



## Weak genetic divergence suggests extensive gene flow at the northeastern range limit of a dioecious *Ficus* species



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### ABSTRACT

Genus *Ficus* (Moraceae) plays a critical role in the sustainability and biodiversity in tropical and subtropical ecosystems. *Ficus* species and their host specific pollinating fig wasps (Agaonidae) represent a classic example of obligate mutualism. The genetic consequence of range expansion and range shift is still under investigation, but extensive gene flow and subsequently low level of genetic divergence may be expected to occur among the populations at the poleward range limit of some *Ficus* species due to long distance gene flow in the genus. In the present study, we focused on populations of *F. sarmentosa* var. *henryi* at its northeastern range limit in southeast China to test whether edge populations were genetically fragile. Consistent with our hypothesis, high level of genetic diversity and weak genetic structure were revealed in *Ficus sarmentosa* var. *henryi* populations, suggesting extensive gene flow at the plant's range limit. Long-distance movements of both pollinators and frugivorous birds were likely to be frequent and thereby predominantly contributed to the extensive gene flow at large scale despite of some magnificent landscape elements like huge mountains.

### 1. Introduction

The species-rich genus *Ficus* (Moraceae) is a significant contributor to the sustainability and biodiversity of tropical and subtropical ecosystems (Shanahan et al., 2001b; Herre et al., 2008). Sexual reproduction of *Ficus* species exclusively relies on their host specific pollinating fig wasps (Agaonidae) that can only utilize ovules inside the syconia (figs) of their host plants to raise offspring, forming a classic example of obligate mutualism (Compton et al., 2010; Cruaud et al., 2012; Liu et al., 2015). *Ficus* species can be divided into monoecious and functionally dioecious species (Cook and Rasplus, 2003). In monoecious plants, the female flowers inside a fig have varying style lengths and after being pollinated, their ovules become either seeds or galls supporting pollinator offspring (Wang et al., 2014). Functionally dioecious species have female plants that only produce seeds and male plants that only support pollinator offspring. Each fig supports a single generation of pollinating fig wasps, with pollinator emergence coinciding with the maturation of pollen.

Like many other organisms (Hewitt, 2000; Hampe and Petit, 2005; Shi et al., 2014), some *Ficus* species have experienced fast poleward range expansion after the last glacial maximum (LGM) and range shifts

driven by the undergoing global climate change, especially for some dioecious fig trees which have extended far north into the subtropical zone (e.g., Chen et al., 2012; Yu and Nason, 2013; Vieira et al., 2015). The genetic consequence of such changes in distribution boundaries is highly variable. Low genetic diversity and high differentiation are observed in edge populations of some species (Excoffier et al., 2009), while similar genetic diversity and lack of differentiation has also been reported between edge and non-edge populations (e.g. Eckert et al., 2008; Shi and Chen, 2012). Generally, the genetic consequences of range expansion on edge populations are mainly determined by the speed of range shifts and the dispersal abilities of the species, with faster range shifts leading to stronger loss of genetic variations while extensive gene flow contributes to the maintenance of genetic diversity and lack of genetic structure (Excoffier et al., 2009; Arenas et al., 2011; Petit, 2011).

Gene flow in plants depends on pollen movement and seed dispersal (Chen et al., 2008). Pollinating fig wasps, the unique pollen dispersal medium of *Ficus* species, are recognized as widely dispersing by drifting in the wind despite of their extremely short life span at adult stage and tiny body sizes (Kjellberg et al., 1988; Herre et al., 2008). Some fig wasps have been shown to disperse pollen over incredibly long

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distances (> 100 km), homogenizing the genetic structure of their host *Ficus* species (Ahmed et al., 2009; Kobmoo et al., 2010; Tian et al., 2015). However, large variation of predicted pollen dispersal distances have been proposed with usually shorter pollen dispersal distances in dioecious *Ficus* species than in monoecious species probably because of their difference in ecology (e.g. Yokoyama, 2003; Harrison and Rasplus, 2006; Wang et al., 2009). Monoecious species usually grow as trees or hemi-epiphytic plants that can reach the forest canopy and their pollinators disperse by drifting in the wind above the canopy while the pollinators of dioecious figs are hardly found above the canopy in agreement with they being mostly understory shrubs (Harrison, 2003; Harrison and Rasplus, 2006; Chen et al., 2011). Nonetheless, wasp mediated pollen gene flow is sufficient to result in weak genetic structure in some dioecious figs (Yu and Nason, 2013; Rodriguez et al., 2017).

Seed dispersal distance in *Ficus* species may be more variable as numerous vertebrates and invertebrates feed on mature figs, including species that are incapable of long-distance dispersals like ants and small rodents while other species have outstanding potential dispersal capacity such as large frugivorous birds and bats capable of flying hundreds of kilometers (Compton et al., 1996; Shilton et al., 1999). Rapid colonization of novel habitats, e.g. newly emerged volcanic islands, have suggested an important role of seed dispersal in large-scale gene flow and range expansions of some *Ficus* species (Thornton et al., 1996; Shanahan et al., 2001a). However, large frugivorous birds and bats are less common in places near the poleward boundaries of *Ficus* species (mainly in subtropical area) than in tropical zone and thereby inter-population gene flow may be weakened (Buckley et al., 2010; Jetz et al., 2012). Here, given the potential for long-distance gene dispersal media, we hypothesized that extensive gene flow existed among populations at the range limit of *Ficus* species, leading to high genetic diversity and low genetic differentiation.

*Ficus sarmentosa* var. *henryi* Corner, a functionally dioecious evergreen perennial root-climber, is one of the seven recorded varieties of *Ficus sarmentosa* (Corner, 1965). It is endemic to China, with its natural distribution covering most tropical and subtropical areas in the south of Yangtze River (Corner, 1965). It mainly grows on bare rocks and walls in open fields and rural areas. *F. sarmentosa* var. *henryi* blooms twice a year, with a spring crop of figs receptive from April to early May and a fall crop of figs, receptive from August to early September. A crop of this species can consist of up to several hundred small figs located in the leaf axils. Receptive figs are of small sizes, usually less than 1.5 cm in diameter (C.-H. Yang, personal observations). Sexual reproduction of *F. sarmentosa* var. *henryi* depends on its host specific pollinating fig wasps, *Wiebesia callida* Grandi. After being either pollinated or colonized by pollinators, figs continue to develop into the mature stage, producing seeds or releasing adult female pollinators carrying pollen. Mature female figs of this plant grow on branches and are small, usually < 2 cm in diameter, and thereby may attract large birds (Lomáscolo et al., 2008, 2010), but other groups of animals like ants and rats were also observed to feed on mature figs (C.-H. Yang, personal observations).

In the present study, we focused on populations of *F. sarmentosa* var. *henryi* at its northeastern range limit in southeast China aiming to evaluate the level of genetic diversity and genetic differentiation among populations and to test whether edge populations have high genetic diversity and low genetic divergence.

## 2. Materials and methods

### 2.1. Sampling

During an extensive survey from May 2013 to May 2014 at the northeastern range limit of *F. sarmentosa* var. *henryi*, we sampled 225 individuals from 11 populations in Anhui, Fujian, Jiangxi and Zhejiang Province in southeast China (Table 1; Fig. 1). Our sampling range covered an area of c. 0.35 million square kilometers with populations

separated by up to 523 km. The minimal interval between sampled individuals was 50 m and their positions were recorded using a portable GPS. Several fresh leaves were collected from each individual, and were dried using silica gel before DNA extraction. Each sampled individual was further identified according to the morphological description in Flora of China (<http://foc.eflora.cn/content.aspx?TaxonId=242322456>) and no individuals from other varieties than *F. sarmentosa* var. *henryi* were found in our samples.

### 2.2. Microsatellite markers and genotyping

Total genomic DNA of each sample was extracted using the DNA Plant Kit (Tiangen Biotech<sup>®</sup>, Beijing) and was dissolved in a 100 µl TE buffer for PCR amplification. Genotyping was carried out using nine polymorphic microsatellite loci (FSH1, FSH2, FSH4, FSH8, FSH13, FSH17, FSH18, FSH21 and FSH23) specifically developed for *F. sarmentosa* var. *henryi* (Yang et al., 2014). The forward primer of each locus was labeled with a fluorescent dye and PCRs were conducted following the protocol proposed by Yang et al. (2014). PCR products were scanned with an ABI 3730 Genetic Analyzer (Applied Biosystems<sup>™</sup>, Foster City, California) and alleles were separated and binned using the software GeneMarker HID v2.0.5 (Holland and Parson, 2011).

### 2.3. Statistical analyses

We tested the neutrality for all loci with the stepwise mutation model in LOSITAN v.1.0 (Antao et al., 2008), to remove from the further analyses any locus that would prove to be under selection. The linkage disequilibrium was analyzed for all pairs of loci eight loci using FSTAT v. 2.9.3 (Goudet, 1995) with sequential Bonferroni correction (Rice, 1989). Departure from Hardy-Weinberg equilibrium was tested for each locus using GENPOP v. 4.0 (Rousset, 2008). Micro-Checker v.2.2.3 (van Oosterhout et al., 2004) was used to test for the presence of null alleles.

The level of genetic variation within each population was quantified by mean number of alleles per locus ( $N_A$ ), allele richness per locus ( $A_R$ ), inbreeding coefficient ( $F_{IS}$ ), and observed ( $H_O$ ) and expected heterozygosities ( $H_E$ ).  $N_A$ ,  $A_R$  and  $F_{IS}$  were evaluated with FSTAT v. 2.9.3 (Goudet, 1995), and  $H_O$  and  $H_E$  were estimated using TPGA v.1.3 (Miller, 1997).

We tested for the presence of recent bottleneck events in the studied populations with a Wilcoxon test using the program BOTTLENECK v.1.2.02 (Cornuet and Luikart, 1996). Simulations were conducted under a two-phase model of mutation (TPM), which is considered as the most appropriate model for the mutations of microsatellites (Liu et al., 2013), with 90% single step and 10% multistep mutations, setting 1000 repetitions.

Genetic differentiation among and between populations was assessed by  $F_{ST}$  using FSTAT v. 2.9.3 (Goudet, 1995). We then calculated  $F'_{ST}$  (the standardized  $F_{ST}$ ) via dividing  $F_{ST}$  by  $F_{ST\ max}$ , which is the maximal  $F_{ST}$  values based on the current data, using RECODEDATA v.0.1 (Meirmans, 2006). Gene flow between populations was estimated using the formula  $Nm = (1 - F'_{ST}) / (4 \times F'_{ST})$ .

We carried out Mantel tests to analyze the relationship between genetic differentiation and geographical distance using the package vegan v.2.4–1 in software R v.3.3.1 (R Development Core Team, 2016). In the analysis,  $F_{ST}$  and  $F'_{ST}$  were used in a calibrated form ( $F_{ST} / (1 - F_{ST})$  and  $F'_{ST} / (1 - F'_{ST})$ ).

The hierarchal distribution of genetic variation was quantified by partitioning genetic variance into within- and among-populations variance using analysis of molecular variance (AMOVA) in GenAlEx v.6.5 (Peakall and Smouse, 2012), setting 1000 randomizations.

A Bayesian clustering approach was conducted to assign all individuals into clusters given their allele frequencies using STRUCTURE v.2.3.4 (Pritchard et al., 2000). We first carried out simulations by replicating 15 runs for each  $K$  value from 1 to 11 setting burn-in length as

**Table 1**  
Sampling information and genetic diversity indices of *Ficus sarmentosa* var. *henryi* populations.

Population	Abbreviation	Location	Sample size	$N_A$	$A_R$	$H_O$	$H_E$	$F_{IS}$
Tongling	TL	N30.858°, E118.026°	15	4.6	3.876	0.486	0.534	0.094
Tianmu	TM	N30.324°, E119.441°	29	6.3	4.442	0.601	0.623	0.036
Huangshan	HS	N30.189°, E118.069°	20	6.0	4.678	0.532	0.634	0.166
Tiantong	TT	N29.805°, E121.793°	28	5.1	4.087	0.531	0.629	0.159
Gutian	GT	N29.415°, E118.225°	9	4.4	4.280	0.492	0.599	0.187
Sanqing	SQ	N28.937°, E118.031°	20	6.7	5.208	0.529	0.653	0.195
Wenzhou	WZ	N28.363°, E121.073°	10	5.7	5.372	0.521	0.716	0.284*
Fuzhou	FZ	N26.257°, E119.159°	29	6.4	4.784	0.468	0.634	0.266*
Tianbao	TB	N25.952°, E117.501°	11	3.6	3.397	0.565	0.549	-0.030
Putian	PT	N25.730°, E119.018°	25	6.0	4.416	0.565	0.600	0.060
Zhangzhou	ZZ	N24.619°, E117.877°	29	6.7	4.751	0.507	0.640	0.210*
Mean			20.5	5.6	4.481	0.527	0.619	0.108

Note:  $N_A$ : mean number of alleles per locus;  $A_R$ : allele richness per locus;  $H_O$ : observed heterozygosity;  $H_E$ : expected heterozygosity;  $F_{IS}$ : inbreeding coefficient.

\* $p < 0.05$ .

10,000 and MCMC repetitions as 20,000. LnP(D) value of each run was extracted for calculating  $\Delta K$  by means of the approach proposed by Evanno et al. (2005). The optimal  $K$  value was determined when  $\Delta K$  reached the maximum, and all individuals were assigned to the  $K$  clusters with a burn-in length of 100,000 and 1,000,000 MCMC repetitions.

### 3. Results

Loci FSH2 and FSH18 showed significant signals for positive selection and therefore were not used in the following analyses (Supplementary Fig. 1). Significant linkage disequilibrium was not observed between any pair of loci (all  $p > 0.05$ ). Departures from Hardy-Weinberg equilibrium were present for all loci except FSH13 in 3–7 populations (Supplementary Table 1). We retained all the seven loci for the following analyses because none of them displayed consistent pattern of null alleles in all population though signs of null

alleles were detected in four loci in some populations (Supplementary Table 1).

#### 3.1. Genetic diversity

Mean number of alleles ( $N_A$ ) and allele richness per locus ( $A_R$ ) varied from 3.6 (TB) to 6.7 (ZZ and SQ) and from 3.4 (TB) to 5.4 (WZ) among *F. sarmentosa* var. *henryi* populations with average values of 5.6 and 4.5, respectively (Table 1). Observed ( $H_O$ ) and expected heterozygosities ( $H_E$ ) ranged from 0.468 (FZ) to 0.601(TM) and 0.534 (TL) from to 0.716 (WZ) among populations with mean values of 0.527 and 0.619, separately (Table 1). Mean value of  $F_{IS}$  was 0.108, and individuals from populations ZZ, FZ and WZ showed significantly high level of inbreeding, reflecting the large difference between  $H_O$  and  $H_E$  in those populations (Table 1).

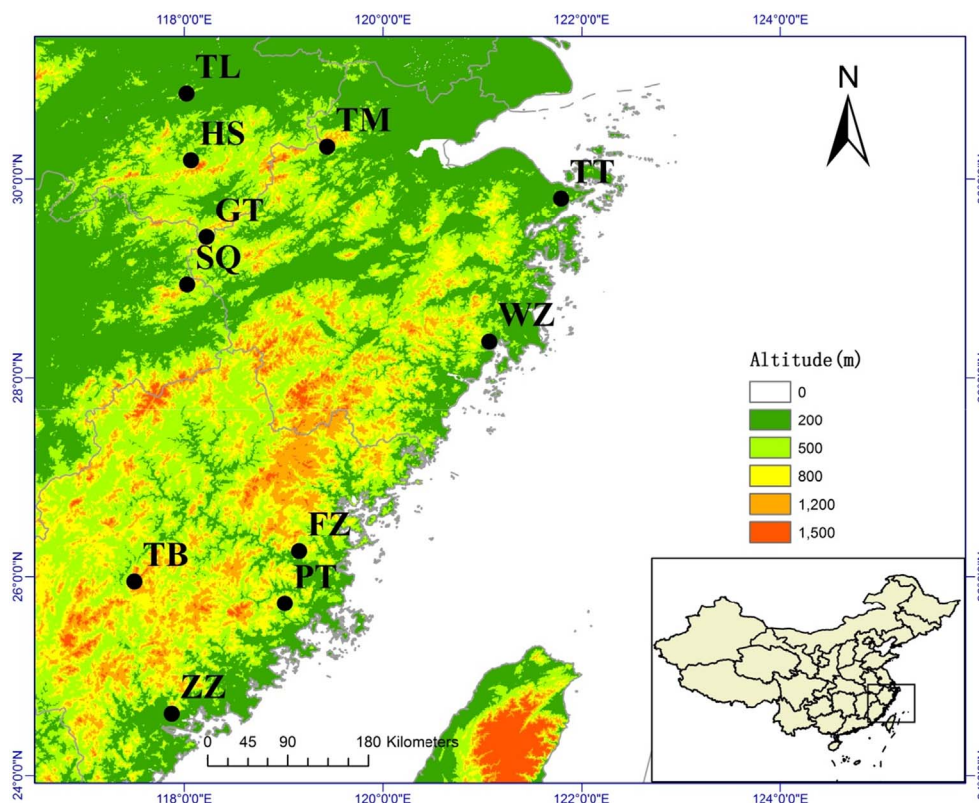


Fig. 1. Location of sampling sites (populations).

**Table 2** Genetic differentiation and estimated gene flow between pairs of populations (upper part:  $F_{ST}$ , lower part:  $F_{ST}$ ,  $Nm$ ). Numerals between brackets are estimated gene flow.

Pop.	TL	TM	HS	TT	GT	SQ	WZ	FZ	TB	PT	ZZ
TL	0										
TM	0.140 (1.533)	0									
HS	0.123 (1.775)	0.147 (1.455)	0								
TT	0.154 (1.370)	0.071 (3.258)	0.158 (1.336)	0							
GT	0.087 (2.607)	0.035 (6.894)	0.021 (11.658)	0.003 (94.525)	0						
SQ	0.192 (1.050)	0.072 (3.233)	0.110 (2.026)	0.083 (2.746)	0.003 (91.000)						
WZ	0.224 (0.866)	0.174 (1.189)	0.181 (1.128)	0.061 (3.845)	0.072 (3.230)						
FZ	0.230 (0.838)	0.195 (1.035)	0.193 (1.048)	0.164 (1.278)	0.085 (2.683)						
TB	0.404 (0.369)	0.237 (0.804)	0.344 (0.476)	0.185 (1.105)	0.203 (0.979)						
PT	0.185 (1.103)	0.106 (2.103)	0.165 (1.268)	0.047 (5.041)	0.009 (27.424)						
ZZ	0.171 (1.211)	0.159 (1.321)	0.143 (1.497)	0.126 (1.731)	0.061 (3.879)	0.131 (1.654)	0.153 (1.387)	0.258 (0.718)	0.213 (0.925)	0.129 (1.682)	0

\*:  $p < 0.05$ , \*\*:  $p < 0.01$ .

### 3.2. Recent bottleneck events

After simulations under TPM, we failed to find any substantial evidence of recent bottlenecks in any population except TT (Table 1), indicating recent dramatic fluctuation in effective population size only in that population.

### 3.3. Genetic structure and gene flow

Moderate genetic differentiation was found among *F. sarmentosa* var. *henryi* populations ( $F_{ST} = 0.055$ ) with  $F_{ST}$  values ranging from 0.001 (GT vs. TT) to 0.184 (TB vs. TL) in pairwise comparisons between populations (Table 2). In addition, we detected significant genetic differentiation ( $F_{ST}$ ) among all populations ( $p < 0.001$ ) and between all pairs of populations except those between GT and TM, TT, PT, SQ, HS and WZ and that between TT and WZ (Table 2). Values of  $F_{ST}$  varied from 0.003 (GT vs. TT) to 0.404 (TB vs. TL) with the corresponding  $Nm$  ranging from 0.408 to 94.525 (Table 2). In addition,  $Nm$  values were larger than 1 in most pairs of populations (80%), suggesting extensive gene flow among populations (Table 2).

Genetic differentiations were independent of geographical distances ( $F_{ST}$ :  $r = 0.236$ ,  $p = 0.072$ ;  $F'_{ST}$ :  $r = 0.236$ ,  $p = 0.068$ ), supported by the absence of isolation by distance (IBD) within our sampling range.

Most genetic variation was present within population, comprising 94% of total genetic variance. Nonetheless, there was significant genetic differentiation among populations ( $p < 0.001$ ) though it only represented 6% of total genetic variance.

Values of  $\Delta K$  reached a peak for  $K = 3$ , and the second highest  $\Delta K$  value occurred at  $K = 2$ , which is much higher than that at other  $K$  values (Fig. 2a). All individuals were therefore assigned into both two and three clusters. For both  $K = 2$  and  $K = 3$ , there was no dominant cluster in all populations except TB, showing no apparent substructure within our study area (Fig. 2b and c).

## 4. Discussion

Consistent with the study by Liu et al. (2013), which focused on the genetic structure of *Ficus pumila* var. *pumila*, a highly related species in the same subsection (subsection *Plagiostigma*, section *Rhizocladus*), our results showed high genetic diversity and weak genetic structure in the edge populations of *F. sarmentosa* var. *henryi*. Lack of genetic structure at large scale must be the consequence of extensive gene flow (Ahmed et al., 2009; Tian et al., 2015; Bain et al., 2016; Rodriguez et al., 2017). Indeed, frequent gene exchange was inferred among most edge populations regardless of potential genetic barriers such as the Wuyi Mountains in the study area. Besides, historical events like human activities and recent colonization followed by fast range expansion could lead to dramatic fluctuations in effective population size, decreasing genetic diversity even in the presence of low immigration rate (Excoffier et al., 2009). *F. sarmentosa* var. *henryi* however is largely exempted from such interference because evidence for recent bottlenecks was only found in one population, also suggesting frequent migration among edge populations.

The intensity of gene flow reflects the performance of gene dispersal agents, i.e., seed dispersers and pollinators. Infructescences of *Ficus* species are an important food resource for numerous animals, many of which are potential long-distance seed dispersers such as large birds and bats (Shilton et al., 1999; Shanahan et al., 2001b; S. G. Compton, personal communication). However, figs relying on different groups of long-distance dispersers have evolved distinct traits with bird syndrome fruits being small and brightly colored whilst bat syndrome fruits are larger and dull colored (Lomáscolo et al., 2008, 2010). Mature figs of *F. sarmentosa* var. *henryi* are likely to be more attractive to birds than to bats due to their small sizes and growing positions (on branches) though their purple color may be less apparent. Besides, *F. sarmentosa* var. *henryi* individuals are easy to locate although it is a climbing plant

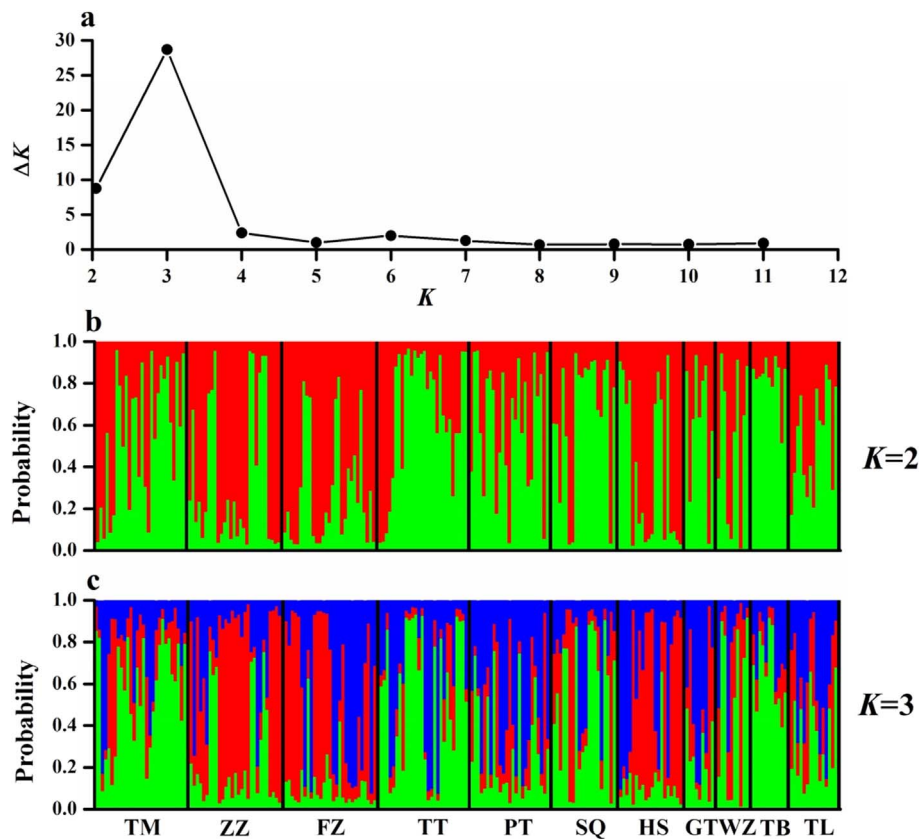


Fig. 2. Genetic structure analyzed using Bayesian clustering approach in software STRUCTURE with  $\Delta K$  value in each  $K$  from 2 to 11 (a) and the results of individual assignments at  $K = 2$  (b) and  $K = 3$  (c).

because it is mainly found in open fields or rural areas rather than inside forests (Chen et al., 2003). Seeds of the studied species could therefore be dispersed over long-distances by birds even in marginal populations, especially as frugivorous bats are not recorded in the north of the tropic of cancer (S.-Y. Zhang, personal communication).

In addition to long-distance seed dispersal, genetic signatures of remarkable pollen dispersal has also been reported in dioecious figs (e.g. Yu and Nason, 2013; Liu et al., 2015). Pollen dispersal is not directly related with the host tree but with insect behavior. Dioecious *Ficus* species are usually shrubs and climbing plants, whose pollinators are seldom caught drifting in the air currents above the canopy probably because their host trees are normally low growth understorey species (Harrison and Rasplus, 2006). However, given that *F. sarmentosa* var. *henryi* mainly grows in open areas where strong wind is expected, *W. callida* is likely to be dispersed by the wind further than the pollinators of dioecious figs inhabiting forests. Moreover, the dispersing efficiency may depend on the ecology and the fruiting phenology of the host plants. Dispersal distance of pollinating fig wasps heavily depends on the local population density and crop size of their host *Ficus* species (Harrison, 2003; Zavadna et al., 2005). Population density of *F. sarmentosa* var. *henryi* was low in most studied populations, and most individuals (both functionally males and females) only produced a small number of receptive figs (from several tens to about two hundred) for each crop (C.-H. Yang, personal observations). This could force local female pollinators to disperse far away in search of receptive figs, thus increasing pollen dispersal distances. Extensive pollen dispersal also effectively prevented inbreeding, which was reflected by nonsignificant  $F_{IS}$  values in most populations.

The flowering phenology of *Ficus* species in subtropical areas usually shows spatio-temporal heterogeneity (Liu et al., 2014; Zhao et al., 2014). Furthermore, an individual fig only remains receptive for a few days, usually less than one week (e.g., Liu et al., 2014). Given the

extremely limited life span (generally shorter than three days) of adult female pollinators (Herre et al., 2008), pollinating fig wasps might hardly find receptive figs even if they successfully arrive to some host populations far away from their natal populations. However, all sampled populations *F. sarmentosa* var. *henryi* flowered at the same time of the year (early May for the spring crop), though details of flowering phenology of this species are still unclear and variation at larger scale is possible. Therefore, flowering phenology is likely to facilitate successful dispersal of pollinating wasps and hence long distance gene flow of the plant.

Population TB appears to be at somewhat different from other populations. The higher  $F_{ST}$  values are probably a simple consequence of its slightly reduced genetic diversity comparatively to other populations due to a recent bottleneck effect. Transient local founder effects that can influence fig population dynamics is likely to result in transient local reduction in genetic diversity. A role for topographic obstacles is unlikely as several large mountains (e.g. the Wuyi Mountains) in our study area failed to disturb gene dispersal among other populations.

In conclusion, high level of genetic diversity and weak genetic structure was detected in populations of *F. sarmentosa* var. *henryi*, suggesting extensive gene flow at the plant's northeastern range limit, despite of the presence of some landscape elements that might have served as obstacles to gene dispersal. Long-distance movements of both pollen and seeds were expected to critically contribute to gene flow at large scale. Future studies focusing on seed dispersal processes, flowering phenology and the strategy of pollinators searching for receptive figs will lead to a better understanding of the maintenance of genetic diversity in *Ficus* species.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.actao.2018.02.002>.

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