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# Fig trees at the northern limit of their range: the distributions of cryptic pollinators indicate multiple glacial refugia

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## Abstract

**Climatic oscillations during the last few million years had well-documented effects on the distributions and genomes of temperate plants and animals, but much less is known of their impacts on tropical and subtropical species. In contrast to Europe and North America, ice-sheets did not cover most of China during glacial periods, and the effects of glacial cycles were less dramatic. Fig trees are a predominantly tropical group pollinated by host-specific fig wasps. We employed partial mitochondrial *COI* (918 bp) and nuclear *ITS2* (462 bp) gene sequences to investigate the genetic structure and demographic histories of the wasps that pollinate the subtropical *Ficus pumila* var. *pumila* in Southeastern China. Deep genetic divergence in both mitochondrial (7.2–11.6%) and nuclear genes (1.6–2.9%) indicates that three pollinator species are present and that they diverged about 4.72 and 6.00 Myr BP. This predates the Quaternary ice ages, but corresponds with the formation of the Taiwan Strait and uplifting of the Wuyi–Xianxia Mountains. The three pollinators have largely allopatric distribution patterns in China and display different postglacial demographic histories. *Wiebesia* spp. 1 and 2 occupy, respectively, the northern and southern regions of the mainland host range. Their populations both underwent significant postglacial spatial expansions, but at different times and at different rates. *Wiebesia* sp. 3 is largely restricted to northern islands and shows less evidence of recent population expansion. Their mainly allopatric distributions and different demographic histories are consistent with host plant postglacial expansion from three distinct refugia and suggest one mechanism whereby fig trees gain multiple pollinators.**

*Keywords*: co-evolution, demographic history, *Ficus pumila*, fig wasp, glacial refugium, phylogeography, *Wiebesia*

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## Introduction

The climate oscillations of the past few million years have had dramatic impacts on north temperate plants and animals, generating extinctions and repeatedly altering the distributions of surviving species (Hewitt 2000, 2004). During glacial periods, temperate species are driven towards the Equator, but then have the opportunity to expand their distributions polewards during warmer interglacial periods like those of the present day. Shifts in distribution are especially obvious in Europe and North America, where the development of massive ice-sheets made large areas of the continents uninhabitable (Reviewed by Hewitt 1996, 2000, 2004; Soltis *et al.* 2006; Shafer *et al.* 2010). The impact of Pleistocene glacial cycles on speciation rates is less clear (Baker 2008). Vicariance induced by the fragmentation

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of populations during glacial periods would be expected to facilitate speciation, but it appears that the repeated admixture of different refugial populations has often prevented the genetic differentiation required for sufficient divergence (Hewitt 2001; Barnosky 2005; Shafer *et al*. 2010).

Unlike Europe and North America, late Quaternary ice-sheets and glaciers were of limited extent in China and only developed on the Qinghai-Tibet Plateau and adjacent high elevation mountains in West China, leaving most of Eastern China free from ice cover (Zhou *et al.* 2004). Cool-adapted plants and animals could therefore survive in Southeastern China throughout glacial periods, and the size, distribution and numbers of these temperate refugia are increasingly well understood (Qian & Ricklefs 2000; Harrison *et al.* 2001; Qiu *et al.* 2011). Recent molecular phylogeographical studies suggest that there were multiple refugia for temperate plants in China, distributed both in the subtropical biome of today and in more northerly latitudes (Qiu *et al.* 2011). The refugia are believed to have contributed to the exceptional diversity of the contemporary temperate flora in China (Qian & Ricklefs 2000; Harrison *et al.* 2001; Qiu *et al.* 2011). In contrast, the subtropical biome, the largest biome in China today (Wu 1980), retreated southwards into Southeast Asia and was largely absent from China during glacial maxima (Yu *et al.* 2000; Ni *et al.* 2010). As a consequence, most populations of subtropical species were lost from China and the species present today have recolonized from southern refugia. Based on patterns of endemism and physiognomy, several such refugia for subtropical species have been inferred (Wang & Liu 1994), but few molecular phylogeographical studies have been conducted to elucidate their location and their roles in population persistence and divergence (Shen *et al.* 2005; Lu *et al.* 2006; Wang *et al.* 2009a).

Fig trees (*Ficus* spp., Moraceae) are of great ecological significance because of the large numbers of vertebrates that feed on the figs they produce (Shanahan *et al.* 2001). The 800 or more species are mainly restricted to tropical regions today, but during warmer periods, they have expanded northwards, colonizing England during the Eocene (Compton *et al.* 2010). In the present study, we describe the postglacial demographic dynamics of the pollinating fig wasps (Agaonidae) associated with *Ficus pumila* L., a common subtropical dioecious fig tree in Southeastern China, and use the distributions of the insects to infer the distribution of their host plant during the last glaciation. Fig wasps and their fig tree hosts represent one of the most tightly co-evolved and ancient plant–insect mutualisms (Weiblen 2002). Fig wasps depend on figs to provide larval feeding sites (in galled ovules), while figs depend on fig wasps to transfer pollen between figs. Previously, it was accepted that each species of fig tree was pollinated by a single species of host-plant-specific fig wasp (Ramirez 1974; Janzen 1979; Wiebes 1979), but molecular markers have detected an increasing number of cases of trees with two or more species of 'cryptic' pollinators that differ only slightly, if at all, in external morphology. One:one relationships may even be in a minority in some fig tree lineages (Molbo *et al.* 2003; Haine *et al.* 2006; Herre *et al.* 2008; Su *et al.* 2008). This has called into question the assumption of strict sense cospeciation between fig trees and fig wasps, where new species of trees and pollinators arise together (Erasmus *et al.* 2007; Herre *et al.* 2008), but leaves unclear the events that lead to fig trees obtaining multiple pollinators. This could result from more rapid speciation in pollinators than their host figs, leading to multiple pollinators sharing same hosts (e.g. Molbo *et al.* 2003; Haine *et al.* 2006; Lin *et al.* 2008) or host switching by fig wasps (leading to unrelated species sharing hosts) (e.g. Compton *et al.* 1991). Local adaptation and speciation are potential consequences of populations becoming isolated in multiple refugia during glacial periods (Shafer *et al.* 2010; Qiu *et al.* 2011), but the responses of fig trees and fig wasps to periods of glaciation have rarely been considered (Lin *et al.* 2008).

*Ficus pumila* is one of the most northerly distributed fig trees. In China, it is at the northern limit of its mainland distribution and also occurs on offshore islands—two situations where it has been predicted that multiple pollinators of fig trees are particularly likely (Janzen 1979). We employed mitochondrial and nuclear genes to investigate the genetic structure and population histories of the pollinators of *F. pumila* in Southeastern China to answer the following questions: Is there more than one species of fig wasp pollinating *F. pumila* in China, and if so, did they diverge during the period of Quaternary glaciations? Are their current distributions sympatric or allopatric? Did they have one or multiple refugia during the last glaciation? If there were multiple refugia, did they facilitate the differentiation of these cryptic species?

## Materials and methods

### *Study species*

*Ficus pumila*, the creeping fig, was placed in subgenus *Synoecia*, section *Rhizocladus*, by Berg & Corner (2005), but recent multilocus phylogenetic studies of both the plant (Ronsted *et al.* 2008) and its pollinating fig wasps (Cruaud *et al.* 2010) suggest that its true affinities lie in subgenus *Ficus*, section *Ficus*, subsection *Frutescentiae*. The natural distribution of *F. pumila* includes Southern

China, Japan and Vietnam (Wu & Raven 1994), but it is widely planted elsewhere. It is a functionally dioecious evergreen shrub that sprawls across old walls, rocks and trees. Large individuals can form dense mats extending for over 10 m. Its immature leaves (bathyphylls) are quite different in size and shape from mature ones (acrophylls). The figs, which are produced in acrophyll axils, are large, reaching over 70 mm in diameter, and contain thousands of flowers. Like other dioecious fig trees, male individuals produce only pollen-carrying fig wasps and females produce only seeds (Wang *et al.* 2009b). Male trees growing in Hong Kong were reported to produce three overlapping crops (Hill 1967a). Further north in China, just one or two crops are reported to be produced annually, maturing in the spring and sometimes additionally in the autumn (Ma & Wu 1989; Luo *et al.* 2000). Two varieties of *F. pumila* have been described, differing in the shape of their figs. *Ficus pumila* var. *pumila* is native to mainland Asia and the island of Taiwan, whereas *Ficus pumila* var. *awkeotsang* was probably originally endemic to Taiwan. Both varieties of *F. pumila* are found in Taiwan, but they have different habitat preferences, with *F. p. awkeotsang* distributed at higher altitudes in central and south Taiwan (usually >1200 m a.s.l.) than *F. p. pumila*. Although *F. p. awkeotsang* has been reported previously to be present naturally at two mainland sites (Wu & Raven 1994), our field surveys have failed to confirm the presence of any native plants. However, in the last decade, *F. p. awkeotsang* and its pollinating wasps have been introduced and cultivated in mainland Fujian (Chen *et al.* 2008; Wu & Chen 2008).

*Wiebesia pumilae* (Hill), described from Hong Kong as *Blastophaga pumilae* (Hill 1967b), is the sole recorded pollinator of the two varieties of *F. pumila* (Hill 1967b; Ma & Wu 1989; He 1991; Wiebes 1994). A recent study in mainland China nonetheless found that experimental introductions into cultivated *F. p. awkeotsang* figs of pollinating wasps that emerged from *F. p. pumila* failed to produce galled ovules, whereas other wasps from *F. p. pumila* that entered *F. p. awkeotsang* figs under natural conditions could successfully pollinate the plants and produce galls (Chen *et al.* 2008). The possibility that two or more pollinator species are associated with the two varieties of *F. pumila* cannot therefore be excluded.

### *Sample collection and DNA preparation*

Between 2005 and 2010, we sampled pollinators from the variety *F. p. pumila*. Figs were collected from 43 natural populations in Southeastern China (Table 1, Fig. 1). Within each population, mature figs were collected from male host trees separated by at least 30 m from each other (to prevent repeat sampling from the same plant). These figs were kept in fine-mesh bags to let the wasps emerge. The pollinating fig wasps that emerged were preserved in absolute ethanol and stored in a refrigerator at 4 °C.

From each population, 7–10 female pollinator fig wasps were used for genetic analysis. Where possible, we chose just one wasp from one fig on each tree (Table 1). In total, 409 individuals from 367 *F. p. pumila* figs were analysed. Genomic DNA was extracted from the whole bodies of single female wasps using a method modified from Sambrook *et al.* (1989), using chloroform:isoamyl alcohol (24:1) to extract DNA, instead of phenol.

### *Sequencing of mitochondrial* COI *and nuclear* ITS2 *genes*

A 918-bp fragment of mitochondrial gene *CO*I was amplified using the universal primer pair Jerry/Pat (Simon *et al.* 1994). The PCR amplification was carried out in 50 μL volumes on PTC-220 DNA engine DYAD (MJ Research, USA). The thermal programme was 94 °C 3 min; 30 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min; followed by a final extension of 72 °C for 4 min. The PCR amplicons were sequenced on ABI PRISM 3730 from both directions using the same primer pair.

A 462-bp fragment of nuclear gene *ITS*2 was also amplified and sequenced. The same primer pair ITS-F/ITSR (Lopez-Vaamonde *et al.* 2001) was used for both PCR amplification and sequencing. The PCR amplifications were performed in 50 μL volumes, and the amplicons were again sequenced on ABI PRISM 3730. Initially, we sequenced the *ITS*2 fragment by randomly choosing samples from which we had sequenced mtDNA *CO*I fragments. However, we found all the sequenced individuals in each *CO*I clade had the same *ITS*2 haplotype. After sequencing 10–15 individuals in each clade chosen randomly, we then chose our *ITS*2 samples based on their *CO*I haplotypes to make sure our samples did have genetic differences. In total, 105 *ITS*2 sequences were obtained from a subset of individuals from each mtDNA clade.

Only sequences where the two-direction chromatograms were well matched were used. Sequences were aligned using ClustalX 2.0 (Larkin *et al.* 2007). The *CO*I haplotypes were also aligned with the *CO*I sequence of *W. pumilae* available in GenBank (AY014995, obtained from a *Wiebesia* sp. collected from *F. pumila* in Kenting, Taiwan. No information on its host variety is available, but the altitude of the sample site suggests *F. p. pumila*) and the *CO*I sequence of a *Wiebesia* sp. we reared from a *F. p. awkeotsang* fig in location ND (Fujian Province,

**Table 1** Sampled populations of pollinating fig wasps from *Ficus p. pumila*

| Code | Population | Geographical coordinates | No. of fig trees | No. of figs | No. of fig wasps | Clade (*CO*I haplotype) distributions* |
|---|---|---|---|---|---|---|
| YZ | Yangzhou | 32°24′N, 119°25′E | 4 | 10 | 10 | 1 (7) |
| SZ | Suzhou | 31°18′N, 120°38′E | 10 | 10 | 10 | 1 (7) |
| SH | Shanghai | 31°05′N, 121°12′E | 6 | 9 | 9 | 1 (7, 58, 59) |
| SS | Shengsi island | 30°42′N, 122°26′E | 6 | 10 | 10 | 1 (1, 2) |
| TM | Tianmushan | 30°31′N, 119°30′E | 8 | 10 | 10 | 1 (7) |
| QS | Qushan island | 30°26′N, 122°21′E | 4 | 5 | 8 | 1 (1, 10) |
| CT | Dachangtu island | 30°14′N, 122°18′E | 10 | 10 | 10 | 1 (1, 3, 4), 3 (35) |
| DJ | Dongji island | 30°14′N, 122°29′E | 6 | 10 | 10 | 3 (33, 34) |
| MA | Maao | 30°08′N, 122°06′E | 7 | 10 | 10 | 1 (1) |
| FC | Fuchi island | 30°05′N, 121°59′E | 3 | 4 | 8 | 1 (1, 5, 11, 16) |
| CG | Cengang | 30°03′N, 121°59′E | 6 | 10 | 10 | 1 (1, 3, 4) |
| JT | Jintang island | 30°03′N, 121°52′E | 10 | 10 | 10 | 1 (1, 17), 3 (33, 34) |
| DH | Dinghai | 30°00′N, 122°07′E | 10 | 10 | 10 | 1 (1, 14) |
| PT | Putuoshan island | 30°00′N, 122°23′E | 8 | 8 | 10 | 1 (1,5), 3 (33) |
| AS | Aoshan island | 29°59′N, 122°08′E | 2 | 2 | 9 | 1 (1, 2) |
| PC | Panzhi island | 29°59′N, 122°05′E | 6 | 10 | 10 | 1 (1, 3, 4) |
| XX | Xixiezhi island | 29°59′N, 122°02′E | 6 | 10 | 10 | 1 (1, 3, 4, 8, 13) |
| QL | Qionglongshan | 29°58′N, 122°18′E | 10 | 10 | 10 | 1 (1,3) |
| DM | Damao island | 29°57′N, 122°02′E | 9 | 9 | 9 | 1 (1), 3 (33, 34) |
| DX | Daxie island | 29°56′N, 121°57′E | 5 | 8 | 8 | 1 (1, 2, 3), 3 (34, 38) |
| ZJ | Zhujiajian island | 29°54′N, 122°23′E | 10 | 10 | 10 | 1 (1), 3 (33, 34, 38) |
| BF | Baifeng | 29°52′N, 121°57′E | 9 | 9 | 9 | 1 (1, 2, 8, 23, 24) |
| DB | Dengbu island | 29°52′N, 122°19′E | 5 | 5 | 10 | 1 (1), 3 (33) |
| TH | Taohua island | 29°48′N, 122°18′E | 10 | 10 | 10 | 1 (3, 6, 7), 3 (33, 34, 38, 40) |
| MS | Meishan island | 29°47′N, 121°59′E | 2 | 3 | 10 | 1 (1) |
| TT | Tiantong | 29°47′N, 121°47′E | 8 | 8 | 8 | 1 (1, 2, 3) |
| FD | Fodu island | 29°44′N, 122°01′E | 9 | 9 | 10 | 3 (33, 34) |
| LH | Liuheng island | 29°44′N, 122°04′E | 8 | 10 | 10 | 1 (1, 3, 15, 18), 3 (33, 34) |
| XS | Xianshan | 29°29′N, 121°50′E | 10 | 10 | 10 | 1 (1, 3) |
| JH | Jinhua | 29°09′N, 119°40′E | 7 | 10 | 10 | 1 (1, 6, 7, 19) |
| XJ | Xianju | 28°56′N, 120°45′E | 9 | 9 | 9 | 1 (1, 12, 20, 22) |
| LN | Linhai | 28°53′N, 121°08′E | 6 | 10 | 10 | 1 (1, 9, 21, 22), 2 (25, 27) |
| QZ | Quzhou | 28°42′N, 118°51′E | 4 | 10 | 10 | 1 (6, 7,19) |
| SR | Shangrao | 28°30′N, 117°52′E | 3 | 7 | 7 | 1 (6, 7, 42) |
| DG | Dongxiang | 28°15′N, 116°37′E | 2 | 5 | 10 | 1 (6, 7, 41) |
| WZ | Wenzhou | 27°54′N, 120°43′E | 6 | 10 | 10 | 1 (6), 2 (25, 53, 54, 56, 57) |
| FA | Fuan | 27°04′N, 119°25′E | 5 | 10 | 10 | 2 (25, 26, 28, 32) |
| ND | Ningde | 26°26′N, 119°20′E | 10 | 10 | 10 | 2 (25, 26, 29, 30, 31), 3 (36, 37, 39) |
| FZ | Fuzhou | 26°09′N, 119°17′E | 8 | 9 | 9 | 2 (25, 26, 46, 52) |
| YA | Yongan | 25°54′N, 117°26′E | 7 | 9 | 10 | 2 (43, 49, 50, 51) |
| TW | Taiwan | 25°02′N, 121°32′E | 1 | 1 | 8 | 2 (60, 61) |
| XM | Xiamen | 24°26′N, 118°08′E | 6 | 9 | 9 | 2 (44, 45, 47, 52) |
| HK | Hong Kong | 22°28′N, 114°11′E | 4 | 9 | 9 | 2 (46, 48, 52, 55) |

*Assigned clades of each population are indicated by numbers, while their *CO*I haplotypes are shown in parentheses.

mainland China, where this variety has been introduced).

### *Phylogenetic groups and their divergences*

Checks for indications of pseudogenes, such as multiple peaks in chromatograms, stop codons or frameshift mutations, were carried out on our mitochondrial *CO*I data according to the procedure suggested by Song *et al.* (2008). The *CO*I sequences were translated using the invertebrate mitochondrial genetic code by MEGA 4 (Tamura *et al.* 2007) and blasted in GenBank using MEGABLAST. No signs of pseudogenes were detected. The *CO*I data were then partitioned into two parts (1 + 2, 3) based on codon positions, with variable substitution rates applied owing to rate differences among codon positions and too little information from the second codon position. We ran BEAST v.1.6.1 (Drummond &

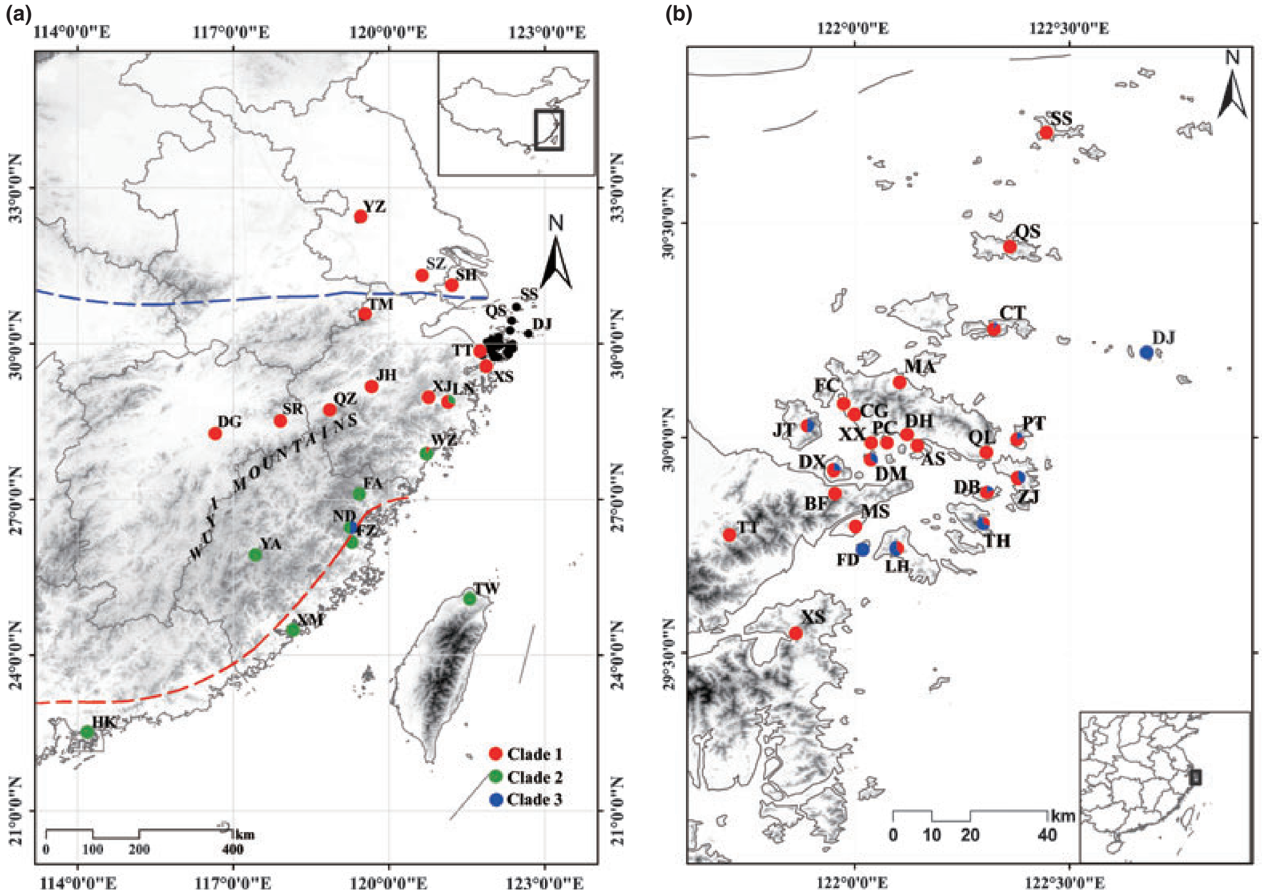

**Fig. 1** Left: Sample sites and distributions of pollinating fig wasp clades from *Ficus pumila* var. *pumila* in Southeastern China. The approximate northern borders of evergreen broadleaf forest zones today (modified after Song 1999) and during the LGM (21 000–18 000 years BP, modified after Harrison *et al.* 2001) are indicated by blue and red dashed lines, respectively. For sites indicated by black circles, consult the right-hand figure. Right: Detail of northern islands, Eastern Zhejiang Province.

Rambaut 2007; Heled & Drummond 2010) to explore the best combinations of substitution, clock and population models, with 10 million generations. Bayes Factors (posterior odds of one model relative to another) were applied (Kass & Raftery 1995; Nylander *et al.* 2004). LnBF (twice the difference in Ln harmonic mean of the likelihood of each model combination) (Suchard *et al.* 2001; Nicholls *et al.* 2010) was calculated with TRACER 1.5 (Rambaut & Drummond 2007) to assess the preferred models, based on the lnBF table of Kass & Raftery (1995). First, HKY and GTR models were compared under three different clock models (strict, uncorrelated exponential and lognormal relaxed clocks). BFs indicated HKY was the best model (with lnBFs from 8.63 to 21.65). Then, the HKY, HKY + I, HKY + G, HKY + I + G models were compared. HKY was again favoured (lnBFs from 2.80 to 4.45). Using the HKY model, we ran BEAST under either a strict clock, uncorrelated exponential or lognormal relaxed clocks, with each of population model (constant size, exponential growth, yule process, birth–death process). BFs indicated that the combination of a relaxed lognormal clock and the exponential process was the best (with lnBFs from 1.71 to 184.82 and most >33).

Phylogenetic relationships of the mitochondrial *COI* haplotypes were reconstructed using BEAST v.1.6.1 under deterministic models (HKY + lognormal relaxed clock + exponential). The data included the GenBank sequence AY014995 and the sequence of a pollinating wasp reared from *F. p. awkeotsang*. Molecular analyses have previously revealed that *Wiebesia* is polyphyletic and includes species that are not closely related (Machado *et al.* 2001; Jiang *et al.* 2006; Jackson *et al.* 2008; Cruaud *et al.* 2010). Among the species included in their analyses, Cruaud *et al.* (2010) indicated that *W. pumilae* was most closely related to *Blastophaga quadrupes*, then *Blastophaga esquirolianae* and *Blastophaga javana*. We failed to align the COI sequences of *B. quadrupes* from GenBank with our sequences, probably due to having different fragments of *COI*. Consequently, we used *COI* sequences of *B. esquirolianae* (GenBank accession number: AY842416) and *B. javana* (GenBank accession number: FJ619191, AY842417) as outgroups. After alignment, 789-bp sequences were obtained for phylogenetic analysis.

Two independent runs of 50 million iterations were performed, with genealogies and model parameters sampled every 1000 iterations. The chain convergence was checked based on ESSs (effective sample sizes) viewed in TRACER. All ESSs for each parameter had to be larger than 200. The phylogenetic tree was summarized by TREEANNOTATOR v.1.6.1, then viewed by FIGTREE v.1.3.1 (Rambaut 2006). The dates to the most recent common ancestor (TMRCA) were scaled by a mutation rate of 1 because no suitable fossil record could be found to calibrate node ages. Estimated divergence times of major nodes were converted to calendar years based on published rates of *COI* sequences for insects in general and net divergences between clades. Node ages were based initially on the net divergence of all three clades (internal nodes, excluding outgroups) and a substitution rate considered to be appropriate for pollinating fig wasps (1.9% per Myr) (Machado *et al.* 2001; Lin *et al.* 2008). They were then compared with node age ranges calculated on the basis of the net divergence of two external nodes (clades 1 and 2) and two extreme *COI* substitution rates for insects (1.2–2.8% per Myr) (Molbo *et al.* 2003).

Because of there being few point mutations in *ITS2* sequences and thus few haplotypes, we used a neighbour-joining tree (NJ tree) to reconstruct the phylogenetic relationships based on all *ITS2* sequences. An NJ tree was reconstructed using MEGA 4 (Tamura *et al.* 2007), and node supports were assessed based on 1000 bootstrap replicates.

Genetic clades were defined separately, according to the *COI* and *ITS2* phylogenetic trees. Both the *COI* and *ITS2* trees defined three monophyletic clades with highly similar compositions. Because phylogenetic networks can provide more information about intraspecific relationships than phylogenetic trees (Posada & Crandall 2001), we also explored the relationship of haplotypes within each clade. Networks of all sampled *COI* sequences in each clade were built separately by TCS 1.21 (Clement *et al.* 2000), using the 95% statistical parsimony criterion as a connection limit. Variation in *COI* sequences among clades, among populations within clades and within populations, was partitioned using hierarchical analysis of molecular variance (AMOVA) implemented in ARLEQUIN 3.01 (Excoffier *et al.* 2005). Significance tests were based on 10 000 permutations. We calculated K2P (Kimura-2-parameter) distances of *COI* and *ITS2* using MEGA 4. K2P distances have proved to be efficient in evaluating intra- and interspecific differences and are frequently used to delimit species, including fig wasps (Song *et al.* 2008; Sun *et al.* 2011).

### *Genetic polymorphism and demographic history*

During glaciations, warm-temperate species were driven towards the Equator and post-LGM populations expanding from southern refugia are expected to harbour higher genetic diversity than those from northern peripheral refugia, which had supported smaller and more isolated populations (Eckert *et al.* 2008). We used DNASP 5.10.01 (Librado & Rozas 2009) to assess the genetic diversity of *COI* sequences in each population and for all samples in each genetic clade. The numbers of haplotypes ($K$), segregating sites ($S$), synonymous substitutions ($dS$), nonsynonymous substitutions ($dN$) were calculated, together with haplotype diversity ($h$) and nucleotide diversity ($\pi$).

We excluded the haplotypes from Taiwan when we inferred the demographic histories of each clade because the limited sample (based on wasps reared from only one fig and comprising potential sibs) could bias the historical reconstruction. McDonald–Kreitman tests (McDonald & Kreitman 1991) implemented in DNASP were performed to inspect the null hypothesis of selective neutrality for mitochondrial *COI* sequences in each clade, using two Taiwan haplotypes and GenBank sequence AY014995 as outgroups. Similar ratios of synonymous/nonsynonymous substitutions within and between each group were expected under neutrality.

Historical population expansions were inferred by two methods. First, Tajima's (1989) $D$ and Fu's (1997) $F_S$ were tested for significance using 10 000 simulations in ARLEQUIN 3.01. Funk & Omland (2003) showed that Fu's test is very sensitive to spatial expansion, whereas Tajima's $D$ test is informative for bottleneck events (Tajima 1989). Then, mismatch distributions (Slatkin & Hudson 1991) were determined using both sudden expansion (pure demographic expansion) (Rogers & Harpending 1992) and spatial expansion (range expansion) models (Excoffier 2004), again in ARLEQUIN. We tested the observed distributions to the models with goodness-of-fit tests based on the sum of squared deviations (SSD) with 10 000 parametric bootstrap replicates.

We used a Bayesian MCMC approach implemented in BEAST to estimate the dates of clade expansions, based on *COI* sequences of all individuals rather than haplotypes in each clade. Data were also partitioned into two parts (1 + 2, 3). BFs were calculated to detect the most suitable clock and skyline models under the HKY substitution model. lnBF indicated that the exponential relaxed clock and piecewise-constant skyline models were the best combination for all three clades (lnBF, 126.85–172.27 for clade 1; 1.55–12.52 for clade 2; and 3.87–12.43 for clade 3). Bayesian skyline plots (Drummond *et al.* 2005) were calculated for each clade separately. Two independent runs of 150 million iterations

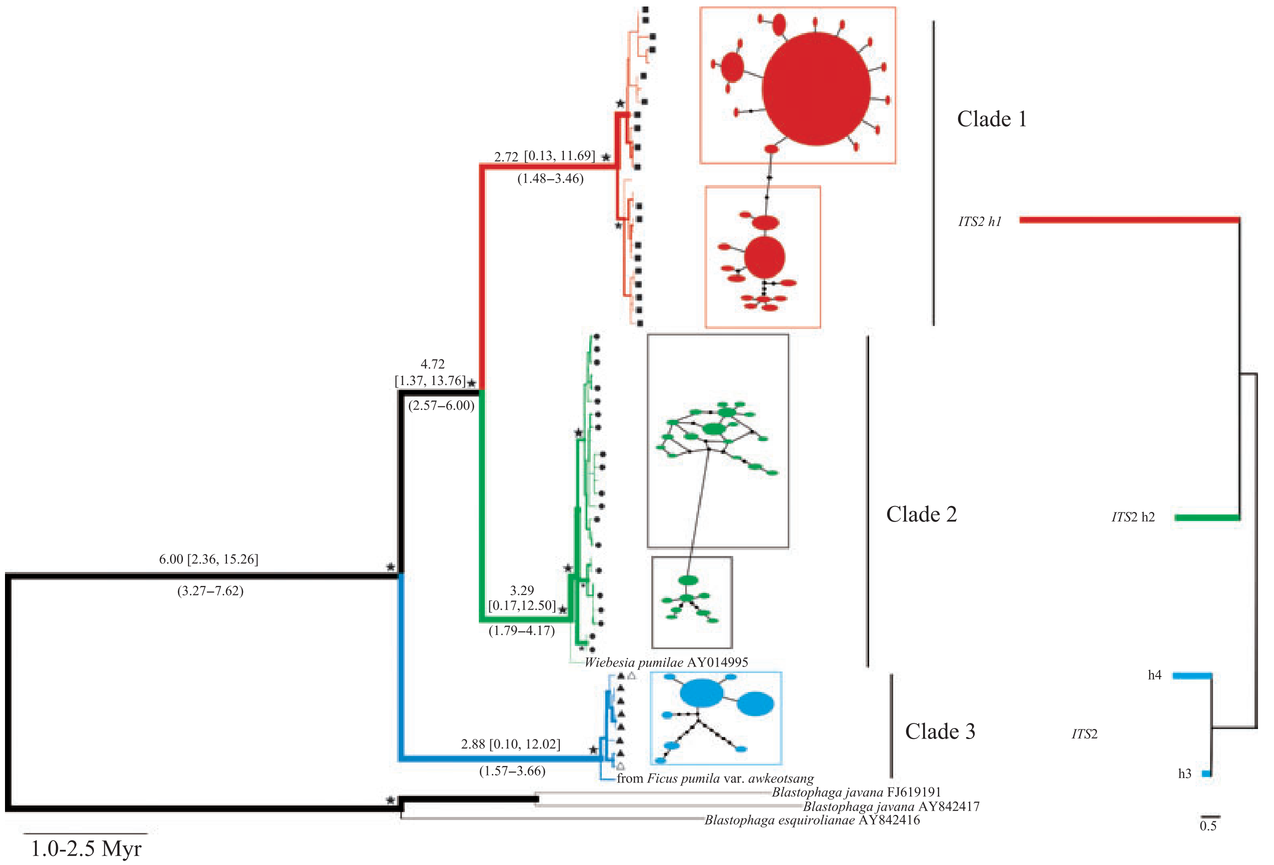

**Fig. 2** Phylogenetic trees and networks of *Wiebesia* pollinating fig wasps from *Ficus pumila* var. *pumila* together with a *Wiebesia* haplotype from *Ficus p. awkeotsang* and three outgroups. Left: Bayesian tree of *COI* haplotypes constructed by BEAST. The putative node age ranges (based on extreme substitution rates of *COI* for insects of 1.2–2.8% per Myr) are shown in parentheses below the branches and node ages based on a typical rate for pollinating fig wasps (1.9% per Myr) (Machado *et al.* 2001; Lin *et al.* 2008) are shown above them, together with 95% highest posterior density (HPD) intervals (in square brackets). Scale bar indicated the time ranges based on extreme substitution rates of *COI* for insects of 1.2–2.8% per Myr. Black stars indicate where posterior probability node supports are larger than 0.95. The corresponding *ITS*2 haplotypes of each *COI* haplotype (h1, h2, h3, h4) are indicated by black squares, black dots, black triangles and an open triangle, respectively. Middle: Networks based on *COI* sequences of individuals within each clade. The areas of each ellipse indicate haplotype frequencies, black dots indicate missing haplotypes. Right: The neighbour-joining tree of *ITS*2 sequences constructed by MEGA.

were performed for each clade separately. Genealogies and model parameters were sampled every 10 000 iterations, and 95% highest posterior densities (HPD) were estimated as credibility intervals. TRACER 1.5 was employed to visualize the results of each run and to reconstruct the Bayesian skyline with a burn-in of 2000. To convert the estimates scaled by mutation rate to calendar years, we applied the substitution rate of 1.9% per Myr.

## Results

### *Genetic divergence in pollinating wasps*

A total of 61 *COI* haplotypes (GenBank Accession Numbers: JN183988–JN184048) (Table S1, Supporting information) and 130 polymorphic sites were identified from the 409 pollinating fig wasp individuals reared from *Ficus p. pumila*. Among polymorphic sites, 118 were parsimony informative, with the most substitutions (82%) observed at the third codon position and the least (1.8%) at the second position.

The Bayesian tree of *COI* haplotypes built by BEAST divided the pollinating fig wasps into three distinct major clades with posterior probability node support of over 0.99. The haplotype of *Wiebesia pumilae* in GenBank (AY014995) was included in clade 2, while the haplotype of the pollinator of *Ficus p. awkeotsang* (GenBank accession number: JN184049) was clustered in clade 3 (Fig. 2). Based on node ages that indicate the date of the most ancient common ancestor of pairs of clades (tMACA) (Hayward & Stone 2006), clade 3 diverged from clades 1 plus 2 about 6.00 Myr BP, with 95% highest posterior density (HPD) intervals of 2.36–15.26 Myr BP. Clades 1 and 2 were estimated to have separated from each other about 4.72 Myr BP (95% HPD = 1.37–13.76 Myr BP).

Differences in *CO*I sequences between any two clades were more than 10 times the average differences within clades. The average pairwise Kimura-2-parameter (K2P) differences between clades ranged from 7.2% to 11.6%, while the mean pairwise differences within each clade were only 0.7% (ranging from 0.6% to 0.8%) (Table S2, Supporting information). The hierarchical AMOVA test verified the highly significant differentiation among these three clades, which contributed a large proportion of the total variance (96.36%, $P < 0.001$).

Four haplotypes (GenBank accession numbers: JN184050–JN184053) and 16 polymorphic sites (including three indels) were detected from the 105 *ITS*2 sequences. As a result of *ITS*2 amplification failure in some individuals, *ITS*2 sequences covered only 46 *CO*I haplotypes (Fig. 2). The nuclear *ITS*2 fragment also showed deep divergence among the three clades. Clades 1 and 2 shared only one *ITS*2 haplotype, while clade 3 had two *ITS*2 haplotypes. The two haplotypes in clade 3 were different at only one site, over a 462-bp sequence, whereas the haplotypes of different clades differed from each other by 7–13 substitutions. The K2P distances between haplotypes of different clades were also much higher than those between haplotypes within clades (Table S3, Supporting information).

### *Genetic diversity within each clade*

The three clades displayed contrasting overall genetic diversities, with clade 2 showing the highest overall genetic diversity ($h = 0.931$, $\pi = 0.004$, Table 2). Haplotype diversity ($h$) ranged from 0 to 1 with a mean of 0.451 in clade 1, from 0.667 to 1 with a mean of 0.791 in clade 2 and from 0 to 1 with a mean of 0.525 in clade 3. In clades 1, 2 and 3, nucleotide diversity ($\pi$) ranged from 0 to 0.005 with a mean of 0.001, 0.001–0.004 with a mean of 0.002, and 0–0.009 with a mean of 0.002, respectively (population data is not shown).

**Table 2** Genetic polymorphism within each *Wiebesia* clade based on *CO*I

| | *n* | *K* | *h* | *π* | *S* | *dS* | *dN* |
|---|---|---|---|---|---|---|---|
| Clade 1 | 279 | 28 | 0.749 | 0.003 | 32 | 30 | 2 |
| Clade 2 | 64 | 23 | 0.931 | 0.004 | 18 | 18 | 1 |
| Clade 3 | 57 | 8 | 0.658 | 0.002 | 20 | 20 | 0 |

*n*, sample size; *K*, number of haplotypes; *h*, haplotype diversity; *π*, nucleotide diversity; *S*, number of segregating sites; *dS*, number of synonymous substitutions; *dN*, number of nonsynonymous substitutions.
Samples collected from Taiwan are not included.

### *Historical demography within each clade*

The three clades have largely allopatric current distributions within the overall geographical range of *F. pumila* in Southeastern China (Fig. 1). Clade 1 was found in the northern host plant populations, and clade 2 occupied the southern host plant populations, including Taiwan. There was a contact zone where both species were present in an area close to the coast (locations LN and WZ). Clade 3 was mostly restricted to the northern islands, but was also reared from both *F. p. pumila* and the introduced *F. p. awkeotsang* at mainland location ND. There are no apparent morphological or genetic differences between the host plants in the three regions pollinated by different pollinator clades, as indicated by our preliminary studies on cpDNA variation among host populations. We sequenced 225 samples from 23 populations in mainland China, 10 samples from Hong Kong with *Wiebesia* sp. 2 as pollinator and 215 samples from 22 island populations with *Wiebesia* spp. 1 and 3 as pollinators. Three cpDNA haplotypes were detected. The Hong Kong population had all three, while all island populations shared one haplotype (F.E. Peng, M. Liu, and X.Y. Chen unpublished data).

Neutral evolution of mitochondrial genes in all three clades could not be rejected by the McDonald–Kreitman tests ($P > 0.05$, with Fisher's exact test, Table 3). The Tajima's $D$ neutrality tests revealed negative values in all three clades, but were only significant in clade 3 ($P = 0.038$). Fu's $F_S$ test showed the converse pattern, being large and significantly negative in clades 1 and 2, but failing to deviate from 0 in clade 3, thereby indicating significant expansion in clades 1 and 2, but not clade 3 (Table 3).

The three clades also showed different expansion patterns when tested under sudden expansion and spatial expansion models. Clade 1 fitted both models well ($P_{(SSD)} > 0.05$), indicating significant demographic and spatial expansion in its recent history. Clade 2 fitted the spatial expansion model ($P_{(SSD)} = 0.297$), but not the sudden expansion model ($P_{(SSD)} = 0.045$). Although clade 3 also showed significant recent demographic expansion ($P_{(SSD)} = 0.430$), but spatial expansion was rejected ($P_{(SSD)} < 0.001$, Table 3). The observed mismatch distribution of clade 2 was typical, being smooth and unimodal, whereas the pairwise differences in clades 1 and 3 were clearly shifted to the lower values, with almost no leading face, indicating recent expansion in all three clades and also

**Table 3** Neutrality tests and mismatch analyses under a sudden expansion model for the *Wiebesia* clades, based on *COI*

| | | | | | | Sudden expansion model | | Spatial expansion model | |
|---|---|---|---|---|---|---|---|---|---|
| | *P* for McDonald –Kreitman tests | Tajima's *D* | $P_{(D)}$ | $F_S$ | $P_{(F_S)}$ | SSD | $P_{(SSD)}$ | SSD | $P_{(SSD)}$ |
| Clade 1 | 0.295 | −1.174 | 0.103 | −9.194 | **0.018** | 0.033 | 0.398 | 0.026 | 0.484 |
| Clade 2 | 1.000 | −0.233 | 0.465 | −10.208 | **<0.001** | 0.008 | **0.045** | 0.007 | 0.297 |
| Clade 3 | 0.726 | −1.556 | **0.038** | 0.076 | 0.568 | 0.047 | 0.430 | 0.035 | **<0.001** |

Significant values ($P < 0.05$) are shown in bold.

bottleneck events in clades 1 and 3 (Fig. S1, Supporting information).

The network of clade 1 showed typical star-like phylogenies, with the two most common haplotypes separated from the remaining haplotypes by 1–5 mutational steps. The network of clade 2 displayed numerous loops without obviously dominant haplotypes. Clade 3 had considerable numbers of absent haplotypes (Fig. 2), indicating genetic drift, bottleneck events or both.

The Bayesian skyline plot (BSP) analysis based on *COI* lineages suggested past increases in effective population sizes of clades 1 and 2, but at different times. Clade 1 showed evidence for a rapid population expansion from about 10 000 to 15 000 years BP, whereas clade 2 expanded from about 20 000 to 30 000 years BP. For clade 3, BSP analysis suggested slight shrinkage, rather than expansion in population size (Fig. 3).

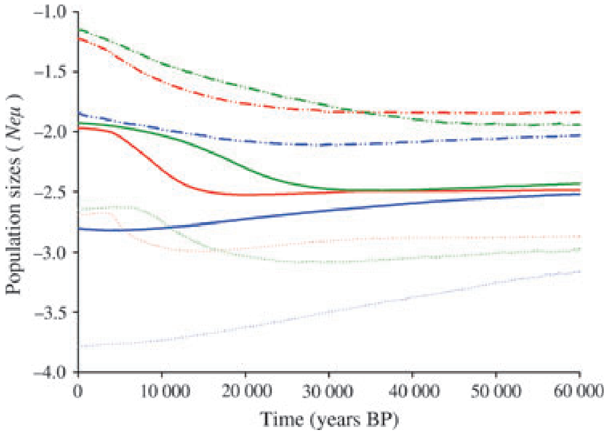

**Fig. 3** Median Bayesian skyline plots of the three clades (clade 1 red, clade 2 green and clade 3 blue) revealing demographic trends in their mitochondrial *COI* lineages. The median estimates are shown by solid lines, the higher and lower 95% HPD limits by dash-dot-dot and dotted lines, respectively. Time estimates are based on a rate of $1.9 \times 10^{-8}$ substitutions per site per year. The *y*-axis units are estimated $N_e\mu$ (the effective population size and the mutation rate), presented on a logarithmic scale.

## Discussion

### How many species of pollinating wasps?

The nuclear *ITS2* gene has previously been shown to be an excellent marker for distinguishing fig wasp species (Xiao *et al.* 2010), as was the case here with the pollinating fig wasps of *Ficus p. pumila* in Southeastern China. The fig wasps showed deep genetic divergence in both this nuclear gene and mitochondrial *COI*, revealing three putative species of pollinating wasps. The K2P distances of *COI* between clade pairs (7.2–11.6%) were more than 10 times the average differences within them and beyond the standard *COI* barcoding threshold for animal species (Hebert *et al.* 2004). The deep mtDNA divergence is also similar to that documented for other 'cryptic' pollinating fig wasp species described previously (Molbo *et al.* 2003; Haine *et al.* 2006; Lin *et al.* 2010).

The *ITS2* haplotypes within each clade were highly homogenous. There was only one haplotype in clades 1 and 2, and the two haplotypes in clade 3 differed from each other by only a single mutation. In contrast, the haplotypes from different clades differed from each other by 7–13 substitutions. This pattern reflects the concerted evolution of ribosomal genes, where intraspecific variation is homogenized, but interspecific variation accumulates (Hillis & Dixon 1991; Smith *et al.* 2006), and is again consistent with there being three putative taxa present.

The preliminary study of the genetic structure of the host *Ficus* revealed no genetic divergence among host populations of the three pollinator clades, with all island host populations sharing the same cpDNA haplotype, irrespective of their associated pollinator (F. E. Peng *et al.* unpublished data). This indicates that the three wasp clades are not associated with parallel divergence in their host plant. All three fig wasp taxa pollinate *F. p. pumila* in China, and they are referred to as *Wiebesia* spp. 1, 2 and 3 in the following discussion, corresponding to clades 1, 2 and 3, respectively.

Detailed examination of the three species has revealed small differences in the external anatomy of both males and females (S. G. Compton unpublished data). The differences are sufficiently small to be overlooked during routine examination, which partly explains why they were not detected previously. *Wiebesia* sp. 2 is likely to correspond to *Wiebesia pumilae* as it was the only species we recorded from Hong Kong, the type locality of *W. pumilae*. These results show that *F. pumila* has joined the increasingly long list of fig tree species serviced by two or more sister-species of pollinator. Examples include *Eupristina* species associated with *Ficus microcarpa* in China (Sun *et al.* 2011), *Ceratosolen* species associated with *Ficus sur* in Africa (Kerdelhue *et al.* 1999) and *Ficus septica* in Asia (Lin *et al.* 2010), *Pleistodontes* species associated with *Ficus rubiginosa* in Australia (Haine *et al.* 2006), and others (Molbo *et al.* 2003; Cook & Segar 2010; Moe *et al.* 2011). Differences in habitat preferences have been demonstrated in, for example, the pollinators of *Ficus ottoniifolia* in West Africa, where one species is associated with forest, another with savanna (Michaloud *et al.* 1985), but few fig tree species have been sampled at numerous locations within their distributions.

Recent studies from America, Australia, Africa and Asia have revealed the complex and diverse histories of many tropical plant and animal species. The origins of many of these species, including numerous insects, date from the Pliocene (reviewed by Hewitt 2004; Beheregaray 2008; Elias *et al.* 2009; Morgan *et al.* 2009). Similarly, we detected a pattern of Miocene and Pliocene, rather than Pleistocene, divergences among the pollinating wasps of *F. p. pumila* in China. *Wiebesia* sp. 3 diverged first, at an estimated date of 6.00 Myr BP (Fig. 2). The divergence between *Wiebesia* sp. 3 and the ancestor of *Wiebesia* spp. 1 and 2 corresponds with the period in the late Miocene when the Taiwan Strait was formed and Taiwan first became an island (Zhao 1982). Species 3 was restricted to island populations except for an isolated record from Fujian Province in an area where *Ficus p. awkeotsang* has recently been introduced from Taiwan, and *Wiebesia* sp. 3 may have been transported there with its host (Chen *et al*. 2008; Wu & Chen 2008). Our very limited initial sample from Taiwan failed to detect *Wiebesia* sp. 3, but more extensive sampling in Taiwan is clearly required.

*Wiebesia* spp. 1 and 2 diverged about 4.72 Myr BP (Fig. 2). Their divergence coincided with a late Tertiary to Quaternary period of major uplift in Southeastern China that resulted in the approximately SW-NE running Wuyi–Xianxia mountain chains. Three major uplifts are believed to have taken place, each of 300–400 m (Chen & Zhou 1993). These would have resulted in the separation of *F. pumila* populations to the north and south of the mountains, as is seen today, where *Wiebesia* sp. 1 is the pollinator to the Northwest and *Wiebesia* sp. 2 is the pollinator to the Southeast. The timing of the separation of *Wiebesia* spp. 1 and 2 is therefore consistent with a vicariance model of speciation.

### *Do contemporary distributions suggest multiple glacial refugia?*

*Ficus pumila* is currently distributed across northern tropical and subtropical eastern Asia, from Indochina to Japan, but is widely grown as an ornamental beyond this range (Hill 1967a; Berg & Corner 2005). In China, its distribution is within broadleaved evergreen forest and warm mixed forest zones, where it extends to approximately 34°N (Wu & Raven 1994; personal observations). Analysis of fossil pollen indicates that during the last Quaternary glacial maximum (LGM), these biomes retreated to the southern coast of China, with a northern limit of 24°N (Yu *et al.* 2000). This represents a southwards shift of around 300–1000 km relative to today (Ni *et al.* 2010). Consequently, *F. pumila* could not have survived in most of its current range in mainland China at that time, but may have survived in coastal areas (Qiu *et al.* 2011) and may also have extended its range further south into Indochina than it is found today.

The three species of pollinators associated with *F. p. pumila* in China had clearly diverged before the LGM, but today have largely allopatric distributions, with only limited areas of overlap (Fig. 1). Their contrasting distributions suggest that they may have had different refugia during the LGM and have only come into secondary contact relatively recently. If this is the case, then they would be expected to display contrasting demographic and colonization histories.

*Wiebesia* sp. 1 populations display a typical pattern of pioneer or leading-edge expansion (Hampe & Petit 2005), as seen in many populations of north temperate species in Europe (see Hewitt 1996, 2000). Significant demographic and spatial expansions are suggested by its unstructured star-like genealogy, significantly negative Fu's $F_S$ value ($P = 0.018$), mismatch distribution analysis under sudden expansion ($P_{SSD} = 0.398$) and spatial expansion models ($P_{SSD} = 0.484$) (Rogers & Harpending 1992; Rogers 1995; Fu 1997; Funk & Omland 2003; Excoffier 2004). Rapid postglacial expansion from a few pioneers or a small refugial population was also reflected in its genetic diversity and haplotype distribution patterns as this species has the lowest within-population genetic diversity among the three pollinator species, despite currently being the most widely distributed. Its two most common haplotypes were at the centre of the network, while other haplotypes were at very low frequencies and linked to the central haplotypes by 1–5 mutations, indicating their recent origin (Fig. 2). All these characteristics are consistent with a rapid postglacial expansion from a small population (Slatkin & Hudson 1991; Rogers 1995; Provan & Bennett 2008).

Based on its demographic history and current distribution, *Wiebesia* sp. 1 may have retreated Southwestwards along the northern edge of the Wuyi Mountains during glacial periods, becoming extinct in the northeastern part of its present distribution, in Zhejiang Province. Further south, Quaternary climate matching based on temperature and pollen data (Qiu *et al*. 2011) suggests that populations could have persisted on the northwestern edge of the mountains. The genetic evidence suggests that this refugium supported a relatively small population of *Wiebesia* sp. 1. After the last LGM, about 20 000 years BP, its population size increased, and then about 15 000 years BP as the climate became much warmer, it rapidly expanded northwards and northeastwards to attain its present distribution.

Genetically, *Wiebesia* sp. 2 populations display a 'phalanx' expansion pattern, implying relatively slow postglacial expansion into its current range. Species with similar attributes have been detected in southern Europe and North America and also the tropics (Hewitt 2004). *Wiebesia* sp. 2 nonetheless displays a significant expansion history, as revealed by a typical unimodal mismatch distribution, and a significant Fu's $F_S$ value ($P < 0.001$), which is thought to be very sensitive to spatial expansion (Funk & Omland 2003). However, only spatial ($P_{SSD} = 0.297$) and no demographic expansion was detected ($P_{SSD} = 0.045$) when populations were simulated under these respective models. *Wiebesia* sp. 2 also has both the highest overall and highest average population genetic diversity, with no obviously dominant haplotype in its phylogenetic network (Fig. 2). Combined with the small and not significantly negative Tajima's $D$ value ($D = -0.233$, $P = 0.465$), which rejected the presence of recent bottleneck events (Tajima 1989), this suggests a relatively large refugium population and slow postglacial expansion.

*Wiebesia* sp. 2 occupies the southern Chinese populations of its host plant. According to pollen data, some of its current area of distribution in China, to the South and East of the Wuyi Mountains, remained covered by broad-leaved evergreen forest during the LGM (Yu *et al*. 2000; Ni *et al*. 2010), and so could have provided a refugium at that time for *F. pumila* and also for this pollinator. Suitable habitat would also have been available further south. The BSP analysis, which suggested that *Wiebesia* sp. 2 populations expanded slowly between 30 000 and 20 000 BP, but then had no rapid postglacial expansion, is consistent with this scenario.

The natural range of *Wiebesia* sp. 3 may have been restricted to islands until recently, but assessment is complicated by uncertainty over whether or not its mainland records result from human transportation. Its sole known mainland location (site ND in Fujian Province) is in an area where *F. p. awkeotsang* and its pollinators have been introduced from Taiwan in recent years (Chen *et al*. 2008; Wu & Chen 2008), and it appears that *Wiebesia* sp. 3 has subsequently become established on both introduced *F. p. awkeotsang* and native *F. p. pumila*. We sequenced 12 pollinating wasps from one *F. p. pumila* fig collected in ND. Seven of them were sp. 2 with four haplotypes, while the other five were sp. 3 with just one haplotype (Y. Chen *et al*. unpublished data).

No significant expansion by *Wiebesia* sp. 3 during any period was detected using Fu's $F_S$ test ($F_S = 0.076$; $P = 0.568$), a goodness-of-fit test under a spatial expansion model (SSD = 0.035; $P_{(SSD)} < 0.001$) or the BSP analysis (Fig. 3). The BSP analysis also indicated a slightly shrunken current population size for *Wiebesia* sp. 3 (Fig. 3). A significantly negative Tajima's $D$ ($P = 0.038$) and the half-unimodal mismatch distribution curve without a leading face indicate that severe bottleneck events have occurred in its history (Tajima 1989; Rogers 1995). Climate matching based on temperature and pollen data (Qiu *et al*. 2011) suggests that areas of warm-temperate evergreen forest may have persisted along the coastal areas of China during the LGM, but in the north, these would have been small and fragmented. With elevated current sea levels, any coastal refugium would now be mainly beneath the sea (Taiwan and the northern islands were connected to the mainland during the LGM). Populations of *F. pumila* and *Wiebesia* sp. 3 may subsequently have colonized Taiwan and the other islands, but failed to successfully colonize the mainland, or may have survived throughout the LGM on Taiwan (Lin *et al*. 2008). The genetic evidence suggests that the glacial refugia supported moderately large populations of *Wiebesia* sp. 3 and that with sea level rise the range of this species has contracted. Currently, *Wiebesia* sp. 3 co-occurs with *Wiebesia* sp. 1 in the northern islands. This is most likely as a result of the postglacial northeasterly expansion of *Wiebesia* sp. 1, which eventually led to their colonization of the islands from the mainland.

Host conservatism (Jackson *et al*. 2008; Jousselin *et al*. 2008), ecological tolerances (Warren *et al*. 2010), changes in climate (McLeish *et al*. 2011) and vicariance events are all likely to have influenced the relationships and distributions of fig trees and their pollinator fig wasps during their 80 Myr of shared history. In the case of *F. pumila*, divergence and speciation among its pollinators took place long before the most recent glacial cycle, but differing physiological tolerances among the fig wasps provides one possible explanation for the retreat of the three species to different refugia during the last glaciation (Wang *et al*. 2009a). The history and extent of divergence of the two host plant varieties still remain to be established, as does their relationship to pollinator performance and divergence.

## Conclusions

As a largely tropical genus, and one which does not have a useful palynological record, the responses of fig trees to climate change have hardly been considered. Based on *COI* and *ITS*2 sequences, we have shown that *Ficus p. pumila* in China is pollinated by three distinct species with largely allopatric distributions. Our population genetic studies revealed that the three species had complex and contrasting histories and that their current distributions probably resulted from isolation in different refugia during the LGM. Although isolation during Quaternary glacial periods may have reinforced ecological differences between the species, they diverged earlier, during periods when major geological events were taking place within the current range of their host plant.

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Y.C. worked on population genetics of fig wasps. S.G.C. has a long-term interest in plant–animal interactions, rainforest regeneration, gene flow in fig trees, insect and plant conservation. M.L. is a Ph.D. candidate studying co-evolution of figs and their pollinating wasps. X.-Y.C. is interested in the genetic consequences of fragmentation, phylogeography and co-evolution of plant–animal relationships.

## Data accessibility

DNA sequences: The DNA sequences have been deposited in GenBank (Table S1, Supporting information; accessions numbers for *CO*I: JN183988–JN184049, *ITS*2: JN184050–JN184053). Details regarding individual samples are available in Table S1 (Supporting information).

## Supporting information

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

**Fig. S1** Mismatch distributions of gene *CO*I in three clades.

**Table S1** All the sequences of mitochondrial *CO*I and nuclear *ITS*2 genes used in the present study.

**Table S2** The average K2P distances within and between clades of *Wiebesia* spp.

**Table S3** The K2P-distances between *ITS*2 haplotypes.