meirmans 2006). Additionally, we calculated estimators of actual differentiation D_{EST} (Jost 2008), which are also considered more accurate than traditional F_{ST} and the analogy, using the software SMOGD version 1.2.5 (Crawford 2010). In addition, we also calculated pairwise and associated global unbiased F_{ST} ($F_{ST(ENA)}$) (Weir 1996) among populations using the ENA (excluding null alleles) method implemented in FREENA (Chapuis & Estoup 2007), a method that corrects for the presence of null alleles.

Gene flow plays an opposite role to genetic drift within small populations generated by habitat fragmentation. To test whether gene flow can balance the effects of genetic drift, we assessed patterns of isolation by distance. A Mantel test was used, based on the regression of pairwise estimates of genetic distances F_{ST} , ($F_{ST}/(1-F_{ST})$), F'_{ST} , D_{EST} or ($F_{ST(ENA)}/(1-F_{ST(ENA)})$) against the corresponding logarithmic (\log_{10}) geographical

separation for the two species. These analyses were conducted with the R package 'vegan' (Dixon 2003), and 10 000 permutations were performed to determine statistical significance.

We evaluated whether the genetic structure of *F. pumila* and the pollinator showed the same pattern by regressing the pairwise genetic distances ($F_{ST}/(1-F_{ST})$) or ($F_{ST(ENA)}/(1-F_{ST(ENA)})$) of the two species and using Mantel tests both at the overall population level and islands-only population level. All analyses were performed in R.

Genetic clustering

To address whether there was geographically related genetic clustering of populations for *F. pumila* and its pollinators, we ordered populations from west to east and then used a Bayesian approach for inferring population structure, in STRUCTURE 2.3.1 (Pritchard *et al.* 2000). We ran the admixture model with correlated frequencies, with 15 independent runs for each *K* (from 1 to 10) performed with 10 000 MCMC repetitions and a burn-in of 10 000. We used the mean maximum estimated logarithm of posterior probability of the data for each *K*, $\text{LnP}(D)$, to identify the most probable number of clusters (Pritchard *et al.* 2000), and we also used ΔK values to obtain the optimal *K* (Evanno *et al.* 2005). After the best *K* was chosen, all individuals were assigned to the *K* populations probabilistically using a burn-in of 300 000 and 1000 000 MCMC repetitions.

Because the fig wasp populations showed a longitudinal gradient in genetic composition, we calculated the genetic distances (F_{ST}) and \log_{10} geographical distances to the most western (population JH) and most eastern (population SS) populations and tested the relationships between genetic and spatial distances using linear regression, aiming to infer the original direction of colonization of the fig wasps into the study region.

Population bottlenecks

Fragmented habitats usually support small populations that have become more prone to local extinction and re-colonization events and therefore exhibit population bottlenecks. We used the program BOTTLENECK version 1.2.02 (Piry *et al.* 1999) to detect the genetic signals of recent population bottlenecks. The two-phase model of mutation (TPM) was chosen, which is believed to be the most appropriate model for the mutation of microsatellites (Di Rienzo *et al.* 1994; Ellegren 2000, 2004), with 80% stepwise and 20% multistep mutations, and a variance of 20. Estimates were based on 1000 replications and Wilcoxon sign-rank tests.

Results

Hardy–Weinberg equilibrium, linkage disequilibrium and presence of null alleles

In total, 644 *F. pumila* and 558 *Wiebesia* sp. 1 individuals were genotyped using polymorphic microsatellites. Linkage disequilibrium was found in two pairs of *F. pumila* loci (FP213 and FP435; FP328 and FP540). Loci FP328 and FP435 were therefore excluded in subsequent analyses. Locus FP327 was found to have null alleles in all populations except in population TM and was also excluded. Except for locus FP213, the other seven loci were all found to have null alleles in one to five populations with an average frequency from 9.49% to 18.95%. Analysis of genetic variation of *F. pumila* was therefore based on these eight loci. Tests of Hardy–Weinberg equilibrium (HWE) per locus in each of the 27 samples showed a significant departure from HWE in 13 of 216 cases (FP9 in JH; FP38 in DM and JH; FP102 in SS; FP134 in JH; FP540 in JH; FP556 in BF, CG, DH, JH, SH and TH; FP601 in JH).

In *Wiebesia* sp. 1, there were eight cases of deviations from HWE (WP294 in DM; WP076 in DM and XX; WP403 in FC; WP554 in XJ and ZJ; WP231 in MS and XJ). No linkage disequilibrium was found in any locus pair in mainland populations of *Wiebesia* sp. 1, allowing all ten loci to be used for further analyses. However, significant linkage disequilibrium in 9 of 900 (1.0%) cases was found in the island populations. All loci showed signs of null alleles from two to eight populations, depending on the locus, with average frequencies varying between 10.86% and 16.93%.

Within-population genetic diversity and its relationship with patch size

Similar levels of genetic diversity were found between mainland and island populations of *F. pumila*. The mean number of alleles (N_A) across all eight of its loci ranged from 3.1 to 9.0, with means of 4.7 and 4.8 for mainland and island populations, respectively (Table 1). Mean observed heterozygosity (H_O) ranged from 0.422 to 0.678 with means of 0.493 and 0.554 for mainland and island populations, respectively. The expected heterozygosity per population (H_E) ranged between 0.406 and 0.689, with means of 0.545 and 0.573 for mainland and island populations, respectively (Table 1). Allelic richness was lowest in population XJ (2.841) and highest in population JH (5.893). ANOVA indicated no significant differences in the four diversity indices among the four groups of *F. pumila* populations (Fig. 2, left panel).

In *Wiebesia* sp. 1, the number of alleles per locus (N_A) ranged between 3.7 and 8.4 with means of 7.6 and 5.7

for mainland and island populations, respectively (Table 1), with H_O and H_E ranging from 0.406 to 0.773 and 0.571 to 0.789. Population ZJ had the lowest A_R (3.123) and population XS had the highest (5.335). Three of the four indices (N_A , A_R and H_E) showed that there was significant differentiation related to patch size in *Wiebesia* sp. 1. The mainland populations had significantly more alleles per locus than the smaller (<100 km²) habitats (Fig. 2, right panel), and the mainland populations also had significantly higher values of A_R and H_E than those of populations on the moderate-sized islands. Although not significant, populations from the large island also had higher levels of genetic variation than those of the smaller ones. However, the observed heterozygosity (H_O) was similar among the four patch size categories (Fig. 2, right panel).

Correlation analysis revealed no significant relationship between the local level of genetic diversity of *F. pumila* and that of *Wiebesia* sp. 1 among the 27 sites (data not shown). Hence, local genetic diversity in populations of the pollinating wasp appears to be independent of local genetic diversity of its specific host.

In general, low values of inbreeding coefficients were found in *F. pumila* and *Wiebesia* sp. 1 (Table 1). Inbreeding coefficients of only one mainland and one island population of *F. pumila* were significantly larger than zero. In contrast, F_{IS} values of four mainland (57%) and seven island (35%) populations of *Wiebesia* sp. 1 were significantly larger than zero.

Genetic differentiation and isolation by distance

Global F_{ST} was 0.094 ± 0.025 , which is significantly greater than zero ($P < 0.05$). F'_{ST} was 0.218; D_{EST} was 0.111; and $F_{ST(ENA)}$ was 0.091 ($P < 0.05$). Significant genetic differentiation among populations was also observed in the pollinators. Global F_{ST} was 0.102 ± 0.06 , again significantly different from zero ($P < 0.05$). F'_{ST} was 0.370, with the overall estimated D_{EST} 0.300 and $F_{ST(ENA)}$ 0.097 ($P < 0.05$).

The genetic differentiation patterns were similar using either of the differentiation parameters (Table S1, Supporting information), so we have just presented the results of ($F_{ST}/(1-F_{ST})$). Genetic and \log_{10} geographical distances were significantly correlated among all *F. pumila* populations ($r = 0.401$, $P = 0.004$; Fig. 3a) but not among island populations alone ($r = 0.230$, $P = 0.061$). In *Wiebesia* sp. 1, significant relationships between genetic and geographical distances were found among all populations ($r = 0.479$, $P = 0.001$) and also among island populations alone ($r = 0.454$, $P = 0.004$), indicating a consistent pattern of isolation by distance within this species (Fig. 3b).

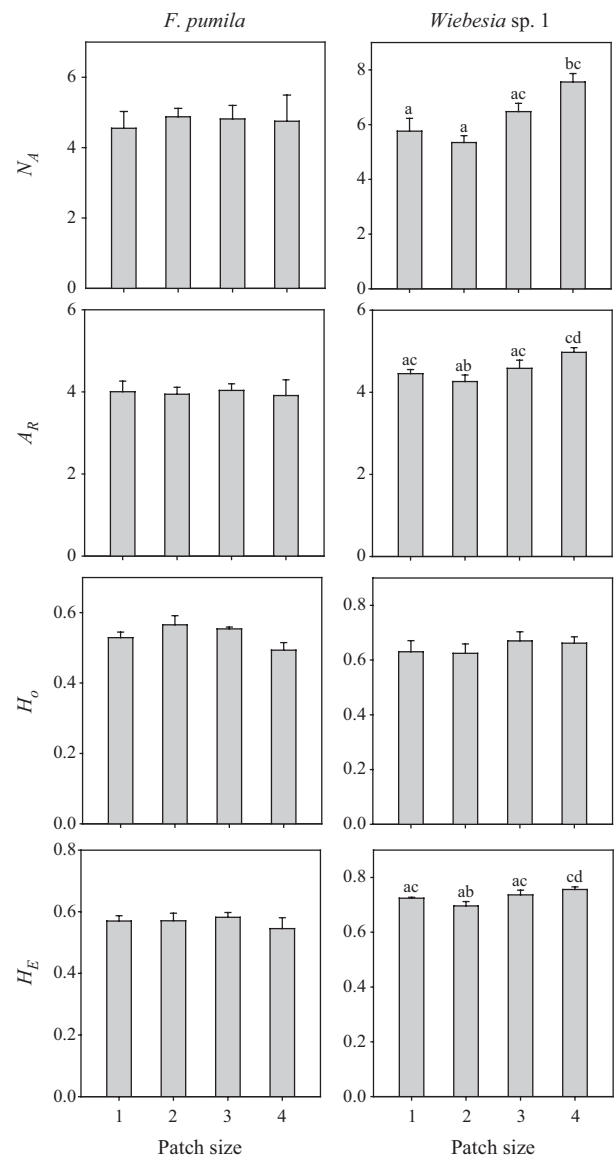


Fig. 2 Genetic variation (N_A , A_R , H_O , H_E) in populations of *Ficus pumila* (left panels) and its pollinator *Wiebesia* sp. 1 (right panels) in habitat patches of different sizes. *Ficus pumila*: N_A (d.f. = 3, $F = 0.080$, $P = 0.970$), A_R (d.f. = 3, $F = 0.037$, $P = 0.990$), H_O (d.f. = 3, $F = 1.812$, $P = 0.173$), H_E (d.f. = 3, $F = 0.261$, $P = 0.853$); *Wiebesia* sp. 1: N_A (d.f. = 3, $F = 10.380$, $P < 0.001$), A_R (d.f. = 3, $F = 4.227$, $P = 0.016$), H_O (d.f. = 3, $F = 0.384$, $P = 0.766$), H_E (d.f. = 3, $F = 3.478$, $P = 0.0323$). 1, 2, 3 and 4 represent small (<100 km²), moderate (10–100 km²) and large (>100 km²) island populations, and mainland populations, respectively. Each value shows mean + SE. Different letters above columns indicate significant ($P < 0.05$) differences between groups (where present) according to post hoc Tukey's HSD.

Mantel tests indicated no significant relationship in the extent of genetic differentiation between *F. pumila* and *Wiebesia* sp. 1, either globally ($r = 0.178$, $P = 0.112$)

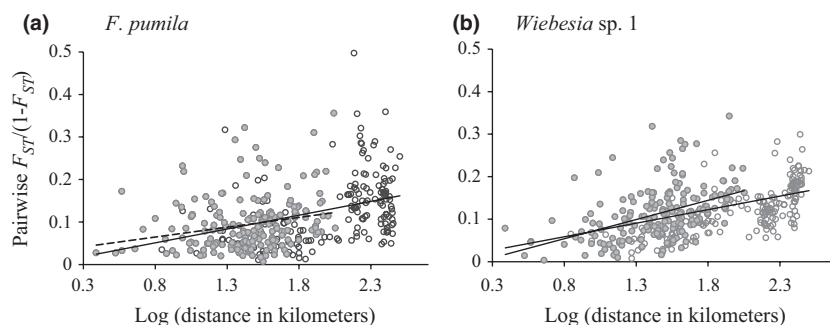


Fig. 3 The relationship between geographical distance and genetic dissimilarity in populations of *Ficus pumila* (a) and its pollinating wasp *Wiebesia* sp. 1 (b). Filled circles indicate distances of island–island populations, and empty circles indicate distances of mainland–mainland populations and mainland–island populations. A solid line indicates a significant relationship ($P < 0.05$), and a dashed line indicates no significant relationship.

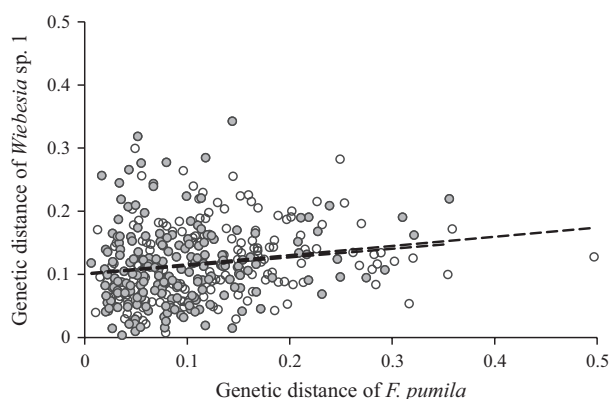


Fig. 4 Relationships between genetic differentiation of *Ficus pumila* and *Wiebesia* sp. 1 populations. Filled circles indicate distances of island–island populations, and empty circles indicate distances of mainland–mainland populations and mainland–island populations. The dashed lines indicate no significant relationship ($P > 0.05$).

or among island populations only ($r = 0.37$, $P = 0.165$; Fig. 4). Populations of *Wiebesia* sp. 1 and its host therefore have different genetic structures.

Genetic clustering

In the analysis of assignment test using STRUCTURE, the most appropriate K for both *F. pumila* and *Wiebesia* sp. 1 was three (Fig. S2, Supporting information), indicating that individuals of both species were most appropriately divided into three clusters. The three clusters of *Wiebesia* sp. 1 showed an obvious spatial structure, with a west–eastern gradient in genetic composition. In contrast, populations of *F. pumila* were much more mixed and provided little evidence of geographical effects (Fig. 5).

Linear regression revealed that there was a significant positive relationship between genetic distance and

geographical distance of other populations from the most westerly population JH ($r^2 = 0.304$, $P = 0.003$; Fig. 6a), but no significant relationship between genetic and geographical distances of other populations with the most easterly population SS ($r^2 = 0.082$, $P = 0.115$, Fig. 6b), a result that is consistent with a west-to-east dispersal pattern of *Wiebesia* sp. 1. However, similar analyses of *F. pumila* populations failed to detect any significant relationships between genetic and geographical distances (Fig. S3, Supporting information).

Population bottlenecks

Bottleneck analysis indicated that one mainland (TM) and three island (DB, MA and QS) populations of *Wiebesia* sp. 1 had experienced recent bottlenecks. However, we found no significant signs of recent bottlenecks in either mainland or island populations of *F. pumila*.

Discussion

Incongruent genetic structure between Wiebesia sp. 1 and its host plant

Population sizes of plant-specific phytophagous insects are closely related to available resources – their host plants' population sizes (Dunn *et al.* 2009). Parallel relationships in genetic structure are therefore also to be expected, yet populations of *Wiebesia* sp. 1 and *F. pumila*, despite being obligate mutualists, display quite different patterns of genetic structure. There was no significant positive relationship in genetic diversity between the two species, and although isolation-by-distance patterns were found in both *Wiebesia* sp. 1 and its host plant, there was no significant positive relationship in genetic differentiation between them. Furthermore, a distinct west-to-east clinal change was found in *Wiebesia* sp. 1, but not in *F. pumila*. Such absences of

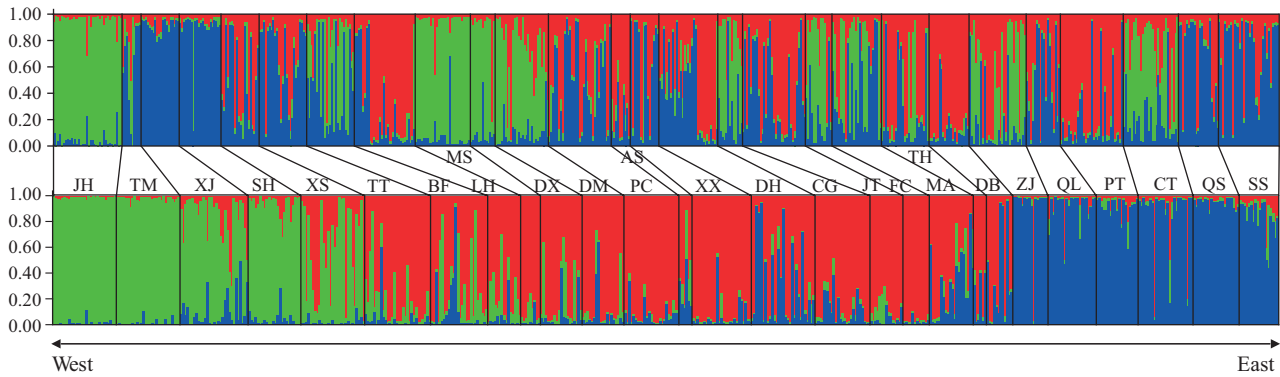


Fig. 5 Genetic clusters of *Ficus pumila* (above) and *Wiebesia* sp. 1 (below) populations assigned by STRUCTURE. The populations are arranged by their longitudes, from west to east. Green, blue and red represent the probability of assignment to the three major clades.

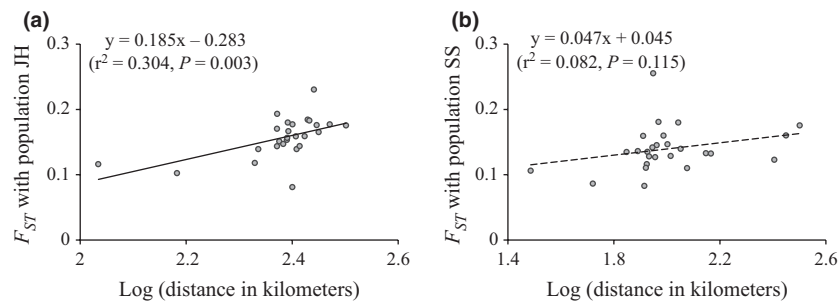


Fig. 6 A linear regression of relationships between genetic distance and geographical distance of other populations to the most westward population JH (a) and to the most eastward population SS (b) of *Wiebesia* sp. 1.

concordance in genetic structure between ecologically related species have been observed earlier (Jones & Britten 2010). Magalhaes *et al.* (2011) similarly detected no significant correlation between the genetic distances of *Silene latifolia* and its pollinator, the moth *Hadena bicruris*. Nevertheless, populations of intimately interacting species do sometimes show similar patterns (e.g. Parker & Spoerke 1998; Smith *et al.* 2011), and in the case of the obligate pollination mutualism between yuccas and yucca moths, shared histories of almost simultaneous population expansion have been detected (Smith *et al.* 2011).

Differences in modes of dispersal are most likely to be responsible for discordant patterns in genetic structure between populations of intimately associated species (McCoy *et al.* 2005; Darvill *et al.* 2010; Jones & Britten 2010). Gene flow in fig wasps is only implemented by short-lived adult female wasps (Kjellberg *et al.* 1988). Unlike wasps associated with large monoecious *Ficus* trees, wasps of small shrubby dioecious species appear to prefer to stay in the understory, where air speeds are low (Harrison & Rasplus 2006; Chen *et al.* 2011). Reflecting this contrast, pollinators of monoecious fig trees such as *F. racemosa* show little population

structuring over a scale of thousands of kilometres (Kobmoo *et al.* 2010), whereas mean pollination distances of only about 200 m were recorded in two dioecious species in India (Dev *et al.* 2011). Restricted gene flow in *Wiebesia* sp. 1 shows that pollen flow is also limited in *F. pumila*. However, the genes of *F. pumila* are also distributed via the dispersal of its seeds and, given that fruit-eating birds can carry seeds over long distances between islands (Shanahan *et al.* 2001a), they are likely to have generated the less structured pattern we observed (Fig. 5), with a relatively small F_{ST} compared with *Wiebesia* sp. 1.

Major fluctuations in environmental conditions can lead to temporary local extinctions of fig tree pollinators, but not of their host plants. Fig wasps in areas of Borneo disappeared temporarily in 1998 due to an extended period of dry weather caused by an El Nino event, during which the trees failed to produce figs (Harrison 2000). Bronstein & Hossaert-Mckey (1995) also reported a local extinction in Florida after Hurricane Andrew, but the pollinators returned quickly, once the trees started producing figs again. Similar events may also have taken place in our study region and led to the contrasts in genetic structure we

observed. Since we began to study *F. pumila* and its pollinators in 2004, several major typhoons have passed over the Zhoushan Archipelago and adjacent mainland, including Typhoon Meihua in 2011 and Typhoon Haikui in 2012. The trees survived, but few figs remained, leading to falls in the fig wasp population sizes. The pollinators populations persisted, but more extreme disruptions in the past are likely to have led to local extinctions. In addition, differing responses of *F. pumila* and *Wiebesia* sp. 1 to habitat fragmentation may also have contributed to the incongruent genetic structures of their populations.

Responses of Wiebesia sp. 1 and its host plant to habitat fragmentation

We did not observe any negative effects of fragmentation on genetic variation in *F. pumila*. This indicates that *F. pumila* populations have not been greatly affected by postglacial sea level rises and the isolation of their populations on separate islands. In contrast, populations of *Wiebesia* sp. 1 occupying larger land masses possessed significantly higher genetic variation than those on the smaller islands. The occurrence of genetic bottlenecks and linkage disequilibrium in island populations in *Wiebesia* sp. 1 suggests that this host-specific pollinating wasp may be more susceptible to habitat fragmentation than its host plant, despite their mutual dependency. A similar conclusion, involving different trophic levels, was reached in a host-parasitoid system involving the specialist parasitoid *Neotypus melanocephalus* and its butterfly host *Maculinea nausithous* (Anton *et al.* 2007). However, the fig wasp populations on larger islands may also have been less likely to go extinct as a result of typhoons and other major perturbations, so it is difficult to isolate the cause of reduced genetic diversity on smaller islands.

The contrasting lifespans and generation times of *F. pumila* and its pollinator are also likely to have played a critical role in the contrasting genetic structures of the two species in the relatively recently fragmented landscape they occupy. Despite its low trophic stature, *F. pumila* is a long-lived woody species, and like other long-lived trees is relatively immune even to long-term fragmentation (Lowe *et al.* 2005). In contrast, *Wiebesia* sp. 1 has two or three generations each year (Liu *et al.*, unpublished data). Fewer generations of the host species have provided less opportunity for genetic drift and inbreeding (Lowe *et al.* 2005) because their populations were fragmented when sea levels rose.

Strong dispersal can link spatially isolated populations into one patchy population, and the dispersal ability of a species therefore contributes to its susceptibility to habitat fragmentation (Darvill *et al.* 2010). As

discussed above, in addition to pollen flow mediated by *Wiebesia* sp. 1, the genes of *F. pumila* are also distributed by fruit-eating birds, which may fly over long distances between islands, and thus, *F. pumila* is more resistant to habitat fragmentation than *Wiebesia* sp. 1.

Demographic factors may also have contributed to the discordance in genetic structuring between *F. pumila* and its pollinator, because populations of the latter are strongly influenced by fluctuations in the resources provided by the plant. *Ficus pumila* is the only host plant for *Wiebesia* sp. 1, and variation in size of the crops produced by male *F. pumila* will be reflected in the sizes of its pollinator populations. Crop sizes on individual *F. pumila* change between seasons of the same year. In the spring, male *F. pumila* produce few if any receptive figs, whereas female trees produce large crops. This ensures pollination of the female figs, but also means that pollinator populations suffer bottlenecks each year (Liu *et al.* unpublished data). Crop sizes also fluctuate considerably across years, leading to drastic change in population sizes of *Wiebesia* sp. 1. These fluctuations in the population sizes of their pollinators are nonetheless unlikely to have significant genetic consequences for host populations, because they are so transient.

The presence of a second pollinating wasp, *Wiebesia* sp. 3, in the Zhoushan Archipelago could mitigate some of the effects of population fluctuations in the primary pollinator, as has been shown in some studies of a different system of plants and their pollinating floral parasites (Thompson *et al.* 2010). However, *Wiebesia* sp. 3 has been found in only nine of the studied islands and has a much lower abundance than *Wiebesia* sp. 1. Its presence would therefore appear unlikely to be responsible for the large-scale genetic patterns found in *F. pumila*.

Conclusion

Even in intimate interactions such as obligate pollination mutualisms, susceptibility to habitat fragmentation can be different between the partners, due to differential species specificity and longevity. Our genetic study of *F. pumila* and its pollinator *Wiebesia* sp. 1 has shown that the pollinator appears to be more susceptible to habitat fragmentation than its host plant, with lower genetic variation in small- and medium-sized island populations, higher genetic differentiation among populations and more bottlenecks than its host plant. *F. pumila* shows no obvious genetic effects as a result of the limited dispersal of its pollinating fig wasp on the islands. However, in the long term, restricted pollen dispersal may affect the persistence of genetic diversity in these fragmented populations of *F. pumila*, especially if the abundance of seed dispersal agents has declined.

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Data accessibility

Sampling information: Table 1. Microsatellite data: DRYAD doi:10.5061/dryad.hp6tb.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 The results of Mantel tests for genetic relationships among geographically separated populations of figs and also of fig wasp.

Fig. S1 The shorelines of studied area since 13 500 BP, when local sea levels were about 70 m lower than today.

Fig. S2 The mean posterior probability ($\ln P(D)$) values (\pm SD) of each K according to Pritchard *et al.*'s (2000) method and the corresponding ΔK statistics based on Evanno *et al.*'s (2005) method for *Ficus pumila* (A) and *Wiebesia* sp.1 (B).

Fig. S3 The linear regression of relationship between genetic distance and geographical distance of other populations to the most westward population JH (A) and to the most eastward population SS (B) of *Ficus pumila*.