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Genetic structure and hybridization in the species group of *Ficus auriculata*: can closely related sympatric *Ficus* species retain their genetic identity while sharing pollinators?

Z.-D. WEI,*† N. KOBMOO,‡§ A. CRUAUD¶ and F. KJELLBERG‡

*Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan, China, †Graduate School of the Chinese Academy of Sciences, Beijing, China, ‡UMR 5175 CEFE, CNRS – Université de Montpellier – Université P. Valéry Montpellier – EPHE, 1919 route de Mende, 34293 Montpellier Cedex 5, France, §Microbes Interaction Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Phahonyothin Rd., Khlong Neung, Khlong Luang, Pathum Thani 12120, Thailand, ¶UMR1062 CBGP, INRA, F-34988 Montferrier-sur-Lez, France

Abstract

Obligate mutualistic nursery pollination systems between insects and plants have led to substantial codiversification involving at least some parallel cladogenesis, as documented in *Yucca*, *Ficus* and Phyllanthaceae. In such systems, pollinators are generally species specific thus limiting hybridization and introgression among interfertile host species. Nevertheless, in the three systems, cases of one insect pollinating several plant species are reported. In most cases, host plants sharing pollinators are allopatric. However, in the case of the species group of *Ficus auriculata*, forms may co-occur over large parts of their range. We show here that the species group of *F. auriculata* is constituted by four well-defined genetic entities that share pollinators. We detected hybrids in nature mainly when both parental forms were growing nearby. Controlled crosses showed that F1 offspring could be successfully backcrossed. Hence, despite sharing pollinators and despite hybrid viability, the different forms have preserved their genetic and morphological identity. We propose that ecological differentiation among forms coupled with limited overlap of reproductive season has facilitated the maintenance of these interfertile forms. As such, establishment of pollinator host specificity may not be a prerequisite for sympatric diversification in *Ficus*.

Keywords: fig, introgression, mutualism, speciation, sympatric

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Introduction

Obligate mutualisms provide model systems to investigate how interactions among species may originate and how species may subsequently evolve together and diversify. Cocladogenesis is mainly documented in mutualisms involving vertical transmission of associates (De Vienne *et al.* 2013). Among obligate mutualisms, obligate pollination mutualisms provide a case of nonvertically transmitted interactions in which codiversification may be expected. Three well-documented nursery pollination systems (systems in which pollinators breed in floral structures of the species they pollinate) have led to important radiations: the *Yucca–Tegeticula* mutualism (50 plant species, Pellmyr 2003), the Phyllanthaceae–*Epicephala* mutualism (500 plant species, Kawakita 2010) and the *Ficus*-pollinating wasp mutualism (750 plant species, Berg & Corner 2005). In these three systems, sufficient numbers of mutualistic associations are available

Correspondence: Finn Kjellberg, Fax: +33 4 67 41 21 38; E-mail: finn.kjellberg@cefe.cnrs.fr

to investigate how diversification proceeds and to investigate variation in the evolutionary processes. For instance, in all three systems, some degree of parallel cladogenesis between host and insect mutualists has been documented in combination with host shifts (Kawakita *et al.* 2004; Althoff *et al.* 2012; Cruaud *et al.* 2012).

Despite the highly coadapted traits of plants and insects in the three systems, new species belonging to other lineages have also joined the mutualism, plants in the case of *Yucca* and Phyllanthaceae (Kawakita 2010) and wasps in the case of *Ficus* (Jousselin *et al.* 2001). In all three systems, two or more insect species may pollinate the same host (Molbo *et al.* 2003; Kawakita & Kato 2006; Althoff *et al.* 2012). Cases of individuals of an insect species sometimes pollinating alternative hosts are also reported for *Yucca* (Smith *et al.* 2009) and *Ficus* (Moe *et al.* 2011). This raises the possibility of genetic introgression among plant species, and indeed, some ongoing introgression among species has been documented in both systems, with an intensity and directionality fitting documented insect behaviour (Smith *et al.* 2009; Moe & Weiblen 2012). However, in *Glochidion* (Okamoto *et al.* 2007) as well as in *Ficus* (Hossaert-McKey *et al.* 2010), receptive flowers or inflorescences of co-occurring species usually produce different odours and the pollinators are attracted exclusively by their host's odour.

More surprisingly, in all three systems, in some cases, one insect species is a main pollinator of several plant species (Leebens-Mack & Pellmyr 2004; Kawakita & Kato 2006; Cornille *et al.* 2012). This is intriguing because it is generally assumed that in these systems, the plants depend on pollinator specificity to avoid genetic introgression, and at least in *Ficus*, a number of experimental crosses have suggested that hybrids were viable (e.g. Moe & Weiblen 2012). However, in the cases documented so far for Phyllanthaceae, *Yucca* and *Ficus*, plant species pollinated by the same insect were either allopatric or parapatric thus limiting potential hybridization (Leebens-Mack & Pellmyr 2004; Kawakita & Kato 2006; Cornille *et al.* 2012). Within the *Yucca* system, *Tegeticula yucassella* pollinates all seven species of section *Chaenocarpa* and receptive flowers of at least six of these species produce similar odour profiles. This odour similarity would facilitate use of all seven species by *Tegeticula yucasella* (Svensson *et al.* 2011). Similarly, Cornille *et al.* (2012) documented convergence in the odours produced by receptive figs of *Ficus burkei* and *F. natalensis*, which share pollinators in KwaZulu Natal (South Africa).

Thus, available data show that in mutualistic nursery pollination systems, several host species may share pollinators by producing similar odours at flower receptivity. Within the *Yucca* system, species of section *Chaenocarpa* are largely allopatric. The situation is more confused in *Ficus*, as, in some cases, species, whose distributions largely overlap, seem to be capable of retaining their identity while sharing pollinators.

The species complex of *F. auriculata* Lour. provides an extreme example of how potentially hybridizing sympatric *Ficus* species seem to retain their genetic and biological identity. This dioecious species complex grows in rainforest, is found from the Himalayan foothills of Pakistan and India, through South China, Burma, Thailand, Indo-China and extends into peninsular Malaysia (Corner 1965). The taxonomy of the complex is unresolved. Corner (1965) distinguished *F. oligodon* from *F. auriculata* and considered *F. hainanensis* as a synonym of *F. oligodon*. Berg in Berg & Corner (2005) considered *F. oligodon* to be a synonym of *F. auriculata* because of the presence of intermediates, which Corner (1978) regarded as results of hybridization. He also recognized *F. hainanensis* as a different species. Later on, Berg (2007) regrouped all forms within *F. auriculata*, as he could not satisfactorily delimit the different taxonomic entities.

While almost all *Ficus* have their species-specific pollinator(s), in south Yunnan and north Thailand, *F. auriculata* and *F. oligodon* seem to share pollinating *Ceratosolen* species (Tarachai *et al.* 2011; Kuaraksa *et al.* 2012), and sequencing suggests that at least three pollinator *Ceratosolen* species are associated with the *F. auriculata* species complex in South-Yunnan (Cruaud *et al.* 2012). Experimental wasp introductions show that wasps emerging from *F. hainanensis* figs will enter figs of *F. auriculata* and reproduce successfully and that the wasps associated with *F. auriculata* reproduce successfully in *F. hainanensis* (Yang *et al.* 2012).

Thus, the *F. auriculata–oligodon–hainanensis* complex may provide valuable insights into how co-occurring species may maintain their identity within a species complex while sharing pollinators within an obligate mutualisitic nursery pollination system. We investigated pollinator specificity and genetic structuring within and among the morphological plant groups that we detected during field studies. Our results show that, even for neutral genes, there is limited genetic introgression among forms as we observe four groups of genotypes, homogeneous over large distances, each corresponding to a morphotype. These distinct groups may coexist in sympatry while retaining their identity and may hence qualify as distinct species. Nevertheless, we document shared pollinators, production of viable and fertile hybrids, and coexistence of a hybrid population with one of its parental species.

Material and methods

Fig tree species description and sampling

As the taxonomy of the species complex is not resolved, no determination key is available. In the following, we use names as we currently understand the taxonomy of the group, trying to interpret correctly the original descriptions of species and the little type material available. We collected 355 fig tree samples from Thailand and South China between 2006 and 2007, and we categorized them in the field into four major forms and what we interpreted as putative hybrids according to morphology (Fig. 1). Quantitative morphological analysis on a subset of 184 trees (60 *F. auriculata*, 26 northern *F. oligodon*, 30 southern *F. oligodon*, 41 *F. hainanensis*, 26 intermediates between *F. oligodon* and *F. hainanensis*) allowed defining simple rules (see below) to recognize the different morphotypes (Z.-D. Wei, unpublished). The quantitative morphological criteria enabled to properly assign 164 trees. Half of the 20 mismatches between field identification and morphometric assignment using the criteria indicated below concerned confusion between northern and southern *F. oligodon*, the two most similar forms. Subsequent genetic analysis showed that categorization in the field was more efficient at identifying form than the morphometric measurements, probably because it was based on a broader, but somewhat more subjective, set of traits. The 355 collected individuals were categorized as follows: (i) *F. auriculata* ($N = 143$): leaves are large and broad (length × width of the lamina ≥580 cm^2; width of leaf/length lamina >0.7), the figs are large and borne on stout, long (>20 cm), leafless branched twigs, the figs are not speckled with white dots, and the flower perianth is white (fresh material). (ii) Southern *F. oligodon* ($N = 85$): leaves are smaller and narrower than those of *F. auriculata*, but not very narrow (length × width of the lamina <330 cm^2; width of lamina/length of lamina ≥0.565), the figs are large, borne on woody cushions on short leafless twigs (<10 cm), the figs are not speckled with white dots, and the flower perianth is white (fresh material). They are present in our southern sampling sites. (iii) Northern *F. oligodon* ($N = 51$): their morphology is very similar to that of southern *F. oligodon* except that leaves are slightly but distinctly broader (330 cm^2 ≤ length × width of the lamina <580 cm^2; width of lamina/length of lamina ≥0.565). They are present in our northern sampling sites, and their geographic distribution does not overlap with southern *F. oligodon*. (iv) *F. hainanensis* ($N = 45$): leaves are smaller and narrower than those of *F. oligodon* (width of lamina/length of lamina <0.565; length × width of the lamina <250 cm^2). The figs are distinctively smaller and are speckled with white dots. The figs are borne on thin elongate leafless branched twigs (>50 cm), and the flower perianth is purple.

We observed three additional types of individuals which we predicted would be of hybrid origin. (v) Atypical *F. hainanensis* ($N = 25$): morphological characters were similar to those of *F. hainanensis* except that figs were produced on much shorter leafless twigs

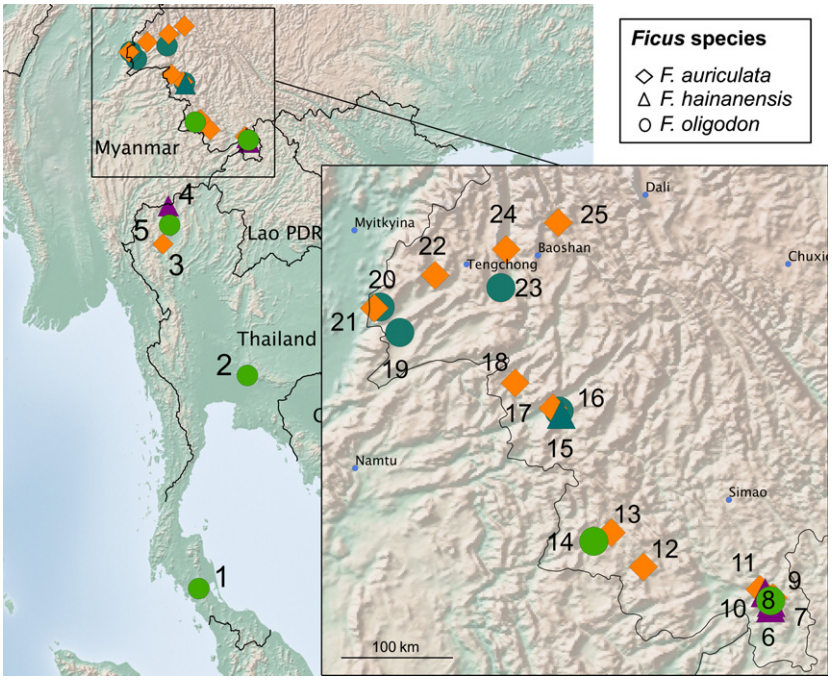

Fig. 1 Sampling locations. Shapes represent species (see Table 1 for further details on morphotypes), and colours represent genetic entities revealed by STRUCTURE (see Fig. 3, same colour code). Population IDs from Table 1 are also reported.

(<50 cm) and the trees were much smaller than typical *F. hainanensis*. They were found in one location growing together with northern *F. oligodon*. (vi) Putative hybrids between *F. auriculata* and southern *F. oligodon* ($N = 5$), as they presented traits of both forms. We found them near the Xishuangbanna Tropical Botanical Garden (XTBG) and at XTBG, growing together with both putative parental species. (vii) One putative hybrid between *F. hainanensis* and southern *F. oligodon*, which presented traits of both species. It was found near XTBG in a place where both putative parents were growing close together.

When possible, we sampled plants in locations where two or three forms were growing close together. Sampling locations are given in Table 1 and Fig. 1. Populations 26 and 27 were constituted by trees growing in the XTBG. Trees in the garden were originally introduced from other botanical gardens, and some of their offspring may have grown subsequently in the garden. When growing in sympatry, the three forms seemed to grow in slightly different habitats. In places of sympatry, *F. auriculata* was generally growing at slightly lower elevations than *F. oligodon*, with a contact zone between the two forms when habitats were continuous. *Ficus hainanensis* grew in very moist places.

Controlled experiments

Controlled crosses were performed near XTBG to demonstrate the viability of crosses within and between morphotypes. Further, these crosses allowed checking for each locus that alleles from both parents amplified properly in hybrids between different forms. Crosses were performed by enclosing fig-bearing branches in fine mesh bags before receptivity and introducing into the bag freshly emerged pollinating wasps from chosen paternal trees. Seeds were extracted from the figs and washed in water. Only seeds which did not float were used for germination. Fifty seeds were deposited per Petri dish on several layers of moistened filter paper,

Table 1 Sampling locations

Locality	GPS coordinates	Morphotype	Population ID
Thailand:			
Trang	E99°48′N7°34′	Southern *oligodon*	1
Khao yai	E101°19′49″N14°20′35″	Southern *oligodon*	2
Chiang mai	E98°42′15″N18°31′06″	*auriculata*	3
	E98°53′47″N19°37′55″	*hainanensis*	4
	E98°52′20″N19°09′28″	*oligodon*	5
China:			
Meng yuan, Meng la, Yunnan	E101°22′52″N21°43′54″	*hainanensis*	6
Meng xing, Meng la, Yunnan	E101°22′55″N21°47′16″	*hainanensis*	7
	E101°22′55″N21°47′16″	Southern *oligodon*	8
	E101°22′55″N21°47′16″	*auriculata*	9
Meng lun, Meng la, Yunnan	E101°18′07″N21°52′26″	*hainanensis*	10
	E101°16′22″N21°55′04″	*auriculata*	11
Meng hai, Yunnan	E100°09′43″N22°08′22″	*auriculata*	12
Meng lian, Yunnan	E99°50′59″N22°27′52″	*auriculata*	13
	E99°41′30″N22°23′11″	Southern *oligodon*	14
Geng ma, Yunnan	E99°22′28″N23°37′47″	Intermediates between *hainanensis* and northern *oligodon* (atypical *hainanensis*)	15
	E99°22′30″N23°38′23″	Northern *oligodon*	16
	E99°19′42″N23°39′11″	*auriculata*	17
Zhen kang, Yunnan	E98°55′39″N23°54′45″	*auriculata*	18
Long chuan, Yunnan	E97°50′36″N24°23′37″	Northern *oligodon*	19
Ying jiang, Yunnan	E97°38′56″N24°37′41″	Northern *oligodon*	20
	E97°35′35″N24°37′22″	*auriculata*	21
	E98°08′20″N24°53′31″	*auriculata*	22
Teng chong, Yunnan	E98°47′50″N24°48′40″	Northern *oligodon*	23
Long yang, Yunnan	E98°52′20″N25°11′03″	*auriculata*	24
Yun long, Yunnan	E99°20′51″N25°26′00″	*auriculata*	25
XTBG, Meng lun, Meng la, Yunnan	E101°15′26″N21°55′43″	*auriculata*	26
	E101°15′26″N21°55′43″	*oligodon*	27

XTBG, Xishuangbanna Tropical Botanical Garden.

and all the Petri dishes were put into incubators with lighting, at 30 °C. The germinated seedlings were transferred to pots containing horticultural soil. Leaves were collected for DNA analysis. The morphotypes used for the crosses were *F. auriculata*, southern *F. oligodon*, *F. hainanensis*, putative hybrid between *F. auriculata* and southern *F. oligodon*, and putative hybrid between southern *F. oligodon* and *F. hainanensis*. When trying to perform the crosses, it became obvious that the different forms tended to fruit at different periods so that we depended on out of season figs.

DNA extraction, Cross-species microsatellite screening and genotyping

One young and healthy leaf of each tree was collected from each tree and immediately dried in silica gel. DNA was extracted from dried leaves using the DNeasy® Plant Mini Kit (QUIAGEN). We used microsatellite pairs of primers developed from *F. carica* (Khadari *et al.* 2001; Giraldo *et al.* 2005), *F. montana* and *F. septica* (Zavodna *et al.* 2005). We first tested for successful amplification by these pairs of primers on 31 individuals (12 *F. auriculata*, 8 *F. hainanensis* and 11 southern *F. oligodon*). The molecular protocols were modified from previous studies using these pairs of primers. Amplification reactions were first carried out separately for each locus and each sample in 10 μL including 0.2 μL of each 10 μM nonlabelled primer, 5 μL of multiplex buffer (QUIAGEN: this solution contains the Taq polymerase), 3.6 μL of pure water and 1 μL of genomic DNA. The PCRs were performed using a PTC 100 thermocycler in the following conditions: 3 min of denaturation at 94 °C and 35 cycles of 30 s of initial denaturation at 94 °C, 45 s of annealing at 57 °C, 45 s of extension at 72 °C and 5 min elongation at 72 °C. The amplification by pairs of primers was detected by electrophoresis on a 1% agar gel with BET. From 35 pairs of primers tested, 28 successfully amplified DNA for every individual, but 3 of them revealed multiple bands. We used a subset of these pairs of primers to analyse polymorphism. The PCR conditions and amplification reactions for detecting polymorphism remained unchanged except for the use of fluorescent-labelled primers for the F side. Genotyping was carried out using an ABI PRISM 310 DNA sequencer, and the genotypes were scored using the program GENEMAPPER. Finally, 10 pairs of primers were used for the genotyping of the whole sampling for their satisfactory polymorphism: MFC3, MFC8 (Khadari *et al.* 2001), LMFC15, LMFC17, LMFC19, LMFC20, LMFC24, LMFC30, LMFC32 (Giraldo *et al.* 2005), FM4-70 (Zavodna *et al.* 2005). Ten pairs of primers were assigned to two multiplex mixes based on PCR conditions: mix one, MFC8, LMFC20, LMFC17, LMFC32, LMFC15, LMFC30, LMFC24 and LMFC19, 35 cycles; mix two, MFC3 and FM4-70 30 cycles. We then mixed the pairs of primers (4 μL of each primer complemented to 200 μL with pure water) and carried out the multiplex PCR for each individual in a solution containing 5 μL of multiplex buffer, 1 μL of primer mix solution, 1 μL of genomic DNA and 3 μL of pure water. Genotype scoring was carried out twice to minimize genotyping errors.

Quality control of the data

Controlled crosses within and between morphotypes were performed at XTBG to verify that alleles segregated according to Mendelian inheritance rules and to assess the presence of null alleles at each locus. Loci found to present many null alleles were discarded. Then, we tested for homozygote excesses using MICRO-CHECKER software version 2.2.3 (Van Oosterhout *et al.* 2004) to evaluate the presence of null alleles at the remaining loci. Moreover, samples presenting more than one locus of missing data were discarded from the data set. After a first analysis using STRUCTURE (Pritchard *et al.* 2000), we removed the individuals inferred to be hybrids from the subsequent analyses (following e.g. Kothera *et al.* 2013).

Detection of genetic clusters and hybrids

To detect the number of genetic clusters and confirm the presence of hybrids, we used STRUCTURE version 2.3 (Pritchard *et al.* 2000; Falush *et al.* 2003) using no prior information on morphological characters and sampling location, setting all the parameters at default values. We conducted several analyses using STRUCTURE for different purposes. (i) To delimitate major genetic clusters and identify hybrids, we used all 355 individuals sampled, including both wild-growing ones and those planted in the botanical garden. To determine the optimal number of genetic clusters (K), we ran STRUCTURE with K varying from 1 to 8, as we had detected 4 main morphological forms and 3 additional morphological forms that we interpreted as potential hybrids. (ii) Then, using the optimal K value, we added all offspring from our controlled crosses as supplementary individuals to evaluate the efficiency of STRUCTURE at detecting known hybrids in our samples. We used the results to determine recognition criteria for hybrids. (iii) To detect finer genetic structure, using the optimal K value, we did further analyses within genetic clusters as proposed by Evanno *et al.* (2005). For these analyses, we only used wild-growing plants and we did not include potential hybrids. We made K vary from 1 to number of populations plus 1. For each analysis mentioned above, we

performed 9 independent runs with a burn-in of 50 000 iterations and a run length of 50 000 iterations following the burn-in; we used the admixture ancestry model, correlated allele frequencies model, and we set all other parameters at default values. We computed ΔK to estimate the best K value (Evanno *et al.* 2005). The obtained K value was validated by checking that every individual was assigned to the same genetic cluster in each independent run.

Delimitation of pollinating wasp species and wasp specificity

To investigate pollinator specificity, we sequenced one mitochondrial marker (cytochrome b, *Cytb*) for 60 wasp specimens (15 that had emerged from figs of *F. hainanensis*, 26 from figs of Southern *F. oligodon* and 19 from figs of *F. auriculata*) and one nuclear marker (F2 copy of elongation factor-1α, *EF1*α) for 17 wasp specimens. Only one wasp was used per fig so as to avoid sequencing sisters. Extraction, amplification and sequencing protocols followed Cruaud *et al.* (2010, 2011). Sequences from both strands were assembled using GENEIOUS version 6.1.6 (Drummond *et al.* 2010). The two gene regions were aligned with MAFFT 6.864 (Katoh *et al.* 2005) using the L-INS-i option. Alignments were translated to amino acids using MEGA 4 (Tamura *et al.* 2007) to detect frame-shift mutations and premature stop codons. This allowed checking that only functional copies of the genes were used in the phylogeny. Sequences were deposited in GenBank under Accession nos KJ523898–KJ523974, and all wasp vouchers are deposited at CBGP. We performed maximum-likelihood (ML) analyses of the two gene regions with MPI-parallelized RAXML 7.2.8 (Stamatakis 2006a) using GTR + Γ models. GTR-CAT approximation of models was used for ML bootstrapping (Stamatakis 2006b) (1000 replicates). Three *Tetrapus* and one *Ceratosolen* species were used as outgroups. Analyses were conducted on the CIPRES Science Gateway (Miller *et al.* 2010).

Results

Delimitation of pollinating wasp species and wasp specificity

On the two phylogenetic trees, the sampled wasps clustered into three distinct clusters (Fig. 2), whose genetic divergences indicated that three species pollinate the *F. auriculata* species complex. In Meng lun, two species were collected. One (white bar, Fig 2) was collected from *F. hainanensis* (13 wasps), southern *F. oligodon* (13 wasps) and *F. auriculata* (four wasps). The second (striped bar) was collected from southern *F. oligodon* (13 wasps) and from *F. auriculata* (15 wasps) but not from *F. hainanensis*. Wasps collected from the same tree could belong to different species. In our limited sampling per tree, this occurred on five trees (2 *F. auriculata* and 3 southern *F. oligodon*), while a single wasp species was obtained from seven other trees of *F. auriculata* and southern *F. oligodon* for which at least two wasps were sequenced (Fig. 2). The third species (black bar) was collected on a tree belonging to population 15, morphologically intermediate between *F. hainanensis* and *F. oligodon* and growing with northern *F. oligodon* trees at Geng ma, more than 100 km away from Meng lun. The three species of *Ceratosolen* evidenced here presented morphological differences, especially in the antennae and mandibulae, allowing identification using a stereoscopic microscope.

Crosses and quality of genetic data

Germination rates were consistently high (>80%) and seedling was grown to a size of about 10 cm with low mortality, although no quantitative analysis was performed. We compared the genotypes of parents and offspring as inferred from microsatellite data for 132 offspring resulting from 23 crosses to establish which loci presented Mendelian disjunction patterns (Table S1, Supporting information). We detected some problems with locus FM4-70. Locus MFC8 did not amplify in *F. hainanensis*. Locus LMFC20 presented frequent dominance problems. So, only the remaining seven loci were included in following genetic analyses. Mendelian disjunctions without any dominance problems were observed at the seven retained loci for all crosses that did not involve *F. hainanensis*. Some dominance and hidden allele problems were apparent in crosses between *F. hainanensis* and other forms.

Twenty-two populations were checked for the presence of null alleles using MICRO-CHECKER (Van Oosterhout *et al.* 2004). Null alleles were found at three loci for one population and at one locus for eight populations. The loci involved varied between populations. Globally, the number of null alleles remained low for interspecific comparisons.

Population structure of the F. auriculata complex revealed by STRUCTURE

When all 355 adult individuals were analysed, we got a very large ΔK value of 253.69 for $K = 4$, that is, a very strong signal in favour of four genetic clusters and total coincidence of each individual's assignations among runs (Fig. 3). All the individuals of atypical *F. hainanensis*, which were growing together with northern *F. oligodon*, were assigned to the genetic cluster of northern

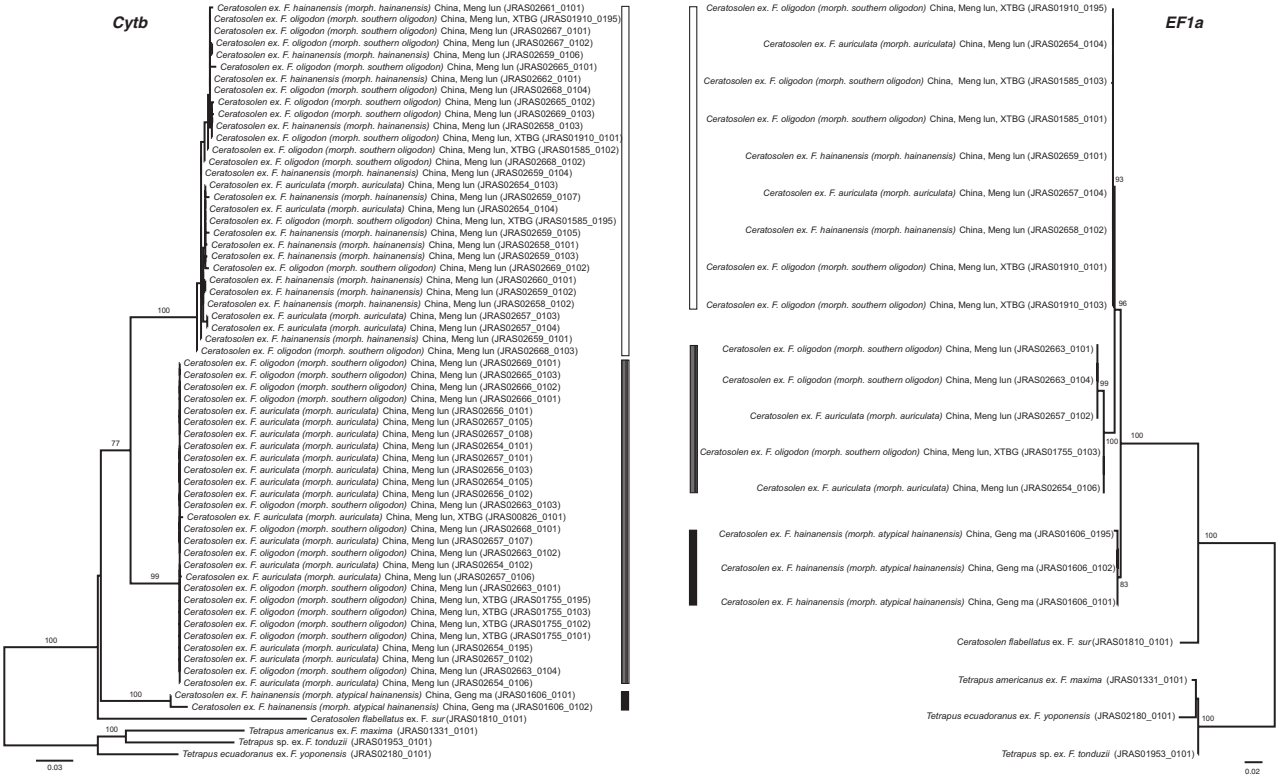

Fig. 2 Pollinating wasp phylogenies. Maximum-likelihood trees from the RAXML analyses of the cytochrome b (left) and elongation factor (right) data sets. Bootstrap percentages (>70) are indicated at nodes. Voucher numbers for sequenced wasps are indicated between parentheses (codes to the left of the underscore indicates fig tree ID; codes to the right of the underscore indicates wasp specimen ID). Bars indicate the three different pollinator species.

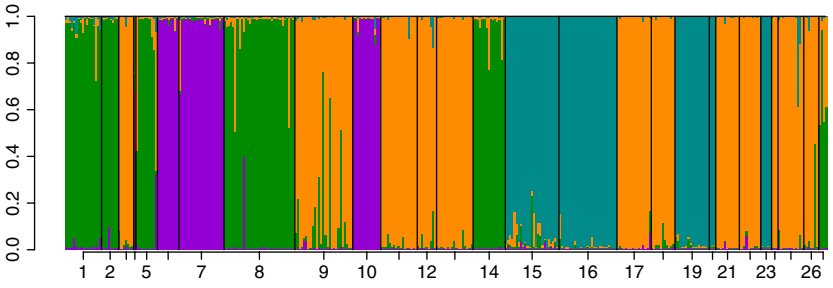

Fig. 3 The five morphological entities were separated into four genetic entities by STRUCTURE in the analysis including all sampled adult trees. Each colour represents one genetic cluster; each individual is represented by a thin vertical line partitioned into *K* coloured segments proportional to its probability of membership in the corresponding genetic cluster. Black lines separate individuals from different populations. Numbers on *x*-axis represent sampled population IDs. The populations are ordered from South to North. Green: Southern *F. oligodon* (populations 1, 2, 5, 8, 14); cyan: Northern *F. oligodon* introgressed by *F. hainanensis* (population 15) and Northern *F. oligodon* (populations 16, 19, 20, 23); orange: *Ficus auriculata* (populations 3, 9, 11, 12, 13, 17, 18, 21, 22, 24, 25); violet: *F. hainanensis* (4, 6, 7, 10). Populations 26 and 27 were collected in the Xishuangbanna Botanical Garden. Population 26 included the trees presenting *F. auriculata* morphotype; population 27 included trees presenting Southern *F. oligodon* morphotype.

F. oligodon. Assignation to the three other genetic clusters corresponded exactly with the a priori morphological assignation to *F. auriculata*, southern *F. oligodon* and *F. hainanensis*. We then analysed each genetic cluster separately to detect further genetic structuring. Analyses with samples from atypical *F. hainanensis* and northern *F. oligodon* gave two alternative optimal *K* values, $K = 2$ ($\Delta K = 10.41$) and $K = 4$ ($\Delta K = 15.86$). For $K = 2$, the genetic clusters corresponded to clear morphological entities (Fig. 4). Almost all samples were

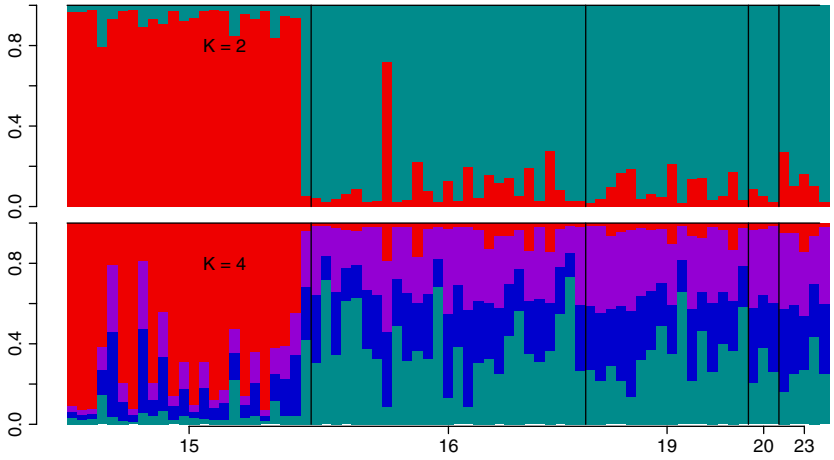

Fig. 4 The population intermediate between *F. hainanensis* and *F. oligodon* is separated from northern *F. oligodon* using STRUCTURE with $K = 2$ and $K = 4$. See Fig. 3 for full figure legend. Numbers on *x*-axis represent sampled population ID: Population intermediate between *F. hainanensis* and *F. oligodon*: 15; northern *F. oligodon*: populations 16, 19, 20, 23.

consistently assigned to one particular genetic cluster. Except for two samples, one genetic cluster corresponded to northern *F. oligodon* and the other to atypical *F. hainanensis*. For $K = 4$, no biologically meaningful genetic clusters could be recognized (Fig. 4). Hence, atypical *F. hainanensis* was separated from northern *F. oligodon* using microsatellite genetic data, even though they were genetically similar. Further analysis within genetic clusters revealed that only southern *F. oligodon* was genetically structured, with $\Delta K = 31.26$ for $K = 3$. Within southern *F. oligodon*, one genetic cluster included one population from Mengxing, South China, another genetic cluster included one population from Menglian, South China, and one from Chiang Mai, north Thailand, and the last genetic cluster included one population from Khao Yai at the limit between the east and the northeast region of Thailand and one population from Trang in the south region of Thailand (Fig. 5). Globally, genetic structuring within southern *F. oligodon* suggested isolation by distance.

Detection and confirmation of hybrids

In all the successful crossing experiments, wasps entered receptive nonhost figs without need for any special manipulation to stimulate them. All the crosses between *F. auriculata*, southern *F. oligodon*, *F. hainanensis*, the *F. auriculata* × southern *F. oligodon* hybrids and the *F. hainanensis* × southern *F. oligodon* hybrid were fertile, producing viable seedlings. Individuals from the northern *F. oligodon* genetic cluster were not included in the controlled crosses as the crosses were performed at XTBG in southern Yunnan where it is absent, and effectively no offspring were assigned to that cluster. Genetic assignment of all offspring was consistent with genetic assignment of their parents: STRUCTURE efficiently detected hybrids. When parents were assigned to different genetic clusters, their offspring were not necessarily assigned with a close to 50% probability to the genetic clusters of their parents (Table S2, Supporting information). This is because the different genetic clusters are genetically similar, and hence, a

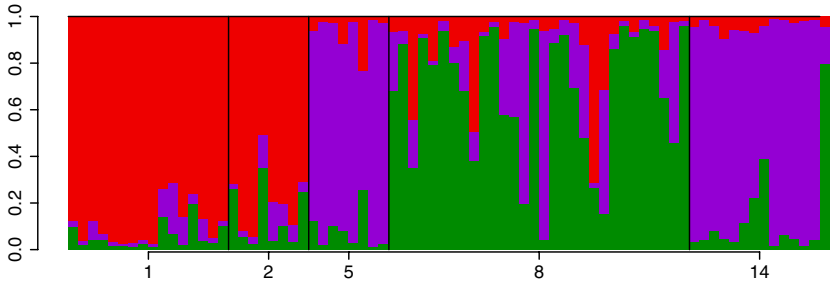

Fig. 5 Genetic clustering of southern *F. oligodon* populations using STRUCTURE for $K = 3$. The two southern populations (south Thailand and east-northeast region of Thailand) cluster together as opposed to the three northern populations (north Thailand and Yunnan). See Fig. 3 for full figure legend. Numbers on *x*-axis represent sampled population ID: 1: Trang; 2: Khao yai; 5: Chiang Mai; 8: Meng xing; 14: Meng lian.

Table 2 Number and type of putative hybrids and nonhybrids detected within the 27 populations using the software STRUCTURE

Inferred assignation	hXa	soXaXno	hXno	soXa	soXno	hXso	aXno	so	no	h	a
XTBG	—	—	—	4	—	—	—	1	—	—	6
Natural populations	2	1	1	10	1	1	1	77	75	43	132
Both parents observed nearby	2	—	—	9	—	1	—	—	—	—	—

Individuals presenting an assignation below 80% to any of these four genotype groups were considered putative hybrids, and their main assignations are indicated. Of 17 hybrids detected in natural populations, 11 co-occurred locally with both their parental species.

h, *Ficus hainanensis*; a, *F. auriculata*; so, southern *F. oligodon*; no, northern *F. oligodon*.

large number of loci are required to get accurate estimates of the actual level of admixture of individuals (Pritchard *et al.* 2000). We chose assignation probabilities below 80% to a genetic cluster as a criterion for deciding that an individual was a hybrid. Using this threshold, no offspring from within form crosses would have been considered as a hybrid and no F1 hybrid would have been assigned as nonhybrid. And most, but not all, backcrossed individuals would have been detected as hybrids. All assignations of parents and offspring are given in Table S2 (Supporting information). The number of nonintrogressed individuals and introgressed individuals is summarized in Table 2.

The five individuals that were a priori classified on morphological grounds as putative hybrids between *F. auriculata* and southern *F. oligodon* (1 in population 8, 2 in population 9, 1 in population 26 at XTBG, 1 in population 27 at XTBG) were recognized by STRUCTURE as such, and the individual morphologically intermediate between southern *F. oligodon* and *F. hainanensis* (population 8) was confirmed to be a hybrid between these two forms. Sixteen additional individuals were classified, based on genetic data, as putative hybrids. Globally, the 21 putative hybrids were distributed between populations 1, 4, 5, 7, 8, 9, 12, 13, 15, 25, 26, 27.

Discussion

We have documented here that although *F. auriculata* co-occurs over large distances with very closely related other forms of its species group, it maintains its genetic homogeneity over the investigated 750 km of co-occurrence. Genetic homogeneity is maintained in *F. auriculata* despite (i) sharing pollinators with southern *F. oligodon* and *F. hainanensis*; (ii) production of viable offspring in crosses between *F. auriculata*, and both southern *F. oligodon* and *F. hainanensis*; and (iii) fertility of F1 hybrids between *F. auriculata* and southern *F. oligodon*. In contrast, *F. oligodon* was subdivided into two vicariant genetic groups, a northern one and a southern one. Some individuals, morphologically intermediate between *F. auriculata* and southern *F. oligodon* and between southern *F. oligodon* and *F. hainanensis*, were detected in places of co-occurrence of parental plants in the field, and hybrid status was confirmed by genetic analysis. Within one stand, typical northern *F. oligodon* individuals were interspersed with individuals presenting intermediate morphological traits between northern *F. oligodon* and *F. hainanensis*. The intermediate individuals were recognized as a separate genetic entity by Bayesian assignation, as confirmed on examination of the genotypes, demonstrating that they constituted an independent reproductive unit. Whether this genetic entity is transient or will survive for longer periods of time is an open question. Finally, *F. hainanensis* seemed to be the genetically most divergent form as one locus did not amplify and as dominance problems appeared in the crosses with other forms. This result suggests that the *F. auriculata* complex can be compared to some other plant species complexes presenting some reticulate evolution through introgression and hybrid speciation such as for instance annual sunflowers (Moody & Rieseberg 2012).

In *Ficus*, the sole other situation in which interspecific hybrids were morphologically detected under natural conditions and were subsequently confirmed to be hybrids using molecular makers involved *F. fistulosa*, *F. hispida* and *F. septica*, which are usually pollinated by different wasp species (Wiebes 1994), and some of the analysed individuals were at least second generation hybrids (Parrish *et al.* 2003). Natural hybridization occurred on small islands and was suggested to result from transient absence of the pollinator of one of the parental fig species. In another situation, molecular data on six co-occurring *Ficus* species (Moe & Weiblen 2012) suggested that <1% of genotyped individuals could be hybrids, and the most convincing results showed the presence of an F1 and an F2 hybrid between two species belonging to a same species group. Interestingly, pollinator typing for the same six *Ficus* species showed that 1.5% of pollinators that had entered figs were the wrong species, thus potentially allowing for gene flow among these *Ficus* species (Moe *et al.* 2011). Further what appeared morphologically to be a hybrid between

two species belonging to another species group produced viable offspring and was regularly pollinated by the pollinators of its two parental species (Moe *et al.* 2011). Hence, there seems to be a potential for genetic introgression among related *Ficus* species. Nevertheless, observed genetic introgression levels range from no recent genetic introgression detected among American figs by Machado *et al.* 2005 and Jackson *et al.* 2008), to the level of introgression documented here within the *F. auriculata* species complex, with sometimes the formation of hybrid entities coexisting with a parental entity. We may suggest that, under natural conditions, hybrids often suffer from reduced fitness due to reduction in the efficiency of the mutualism as observed in other mutualistic systems (Léotard *et al.* 2008). For example, in *Ficus*, when parental species host different wasp species, a hybrid may be less efficient at attracting and/or breeding pollinators resulting in reduced fitness.

The observation that the different forms within the interfertile *F. auriculata* complex preserve their genetic identity is somewhat paradoxical according to what is known about pollinating fig wasp dispersal. Indeed, several sets of data suggest that fig pollinating wasps may disperse regularly over large distances (Nason *et al.* 1996; Harrison & Rasplus 2006; Ahmed *et al.* 2009; Kobmoo *et al.* 2010), and in agreement with long distance pollinator dispersal, it has been shown that a species of dioecious fig presented limited spatial genetic structure over its range (Yu *et al.* 2010). Nevertheless, three sets of data suggest that pollinator dispersal and/or gene flow may be quite local in some dioecious *Ficus* (Valizadeh *et al.* 1987; Harrison 2000; Chen *et al.* 2011), and, in our situation, hybrids between *F. auriculata* and *F. oligodon* were observed in places of local co-occurrence of the parental forms (Table 2).

Another set of data on dioecious *Ficus* shows that in the Ogasawara Islands, *F. nishimurae* (a forest understory species) has diversified locally into two additional entities, *F. boninsimae* (an open habitat species) and form '*higashidaira*' (Yokoyama 2003). The situation is highly suggestive of sympatric speciation. Y tube tests showed that the pollinator of *F. boninsimae* is equally attracted by receptive fig odours of *F. boninsimae* and *F. nishimurae* a trait that could facilitate gene flow between species (Yokoyama 2003). Hence, data on both the *F. auriculata* complex and the *F. nishimurae* complex suggest that strong pollinator specialization may not be necessary to preserve species identity in sympatric closely related *Ficus* species. To our knowledge, these are the first demonstrations of such a situation in a nursery pollination system. In both cases, co-occurring forms inhabit somewhat different habitats, and in the case of *F. auriculata–F. oligodon* hybrids were only observed in places where the parental forms were encountered close together. Hence, habitat specialization may limit intensity of hybridization due to limited pollinator dispersal. Outbreeding depression could also be involved, if hybrids are ecologically unsuited to either habitat.

In the case of the *F. auriculata* species group, fruiting phenology could be an additional factor limiting hybridization and this is quite exceptional for *Ficus*. In all monoecious species of *Ficus*, individual figs are strongly protogynous as pollinating wasps oviposit within figs when the female flowers are receptive, and their offspring load pollen before leaving their natal fig several weeks later. Because of this strong protogyny, fruiting in monoecious *Ficus* is spread out throughout the year. In dioecious *Ficus*, male fig trees breed wasps in their figs, so that they can be receptive to pollinators several weeks before the female trees are receptive to pollen: strongly seasonal fruiting phenology becomes possible. In a limited number of dioecious *Ficus* species, fruiting phenology is very strongly synchronized among trees within sex. Male trees produce a major crop releasing pollinating wasps when the major crop of most female trees is receptive (Kjellberg *et al.* 1987). *Ficus auriculata*, southern *F. oligodon* and *F. hainanensis* seem to present a seasonal fruiting phenology, reminiscent of the fruiting phenology of *F. carica*. The timing of peak pollinating wasp release is different in *F. auriculata* and *F. oligodon* as reported from north Thailand (Kuaraksa *et al.* 2012), and this seems to be the case for these two forms and for *F. hainanensis* in South China (Z.-D. Wei unpublished). This feature very much complicated the artificial crosses between forms as the main periods of pollinating wasp production on one form never corresponded to the main production of receptive female figs on other forms. Separation of reproductive periods between plants sharing pollinators is a classical mechanism known to limit interspecific crosses (e.g. Stone *et al.* 1998), but this is the first documentation of the feasibility of such a mechanism in *Ficus*. Nevertheless, separation among species of pollination periods is not required for the persistence of related *Ficus* species sharing pollinators. Indeed, the closely related monoecious *F. burkei* and *F. natalensis* present continuous fruiting among trees throughout the year and share pollinators in South Africa (Cornille *et al.* 2012). However, separation of pollination periods may allow the much more intricate pattern of co-occurrence of forms observed in *F. auriculata* complex comparatively to what has been observed in monoecious figs.

Thus, in nursery pollination systems, despite the prevalence of flower scent differences acting as prezygotic barriers to hybridization among co-occurring species (Hossaert-McKey *et al.* 2010), such barriers are not obligatory, they are leaky, and they may arise after

separation of the species. Hence, speciation in *Ficus* may follow the classical pattern observed in plants, involving mainly ecological and/or geographic isolation, with pollinator isolation acting less frequently or as a second step (Rieseberg & Willis 2007).

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Z.D.W. and F.K. designed the study. Z.D.W. performed the field work. Z.D.W. and N.K. performed the laboratory work, Z.D.W., N.K., A.C. and F.K. analysed the data. Z.D.W., A.C. and F.K. wrote the article.

Data accessibility

Supplementary material and data/tree files can be found in the Dryad data repository at http://datadryad.org,

doi:10.5061/dryad.v0n20. They include the following items in Supporting information.

Supporting information

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

Tables S1–S3 *Ficus* genotypes. Table S1 Genotypes of parents and offspring in controlled crosses. Table S2 Parent and offspring probability of assignation to genetic cluster using Structure. Table S3 Genotypes at the seven retained loci of the 355 individuals used for the global analysis.

Table S4 Wasp Sampling: List of wasp specimens included in this study with GENBANK accession numbers.
Cytb sequences for phylogenetic inference (Agaonidae).
EF sequences for phylogenetic inference (Agaonidae).
ML tree from the analysis of Cytb sequences.
ML tree from the analysis of EF sequences.