## **ORIGINAL RESEARCH**

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# Endangered but genetically stable—*Erythrophleum fordii* within *Feng Shui* woodlands in suburbanized villages

Zheng-Feng Wang<sup>1,2</sup>  $\square$  | Hai-Lin Liu<sup>3,4,5</sup> | Se-Ping Dai<sup>6</sup>  $\square$  | Hong-Lin Cao<sup>2</sup> | Rui-Jiang Wang<sup>2</sup> | Zhang-Ming Wang<sup>2</sup>

<sup>1</sup>Center of Plant Ecology, Core Botanical Gardens, Chinese Academy of Sciences, Guangzhou, China

<sup>2</sup>Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China

<sup>3</sup>Environmental Horticulture Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China

<sup>4</sup>Key Lab of Ornamental Plant Germplasm Innovation and Utilization, Guangzhou, China

<sup>5</sup>University of Chinese Academy of Sciences, Beijing, China

<sup>6</sup>Guangzhou Institute of Forestry and Landscape Architecture, Guangzhou, China

#### Correspondence

Se-Ping Dai, Guangzhou Institute of Forestry and Landscape Architecture, Guangzhou, China. Email: daiseping@gmail.com

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## Abstract

Feng Shui woodlands are naturally or artificially formed green areas in southern China. They are precious for maintaining ecosystem balance in modern semiurban environments. However, they are generally small and geographically isolated from each other, and the status of genetic diversity of the plant species within them has been almost neglected. Therefore, we studied the genetic diversity of the endangered Erythrophleum fordii in eight Feng Shui woodlands (a total of 1,061 individuals) in Guangzhou, a large city in southern China, using microsatellites. For comparison, one population with 33 individuals sampled in a nature reserve was also studied. Although our results indicate that significant demographic declines occurred historically in E. fordii, such declines have not resulted in consistent reductions in genetic variation over generations in Feng Shui populations in the recent past, and the levels of genetic variation in these populations were higher than or comparable to the genetic variation of the population in the nature reserve. In addition, our parentage and paternity analyses indicated widespread and potential long-distance pollen flow within one Feng Shui woodland, indicating the presence of an unbroken pollination network, which would at least partially alleviate the genetic erosion due to habitat fragmentation and the unequal gene contributions of E. fordii parents to their progenies when favorable recruitment habitats are absent under most of the parent trees. Overall, our results suggest that E. fordii in Feng Shui woodlands may not be driven to extinction in the near future. Nevertheless, uncontrolled fast urban development with a lack of awareness of Feng Shui woodlands will cause the local extinction of E. fordii, which has already happened in some Feng Shui woodlands.

#### KEYWORDS

bottleneck, demographic history, genetic diversity, microsatellites, parentage analysis

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# 1 | INTRODUCTION

Culturally protected forests (CPFs, Hu, Li, Liao, & Fan, 2011) are formed through religious or traditional belief (Avtzis et al., 2018). They are important rural/urban forest types worldwide including Asia, Africa, Europe, and Latin America. They are known as *Feng Shui* woodlands in China (Ye, Xu, Wu, & Cao, 2013) and sacred groves (Avtzis et al., 2018; Bossart & Antwi, 2016) and village groves (Lee, Hong, & Kim, 2019) in the other countries. They are generally small in size but can serve as refuges for a large number of regional species (Avtzis et al., 2018; Bossart & Antwi, 2016; Hu et al., 2011; Lee et al., 2019; Martinez & Amar, 2014).

Feng Shui woodlands have existed in China for more than 2000 years (Coggins, 2003; Guan, 2002; Hu et al., 2011; Ye et al., 2013). Literally, "Feng" means wind and "Shui" means water in Chinese. Following these definitions, Feng Shui is thought to create harmony, and promote health and wealth in indigenous communities. In addition to maintaining biological diversity, they are valuable for regulating the climate, cleaning the air, and protecting soil and water, and they play important roles in cultural heritage, leisure activities, and the local economy.

*Feng Shui* woodlands can be categorized into three types according to their location: village, cemetery, and temple *Feng Shui* woodlands, and among them, village *Feng Shui* woodlands are the most common (Hu et al., 2011; Ye et al., 2013). With the recent development and expansion of cities in southern China, some of these woodlands have become part of cities, such as Guangzhou, which is an ever-growing megacity in southern China. Currently, there are 156 *Feng Shui* woodlands in and around Guangzhou city with a total of area 521.07 ha (Ye et al., 2013). However, because most of these are not included in the local conservation projects of the city government, relentless construction (such as housing developments and road construction) in the city is a constant threat to the woodlands, shrinking their areas and isolating them more from each other. Thus, urbanization contributes greatly to habitat destruction and fragmentation, altering the plant and animal communities of *Feng Shui* woodlands.

The negative effects of urbanization on species genetic diversity have been documented in depth (Johnson & Munshi-South, 2017; Johnson, Thompson, & Saini, 2015) and investigated worldwide (Bartlewicz, Vandepitte, Jacquemyn, & Honnay, 2015; Dubois & Cheptou, 2017; Hermansen, Roberts, Toben, Minchinton, & Ayre, 2015; Nagamitsu, Kikuchi, Hotta, Kenta, & Hiura, 2014; Vranckx et al., 2014; Wang, Sork, Wu, & Ge, 2010). However, in a review of such effects on tree populations, it was found that they do not automatically lead to the extinction of tree species on a large scale. Prolonged clonal growth, long generation times, and long-distance pollen dispersal help species temporarily escape or postpone extinction (Honnay & Bossuyt, 2005; Low, Cavers, Boshier, Breed, & Hollingsworth, 2015), which results in an undetectable erosion of plant species genetic variation, making it less likely for people to start immediate conservation efforts.

So far, only two plant genetic diversity studies in *Feng Shui* woodlands in China have been carried out (Ge, Liu, Shen, & Lin,

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2015; Wang, Ye, Fu, Ren, & Peng, 2008), and their results generally confirmed the results of Honnay and Bossuyt (2005) and Low et al. (2015) and clearly demonstrate the harmful effects of urbanization on species' genetic health. In the work of Wang et al. (2008), they compared the genetic diversity of a common species Cryptocava chinensis in two Feng Shui woodlands and four natural reserves in the lower subtropical region of southern China. Their results revealed unexpected extensive clonal growth of C. chinensis in two Feng Shui woodlands due to severe fragmentation and the small population sizes in suburban areas. The clonal growth in Feng Shui woodlands maintained a substantial proportion of the genetic variation of the initial populations, and the small sizes of the woodlands did not result in significant genetic differentiation from the larger reserve populations. However, as McDonald, Rice, and Desai (2016) found out that asexual population maintained genetic variation at the cost of fixing substantially deleterious mutations while sexual population allowed natural selection to more efficiently sort beneficial from deleterious mutations and speeded adaptation, extensive clonal growth may threaten the long-term adaptation of C. chinensis in Feng Shui woodlands. In the work of Ge et al. (2015), they compared the genetic diversity of Phoebe bournei in three Feng Shui woodlands and three natural reserves in southern China. Their results showed that the genetic diversity of P. bournei was clearly lower in Feng Shui woodlands than in the reserves, which could be related to its low regeneration rate in Feng Shui woodlands.

Given that some of plant species in *Feng Shui* woodlands are even endangered, studies on their conservation are thus needed to improve our ability to make relevant recommendations on ways to alleviate the negative impacts of urban development on native species. Therefore, our objectives were to study the genetic diversity and within population gene flow of the endangered species *Erythrophleum fordii* in *Feng Shui* woodlands in Guangzhou, China.

Due to severe human disturbance and the small population size, we would expect the genetic diversity of E. fordii in Feng Shui populations to have decreased. Furthermore, because gene flow is crucial to maintain genetic diversity in plant populations, by examining the present gene flow pattern via parentage analysis, we aimed to investigate the relationship between genetic diversity and gene flow patterns. Currently, there are contrasting results regarding such a relationship in the literature. Theoretically, human disturbances change pollen mutualisms in Feng Shui woodlands and, subsequently, alter a population's genetic variation. However, empirical evidence does not always support this in other disturbed habitats (Giombini, Bravo, Sica, & Tosto, 2017; Noreen, Niissalo, Lum, & Webb, 2016; Rosas, Quesada, Lobo, & Sork, 2011). Therefore, the genetic consequences for a small population or due habitat disturbance are both species and location specific (Owusu, Schlarbaum, Carlson, & Cailing, 2016; Schwarcz et al., 2018). In particular, we also examined the demographic history of Feng Shui populations of E. fordii to study how the current level genetic diversity is related to historical events such as bottlenecks. As a valuable timber tree, the endangered status of E. fordii is believed mainly to be the result of large-scale logging in **VILEY**\_Ecology and Evolution

## **TABLE 1** Studied populations of Erythrophleum fordii and their genetic diversities

Populations in different places and their abbreviation <sup>a</sup>	N	A <sub>R</sub>	A <sub>P</sub>	Н <sub>о</sub>	H <sub>E</sub>	f	Sampling year
Tangbei (TB) village	346	3.2224	0.1717	0.4999	0.4832	-0.0346*	2017
Adult	18	3.1377	0.2318	0.5058	0.5000	-0.0120	
Juvenile	71	3.0376	0.2121	0.5157	0.4903	-0.0522*	
Seedling	257	2.8843	0.1033	0.4951	0.4756	-0.0412*	
Wayaogang (WYG) village	60	2.8472	0.0268	0.4632	0.4007	-0.1647*	2018
Adult (6) + Juvenile (12)	18	2.4845	0.2345	0.4561	0.4007	-0.1431*	
Seedling	42	2.6140	0.3639	0.4704	0.4008	-0.1757*	
Liantang (LT) village	130	3.2493	0.0475	0.4940	0.4865	-0.0134	2018
Adult (9) + Juvenile (75)	84	3.3737	0.3391	0.5063	0.4929	-0.0272*	
Seedling	46	3.3084	0.2738	0.4817	0.4801	-0.0033	
Zhongtou (ZPT) village	164	3.2691	0.0640	0.4995	0.4774	-0.0509*	2018
Adult	59	3.1118	0.0852	0.4888	0.4520	-0.0822*	
Juvenile	27	3.2538	0.1350	0.4893	0.4852	-0.0086	
Seedling	78	3.2099	0.1132	0.5202	0.4950	-0.0513*	
Zhongling (ZL) village	82	3.1667	0.0605	0.5452	0.4982	-0.0958*	2018
Adult	65	2.8857	0.2218	0.5425	0.4904	-0.1072*	
Juvenile (5) + Seedling (12)	17	2.9813	0.3174	0.5480	0.5061	-0.0857*	
Shuikouying (SKY) village	276	3.3219	0.0592	0.5467	0.5330	-0.0285*	2018
Adult	155	3.3291	0.1015	0.5474	0.5348	-0.0236	
Juvenile	34	3.2169	0.0435	0.5310	0.5262	-0.0091	
Seedling	87	3.3611	0.1158	0.5617	0.5379	-0.0446*	
Yangchengang (YCG) village	1	-	-	-	-	-	2018
Xiaodong (XD) village	2	-	-	-	-	-	2018
Dinghu (DH) Mountain	33	3.1533	0.3089	0.4631	0.4518	-0.0244	2018
Adult	33	3.1533	0.3089	0.4631	0.4518	-0.0244	
Overall	1,094			0.5125	0.5461	0.0615*	

Abbreviations:  $A_{p}$ , Private allelic richness;  $A_{R}$ , Allelic richness; f, inbreeding coefficient;  $H_{E}$ , unbiased expected heterozygosity;  $H_{O}$ , observed heterozygosity; N, sample size.

<sup>a</sup>The letters in parentheses show the abbreviated population name, the numbers in parentheses indicate the sample size of the life stage. \*p < .05 after the Bonferroni correction.

the past. Consequently, it will leave a clear signal of a population decline in genetic diversity.

# 2 | MATERIALS AND METHODS

## 2.1 | The species

Zhu, Wang, Ye, and Cao (2013) and Zhu, Wang, Ye, Cao, and Saravanan (2013) have described *E. fordii* in detail. Briefly, *E. fordii* is a legume species, belonging to the family Fabaceae. It occurs naturally in China and Vietnam. Due to the hardness of its wood, it is commonly known as the "ironwood" tree in China. In the past, overexploitation has made it endangered in the wild (IUCN<sup>1</sup>). At present, it is under second-class national protection in China. It is a typical outcrossing species with pollen dispersed by many kinds of insects, such as beetles, butterflies, bees, and wasps (Zhu et al., 2013). However, self-fertilization could happen in *E. fordii* (Zhu et al., 2013), and it was also reported in the congeneric species *E. suaveolens* (Duminil et al., 2016). Its seed is flat and ovate-shaped, large and heavy with 1.54–1.67 cm long, 1.28–1.43 cm wide, 0.784–0.917 g in weight (Zhao et al., 2009). It is inedible and has no wing and no particular attributes to attract animals; therefore, it is believed to be dispersed by gravity (Zhu et al., 2013).

## 2.2 | Sample collection

According to Ye et al. (2013), *E. fordii* can be found in more than 30 *Feng Shui* woodlands in Guangzhou city. By comparing their geographic locations and population sizes, we sampled eight of them (Table 1, Figure 1, Figure S1) during 2017 and 2018. During sampling, we carefully examined within and around each population to make sure we would not miss *E. fordii* populations or individuals nearby.

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Erythrophleum fordii individuals in TB village in Guangzhou city, China. Red: adults; cyan: juveniles; and yellow: seedlings. Only adults are given proportional circles corresponding to their DBH values. An additional 44 seedling samples were collected from around the adult numbered 303. Since these seedlings grew on the roof of an abandoned hut beside this adult tree, their positions were not recorded by GPS (indicated in Figure S2) and therefore, these 44 seedlings are not shown on the map. According to the plate tagged by the local Forest Management Department in 2013, the individual numbered 1 is more than 200 years old (Figure S3). The photographs of the two individuals numbered 35 and 276 are shown in Figure **S**4

FIGURE 2 Spatial distribution of

For comparison, one population from Dinghu Mountain Nature Reserve (DH Mountain) in Zhaoqing city, China, was also sampled. For convenience, we will use the abbreviations of the village names (Table 1) for the populations. The pairwise geographic distances between populations are shown in Table S1.

Except for SKY village, the initial colonization history of E. fordii (natural vs. artificial) in the other seven woodlands and DH Mountain are unknown. According to records, E. fordii was planted in SKY village in 1368 A.D. by a troop stationed there that used this tree to make arrows. However, in TB village, one E. fordii tree is tagged with a plate which estimates it to be more than 200 years old (Figure S3).

We sampled all the individuals that we could find in the populations of TB, WYG, XD, YCG, ZL, and LT villages (Figure 2, Figure S1). Unfortunately, due to human interference, the populations of YCG and XD villages, which were previously recorded as having large population sizes, had shrunk to one and two individuals, respectively. Since both ZPT and SKY villages contained hundreds of newly sprouted seedlings, which was more than we could genotype with our budget, we randomly sampled only some of the seedlings but all of the individuals with DBH (diameters at breast height) ≥1 cm or height  $\geq 1$  m.

We collected one to three leaf samples per E. fordii individual and put them into sealed plastic bags containing silica gels. We determined the locations of the sampled individuals using GPS, and measured and recorded their DBH if the DBH ≥1 cm, or height if the DBH <1 cm.

In April 2018, we revisited the sampling site in TB village and recorded the flowering status of the individuals. We observed that NIL FY\_Ecology and Evolution

only individuals with DBH >20 cm could flower. By combining flowering status and DBH and/or height information, we classified all of our sampled individuals into three cohorts: adult (DBH  $\geq$ 20 cm), juvenile (1 cm  $\leq$  DBH < 20 cm), and seedling (DBH <1 cm).

DH Mountain was the first nature reserve in China (Zhu et al., 2013), and the *E. fordii* population in it is believed to be well preserved. According to Zhu et al. (2013), there were a total of 528 *E. fordii* individuals including 78 with DBH  $\geq$ 15 cm. Therefore, we used it as the basis to compare the highly disturbed populations in the *Feng Shui* woodlands. However, similar to the populations in *Feng Shui* woodlands, it is in a small area of approximately 2 ha, although there are no obvious environmental limitations that might have prevented it from growing in the surrounding areas (personal observation, WZF). In this study, we resampled 33 individuals with DBH >30 cm and genotyped them using not only the previously isolated loci, but also our newly developed ones (Table S2).

## 2.3 | Microsatellite isolation and genotyping

Since only nine microsatellites in *E. fordii* were previously isolated and characterized (Zhu et al., 2009), to increase the discrimination power in parentage analysis, we used the restriction site-associated DNA sequencing (RAD-seq) method to obtain some new microsatellite markers in *E. fordii*.

By using two E. fordii individuals from the South China Botanical Garden, we constructed two RAD-seq libraries according to the methods described by Baird et al. (2008). Briefly, whole genome DNA of E. fordii was digested using the restriction enzyme EcoRI (Takara). The digested DNA fragments were then ligated to adaptors and PCR amplified. Approximately 300-500 bp fragments were subsequently selected and sequenced on Illumina HiSeg X Ten genetic analyzer (Illumina) to produce 150 bp paired end sequencing reads. After sequencing, we obtained a total of 49,971,692 bp of raw reads for one individual and 39,494,352 bp for the other. The raw sequence data are available in the NCBI SRA database with accession numbers SRX5010692 and SRX5010693. Filtering for PCR duplicates and low-quality reads resulted in 16,662,656 and 10,993,602 bp of useful reads for the two individuals, respectively. These reads were assembled using Rainbow 2.0.4 (Chong, Ruan, & Wu, 2012), and the assembled contigs were combined and re-assembled using CAP3 (Huang & Madan, 1999). We used Msatcommander 0.8.2 (Faircloth, 2008) to screen for microsatellites in the re-assembled contigs. In particular, we only chose sequences with at least eight and seven dinucleotide and trinucleotide motifs repeats for the two individuals, respectively. We then randomly chose 35 microsatellite sequences to perform PCRs to test their availability.

We followed the PCR procedures described by Zhu et al. (2009) but with an annealing temperature of 53°C for all the microsatellite loci. We ran the PCR products on 2% agarose gels which revealed that 29 microsatellites could be successfully amplified to produce fragments of the correct size. We then used six individuals from TB village to perform PCRs to study the polymorphism of the 29 microsatellites. After PCR amplification and running the PCR products on an ABI 3730 sequencer, we identified 16 polymorphic microsatellites with clear electrophoretic profiles of alleles in the six individuals. Using these six individuals, we also tested the seven microsatellites previously isolated by Zhu et al. (2009) and shown to be in Hardy-Weinberg equilibrium (HWE). However, only five of these could be successfully amplified in all of our present samples. Thus, we used these 5 together with the 16 newly identified microsatellites (Table S2) to genotype 346 individuals from TB village. We tested the HWE of the microsatellites and found that the locus *EF-32* not only showed a significant deficit in heterozygosity at the population level, but also in the seedling life stage (Table S3). Therefore, we did not use this locus in our study to avoid null allele errors and instead used a total of 20 loci for all population genotyping and data analyses.

## 2.4 | Data analysis

Since only one and two *E. fordii* individuals were found in YCG and XD villages, respectively, these three individuals were only used to estimate the overall genetic diversity in *E. fordii* but excluded from the other data analyses.

We first estimated null allele frequencies at 20 loci in each population with INEST v2.2 under the individual inbreeding model (IIM) with default parameters (Chybicki & Burczyk, 2009). IIM was implemented by a Bayesian approach which showed better statistical properties than maximum likelihood and the other approaches (Chybicki & Burczyk, 2009). We then calculated genetic diversity parameters, observed and unbiased expected heterozygosity ( $H_{0}$ , H<sub>E</sub>) using GenAlEx 6.501 (Peakall & Smouse, 2012), and the inbreeding coefficient (f) using GENEPOP 4.3 (Rousset, 2008). Since the number of alleles depended on the sample size, which made the allelic richness  $(A_p)$  and private allelic richness  $(A_p)$  results difficult to compare among populations or life stages, we used the ADZE 1.0 program (Szpiech, Jakobsson, & Rosenberg, 2008) to compute rarefied allelic richness and private allelic richness by controlling for the smallest sample sizes. That is, for population comparison, we computed  $A_{p}$  and  $A_{p}$  using the smallest sample of DH Mountain (N = 33), while for life stage comparison within population we computed  $A_{R}$ and  $A_{P}$  using the smallest sample of life stages in each population. Nonparametric Wilcoxon tests were used to test for differences in diversity estimates ( $H_{O}$ ,  $H_{F}$ ,  $A_{R}$  and  $A_{P}$ ) between populations and life stages within population. The Wilcoxon tests were performed with wilcox.test function in R software and one-sided p-values ("less" or "greater") were reported.

We also used GENEPOP 4.3 to assess the deviation from HWE and genotypic linkage disequilibrium (LD) among all pairs of loci. The levels of significance for HWE and LD were adjusted by using the sequential Bonferroni correction (Holm, 1979). At the population level, since the association analysis between loci via the LD tests may be strongly influenced by any family structure (Flint-Garcia, Thornsberry, & Buckler, 2003) present in our data, we only used adults or adults and juveniles (in WYG and LT villages) in the LD tests for our studied loci. At locus *Gm2024*, some individuals produced abnormal alleles whose sizes did not follow the rule for the gain or loss

TABLE 2 Bottleneck analyses in Erythrophleum fordii populations

Population	Number of individuals	p-value based on heterozy- gosity-excess method	p-value based on M-ratio method
ТВ	89 (Adult + Juvenile)	.0274	.0020
WYG	60 (all individuals)	.2980	.0032
LT	84 (Adult + Juvenile)	.0102	.0003
ZPT	59 (Adult)	.0476	.0024
ZL	65 (Adult)	.0054	.0037
SKY	155 (Adult)	.0004	.0044
DH	33 (Adult)	.2445	.0010

*Note*: The numbers in **bold** are p < .05; refer to Table 1 for the population names.

of repeated unit; therefore, we treated these alleles as missing values for those individuals in subsequent bottleneck and demographic history inference analyses, but not for other analyses.

Since only TB village was sampled thoroughly, especially for seedlings, we only performed a parentage analysis in this population using the Cervus 3.07 program (Kalinowski, Taper, & Marshall, 2007). Before performance parentage analysis, the power of exclusion for the microsatellite loci was estimated by Cervus. The cryptic gene flow was then assessed by  $1-(1-P_{parent-pair})^{Na}$  following Dow and Ashley (1996), where  $\textit{P}_{\text{parent-pair}}$  was combined nonexclusion probability of parent pair, and Na was the number of adults used for parentage analysis (18 in this study). After determining the power of exclusion for the microsatellite loci, using the allele frequency data calculated from all the samples in this population, we ran a simulation to estimate the critical Delta scores necessary for parentage assignments at a 95% confidence level. The simulation parameter values were as follows: 100,000 tests, 18 for the candidate parents (the adults in our samples of TB village), 0.9 for the proportion of candidate parents sampled, 1 for the proportion of loci genotyped, 18 for the minimum genotyped loci, self-fertilization was allowed, and the default setting were used for the other parameters. Although we could identify the parents at a 95% confidence level for most of the seedlings, there were some seedlings that we could not identify the parents of. Considering the restricted seed dispersal ability of E. fordii for the rest of the seedlings, we assumed the mother tree to be the nearest tree to the seedlings geographically, and then used the same program to perform paternity analysis. In this analysis, we used the same simulation parameters as in the above parentage analysis. After parentage and paternity analyses, the actual pollen immigration rate was estimated as number of seedlings with undetermined paternity/total number of seedlings. The effective pollination neighboring area ( $A_{ep}$ ) was calculated by  $A_{ep} = 2\pi\sigma^2$ , where  $\sigma^2$  was the variance of the pollen dispersal distance (Levin, 1988).

We also estimated the pollen immigration using the spatially explicit neighborhood model (Burczyk, Adams, Birkes, & Chybicki, 2006) in the NM+ 1.1 (Chybicki & Burczyk, 2010) which simultaneously estimated seed immigration. Using maximum likelihood, we estimated the self-fertilization rate (*s*), the pollen immigration rate (*mp*), pollen dispersal distance (*dp*), the seed immigration rate (*ms*), and seed dispersal distance (*ds*). If initial parameter values were far from the true values, the maximum likelihood algorithm could fail to reach convergence. After trying different initial parameters, the final parameter settings used for estimation were as follows: exponential-power dispersal kernel for both seed and pollen, genotyping error rates 0.01 for all loci, seed immigration rate 0.01, average seed dispersal distance 4.57, shape parameter of seed dispersal kernel 0.42, pollen immigration rate 0.134, average pollen dispersal distance 160, shape parameter of pollen dispersal kernel 1.6, selfing rate 0.12, and default for the other parameters.

We used both heterozygosity-excess (Cornuet & Luikart, 1996) and M-ratio methods (Garza & Williamson, 2001) implemented in INEST v2.2 (Chybicki & Burczyk, 2009) to test for recent population bottlenecks. For both methods, we used Wilcoxon's signed-rank test (10,000 permutations) under a two-phase mutation (TPM) model in INEST v2.2 to determine the significance of the bottlenecks. The parameters for TPM were 3.1 for average size of multistep mutations and 0.22 for proportion of multistep mutations.

To infer the population demographic history, we used DIYABCskylineplot 1.0.1 (Navascués, Leblois, & Burgarella, 2017) to detect and characterize past contractions or expansions using microsatellites. This program uses coalescent theory to estimate the population size changes with generations. After initial trials, we set the following parameter values for DIYABCskylineplot analysis: num\_of\_points = 100 (number of points to draw skyline plot), prior\_THETA\_min = 0.1 (THETA, denoted by  $\theta$ , is the population size and measured by  $4N\mu$ , where *N* is the effective population size and  $\mu$  is the mutation rate per generation), prior\_THETA\_max = 10, prior\_GSM\_min = 0.1 (GSM is the generalized stepwise mutation model for microsatellites), prior\_GSM\_max = 0.8, the repeat size for each locus was specified, and all other options and priors were set to default values.

To perform the bottleneck analysis and infer the population demographic history, we used only the adult individuals for the populations of ZPT, ZL, SKY villages, and DH Mountain; for the populations of TB and LT villages which contained only a few adults, we used both adult and juvenile individuals, and for the population of WYG village, the number of adult and juvenile as only 18 (Table 1); therefore, we used all of the individuals (Table 2).

We finally examined genetic structure among populations by STRCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000). Assuming admixture model with correlated allele frequency, twenty independent runs were performed for each possible cluster (*K*, from 1 to 8) using a  $5 \times 10^5$  Markov Chain Monte Carlo (MCMC) iterations after a burn-in period of  $5 \times 10^5$  on total multiloci genotypes of adults without prior concerning of their origin populations. The choice of the probable *K* value was made both as recommended in STRUCTURE user's manual and by  $\Delta K$  method (Evanno, Regnaut, & Goudet, 2005). For the inferred *K*, we used CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) to calculate the average membership coefficient for each individual by combing the results of 20 runs. All these analyses were

performed by a combination of functions from the StrataG 2.1 (Archer, Adams, & Schneiders, 2017) in R package.

## 3 | RESULTS

The number of alleles per locus varied from 2 to 11 (Table S3) among the 20 microsatellite loci studied for the populations of *E. fordii*, and the locus *EF-33* had the highest number of alleles. Nineteen of the loci showed very low null allele frequencies (<0.005) in all populations, and locus *EF-28* displayed null allele frequency higher than 0.05 in five populations (Table S4). Therefore, locus *EF-28* was excluded from all subsequent analyses. None of the retained 19 loci showed a consistent deviation from HWE in the populations or different life stages, and all locus pairs were in equilibrium linkage.

The population of SKY village had the highest genetic variation with  $A_{\rm R}$ ,  $H_{\rm O_i}$  and  $H_{\rm E}$  values of 3.3219, 0.5467, and 0.5330, respectively, and the populations of DH Mountain had the highest  $A_{\rm p}$  value of 0.3089. The population of WYG village had the lowest genetic variation with  $A_{\rm R}$ ,  $A_{\rm p}$  and  $H_{\rm E}$  values of 2.8472, 0.0268, and 0.4007, respectively, and the population of DH Mountain had the lowest  $H_{\rm O}$  value of 0.4631 (Table 1). One-sided Wilcoxon test indicated that all genetic variations ( $A_{\rm R}$ ,  $A_{\rm P}$ ,  $H_{\rm O}$ , and  $H_{\rm E}$ ) were not significantly different between populations except for WYG and SKY villages in  $H_{\rm E}$  value and WYG village and DH Mountain in  $A_{\rm p}$  value (Table S5). The inbreeding coefficients (f) in the populations ranged from -0.1647 to -0.0134, and almost all the populations showed a significant deviation from zero due to heterozygosity excess except LT village and DH Mountain (Table 1).

Analysis of the different life stages revealed the populations of LT villages had higher genetic variation of all  $A_{\rm R}$ ,  $A_{\rm p}$ ,  $H_{\rm O}$ , and  $H_{\rm E}$  values in older generations (adult and juvenile) than in seedlings, but the populations of WYG, ZL, and SKY villages had the highest in seedlings, the youngest generation (Table 1). However, for each *Feng Shui* population, the differences in genetic variation among generations were not significant (Table S6). All *f* values in the life stages of the populations were smaller than zero, but not all of them significant deviation from zero (Table 1).

The combined nonexclusion probabilities across the 19 microsatellite loci for the first parent, second parent, and parent pairs were 0.0407078, 0.00204951, and 0.00003035, respectively. The combined nonexclusion probability of identity was  $1.654 \times 10^{-10}$ . The probability of cryptic gene flow was 0.0005462. All these results indicated the microsatellites used here had high power of exclusion and were optimal for parentage and paternity analyses. Then, parentage analysis successfully identified the parents for 206 (80.16%) of the 257 seedlings in the population of TB village at a 95% confidence level. We assigned a mother trees for 49 of the 51 remaining seedlings according to their geographic position. Paternity analysis then successfully identified the pollen donating trees for 12 of the 49 at the 95% confidence level. Therefore, the parents of 218 seedlings were identified. The distances of the parent-offspring pairs detected ranged from 2.275 to 457.959 m with mean of 160.085 m (Figure 3), excluding 30 self-fertilization cases. The self-fertilization rate was 0.117 (30/257). The actual pollen immigration rate was 0.144 (37/257), and the effective pollination neighboring area  $(A_{en})$ was 11.241 ha.



**FIGURE 3** Picture showing pollen flows among adult individuals. The width of the lines between two adult trees corresponds to the amount of pollen flow

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**FIGURE 4** Bayesian skyline plots showing the demographic history of *Erythrophleum fordii* for different populations. The black line shows the median estimates of historical population size ( $\theta$ ), and the gray lines around the median estimates show the 95% highest posterior density estimates of the historical population size. Note that the most recent time is on the left of the *x*axis. *N* is effective population size,  $\mu$  is the mutation rate per generation, and *T* is the time in generations



**FIGURE 5** Membership probability results of the STRUCTURE analyses based on adults for seven *Erythrophleum fordii* populations. STRUCTURE shows genetic group K = 2, 3, and 6. In the plot each vertical bar represents one individual and the colors in the bar show the assignment probability to the genetic clusters

For the population of TB village, the neighborhood model showed the self-fertilization rate was 0.122 (SE 0.021), the pollen immigration rate 0.134 (SE 0.023), the mean pollen dispersal distance 220.910 m (SE 24.836), the seed immigration rate 0.010 (SE 0.007), and the mean seed dispersal distance 4.666 m (SE 4.724).

With the exceptions of the populations of WYG and DH Mountain using the heterozygosity-excess method, both heterozygosity-excess and M-ratio methods indicated that all the *E. fordii* populations analyzed were likely to have experienced a past bottleneck at p < .05 (Table 2). Furthermore, DIYABCskylineplot detected a clear demographic decline in recent history in all the populations tested (Figure 4).

The mean Log-likelihood values in STRUCTURE analysis indicated K = 6 was the "optimal" genetic clusters (Figure S5) because at K = 6 the Log-likelihood values began to reach "more-or-less plateaus" according to the STRUCTURE manual. The  $\Delta K$  showed two obvious peaks with the highest at K = 2 and the second highest at K = 6. However, because  $\Delta K$  value was smaller at K = 6 than at K = 3, we then illustrated these three K results (Figure 5). At K = 2, the population of SKY village formed a distinctive cluster separated from the other populations. At K = 3, the population of SKY village remained distinctive, and the populations of ZPT and ZL villages were separated from the rest four populations. At K = 6, all the populations formed their own distinctive clusters except the populations of TB and WYG villages, and the population of SKY village showed clear genetic admixtures.

# 4 | DISCUSSION

#### 4.1 | Genetic diversity

Among approximately 15 species in the *Erythrophleum* genus, which is mainly found in Africa, only *E. fordii* is found in China. Since it is an endangered species, it is valuable to compare its level of genetic diversity to that of its congeners (Cole, 2003; Gitzendanner & Soltis, 2000). However, few studies have reported the genetic diversity of *Erythrophleum* species. The only study was by Duminil et al. (2016) who examined the genetic diversity of *E. suaveolens* in Africa to estimate the impact of logging on population dynamics. Similar to our study, this study also investigated the genetic variation in different life stages of *E. suaveolens*. It reported  $H_0$  and  $H_E$  values ranging from 0.461 to 0.531 and 0.535 to 0.658, respectively. Therefore, the genetic variation of *E. fordii* in the different life stages in our *Feng Shui* populations had lower  $H_E$  values, which ranged from 0.4007 to 0.5379 (Table 1) than *E. suaveolens*, but comparable  $H_0$  values to *E. suaveolens*.

We previously investigated the genetic diversity of *E. fordii* in one well-preserved population using nine microsatellites in the DH

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Mountain, a nature reserve. Although our previous study reported higher genetic diversity in the DH Mountain (a  $H_0$  of 0.606 and  $H_E$ of 0.586, Zhu et al., 2013) than all the *Feng Shui* populations we investigated here, our present study using a new set of microsatellites indicated this was not the case (Table 1). In fact, the genetic diversity of the population of DH Mountain was only significantly higher than that of the population of WYG village in  $A_p$  value (Table S5). This implies, on one hand, that *E. fordii* in *Feng Shui* woodlands are not deprived of genetic diversity; on the other hand, that at population level, the genetic diversities within populations measured using different sets of markers should be compared with caution.

We observed that among all the populations, SKY harbored the highest genetic diversity but not the highest number of private alleles (Table 1). A possible reason for this may be that this population was artificially established with high numbers from many different resources which already somewhat depleted in alleles. Compared to the other populations, substantial admixture revealed by STRCTURE analysis (K = 6) confirmed the admixture. However, to our surprise, these sources could not be from nearby populations we sampled. In ancient times, without efficient transportation tools, the troop stationed in SKY that planted the trees might not have been able to introduce E. fordii individuals from places far away. Since there are no the other E. fordii populations geographically closer to the population of SKY village than the populations we sampled, a possible explanation is that the source population(s) of SKY village have been extinct under urbanization. Nevertheless, this hypothesis needs further studies with more extensive sampling than ours in the future.

For the other genetic diversity parameter, inbreeding coefficients (f values), Duminil et al. (2016) observed high f values in young life stages (seed and/or seedling) of E. suaveolens, regardless of the population densities (ranging from 0.7 to 1.72 individuals per ha with DBH >30 cm), which indicates that their populations had high selfing and inbreeding rates. Our Feng Shui populations of E. fordii did not seem to have suffered from inbreeding because all life stages had near zero f values (Table 1), which indicates random mating. Such random mating also existed in the well-preserved population of DH Mountain whose f value was 0.0082 (Table 1). This is consistent with our previous data with f values of -0.058 to 0.022 in different age classes in the population of DH Mountain (Zhu et al., 2013). Since E. fordii and E. suaveolens live in two geographically distant continents and may have undergone very different evolutionary processes, including pollen mutualisms, the reason behind the difference in their mating patterns, revealed by their f values, may be difficult to interpret currently. In fact the high f value in the early life stage of E. suaveolens was uninterpretable by Duminil et al. (2016).

# 4.2 | Pollination and pollen flow

Our parentage analysis for the TB village population (Figure 3) further supports the conclusion of random mating in *E. fordii*. Although this figure shows that mating events seem to be biased to a few individuals, our field investigation indicated this may not be true in nature. TB village is an environment with lots of human interference, and for most of the mother trees, their existing environment is clearly unsuitable for seedling survival (Figure S4). Below these trees, few or no recruits were found (Figure 2). Since our parentage analysis was based on seedlings collected in the field, these few mother trees would display higher mating events than most of the others, resulting in skewed mating patterns in the population (Figure 3). Given this, we believe that the pollination network required random mating in *E. fordii* populations, at least in the TB village population, was not destroyed by urbanization.

According to the review by Senapathi, Goddard, Kunin, and Baldock (2017), pollinator abundance and composition in urbanized areas are not always inferior to less disturbed areas. The flowers of *E. fordii* can attract a diverse range of pollinators (Zhu et al., 2013). Therefore, the positive effects of the pollinators may compensate for the negative effects of urbanization to maintain a stable random mating system in *Feng Shui* populations of *E. fordii*, even if the pollinator fauna or composition among disturbed and less disturbed populations is different. For example, by comparing the genetic variations of *Syagrus romanzoffiana* between continuously protected and nearby fragmented forests in South America, Giombini et al. (2017) did not find that pollen donors were affected in the latter. However, further pollinator fauna observations are needed to better interpret our results.

In this study, we found pollen immigration rate (mp) of 0.144 and 0.122, and mean pollen dispersal distance (dp) of 160.085 and 220.910 m based on parentage assignments and neighborhood model, respectively, in the population of TB village. Effective pollination neighborhood (Aen) for E. fordii was 11.24 ha between seed trees, equivalent to a circle with a radius of 189.21 m around a seed tree. Hence, considering the very limited seed immigration rate of 0.010 and dispersal distance of 4.666 m we observed, long-distance gene flow in E. fordii is primarily by pollen dispersal. As both mp and dp are directly related to the sizes and degrees of isolation of areas, they should vary among species and populations within species with similar pollination insects (Braga & Collevatti, 2011; Manoel et al., 2012; Monthe, Hardy, Doucet, Loo, & Duminil, 2016; Noreen et al., 2016; Sebbenn et al., 2011; Tambarussi, Boshier, Vencovsky, Freitas, & Sebbenn, 2015). For example, for a Neotropical tree Copaifera langsdorffii in a highly isolated and fragmented forest fragment (4.8 ha), Manoel et al. (2012) found its mp and dp values were 0.08 and 66 m, respectively; while for a tropical tree Entandrophragma cylindricum in a relatively large and continuous forest, Monthe et al. (2016) found its mp and dp values were 0.32-0.40 and 506-540 m, respectively. Therefore, we consider the pollen flow in the population of TB village moderate. Because pollination insects, such as bees, could carry pollen to very long distances (Braga & Collevatti, 2011; Dick, Etchelecu, & Austerlitz, 2003; Manoel et al., 2012; Noreen et al., 2016; Sebbenn et al., 2011; Tambarussi et al., 2015), it is possible to find larger mp and dp in larger and more continuous populations of E. fordii than those in our study, based on pollen-mediated gene dispersal capacities of E. fordii. Because our study here is mainly focused on local pollination dynamics, we provide no indication of the potential pollen sources for immigrated pollens. Continued research NIL FY\_Ecology and Evolution

including more surrounding populations on characterizing within and among population pollen flow patterns is a priority in this system in the future.

## 4.3 | Demographic history

Our data indicate that all of our populations clearly suffered from bottlenecks. It is possible that such bottlenecks might have been caused by recent (approximately 100 years ago) wood demands due to long periods of war and poverty. However, the results from skyline plots (Figure 4) indicate that dramatic declines in population size could be dated back 1,000 years ago. The skyline plot is based on an ABC (Approximate Bayesian computation) framework and is mostly likely to detect large events which influenced population size dramatically, whereas demographic changes of small magnitude and close to the present are the hardest to detect (Navascués et al., 2017).

Among the populations, if we consider the point at 0.3 on the x-axis (measuring the time scale) when the skyline plot results suggest the most recent ancestral decline with a high magnitude started (Figure 4), 20–25 years per generation for E. fordii (Tang et al., 2015), and  $5 \times 10^{-4}$  mutations per locus per generation for all microsatellite loci (Melo, Freitas, Bacon, & Collevatti, 2018), we could convert the time scale to 12,000-15,000 years, which is when the last ice age started to end and humans entered the New Stone Age. However, an accurate prediction of a major historical scenario is still challenging and influenced by many factors (Navascués et al., 2017). Therefore, the above time period related to the demographic declines in E. fordii populations should be interpreted with caution, and earlier or later historical events might also have played a role. According to Meng, Wang, Hu, Zhang, and Lai (2017), southern China underwent a short period of cold weather about 8,500 years ago, which had a big impact on the tropical rainforest trees. Thus, as a thermophilic species, E. fordii populations might have shrunk since then.

How the demographic decline in E. fordii was caused by human activities in the past is unclear. However, due to its high quality timber, E. fordii had been in high demand in historical markets and was recorded as one of the "four famous woods" in the Ming dynasty (established in 1368 A.D.) in China (Liang, 2014). According to historical records, the early use of E. fordii can be dated back to the Qin dynasty (established 221 B.C.), and more records of its use have been found since the Song dynasty (established in 960 A.D.) (Zhou, 2007). One famous use of E. fordii in China was to build Zhenwu Pavilion, which was completely built using approximately 3,000 pieces of E. fordii timber in 1573 A.D. In Vietnam, E. fordii has also been reported to be used in many historical buildings (Nguyen et al., 2018). Nevertheless, the high wood demand might have prompted people historically to establish artificial plantations such as the E. fordii population in SKY village, which would alleviate the shortage of naturally grown E. fordii timber. This could be why E. fordii is frequently found in Feng Shui villages where human activities are intensive. In the future, the use of large-scale sampling and whole genome information for E. fordii will more clearly reveal its historical evolutionary dynamics.

## 4.4 | Conservation implication

Our results show that despite moderate gene flow via pollen, the limited seed dispersal distance may result in significant relatedness among *E. fordii* individuals at short distances. Furthermore, they reveal clearly disproportionate contributions of adults to the recruit landscape due to absence of suitable recruitment environment under some adults, which could remain across reproductive cycles if present unfavorable conditions for seedling establishment continue. Together with substantial self-fertilization which is often associated with isolated and fragmented small populations (Cheptou, Hargreaves, Bonte, & Jacquemyn, 2017), all these factors may increase the rates of mating among relatives, producing negative fitness effects in future generations, and slowing down adaptation in the face of climate change.

Seed collection for ex-situ conservation of *E. fordii* should include *Feng Shui* woodlands as high genetic diversity harbored in most them and contain many trees as many trees as possible. The mean pollen dispersal distance suggests that seed trees must be separated by at least 220 m. In addition, since the gene pool of the population of SKY village is different from the others, seeds collected from it should be separated and only mixed with those from other populations after being sure no outbreeding depression effects happening.

## 5 | CONCLUSION

Feng Shui woodlands, as part of CPFs, are valuable supplements for urban forests in southern China, especially with the present policy aiming to build national forest cities, because they are more natural and have higher species diversities than modern artificial green lands (Ye et al., 2013). Furthermore, they can provide a source for rural afforestation and play a key role in ecological networks. Globally, CPFs are distinctive elements of worldwide vegetations, and they naturally and seminaturally distribute in and around urban areas (Avtzis et al., 2018; Bossart & Antwi, 2016; Hu et al., 2011; Lee et al., 2019). Similar to Feng Shui woodlands in China, most of the plant species in CPFs are not considered being threatened in the near future. However, monitoring their gene pools is essential to prevent genetic erosion caused by anthropogenic effects. Therefore, an extension of this study to other species with different life stages and different landscape configures in CPFs would be recommended to know their gene flow and how such flow determines the microevolutionary changes in them.

Overall, our results suggest that *E. fordii* may have suffered serious demographic declines before large-scale human settlements in southern China and has not recovered at the present time due to consistently high demands for its high quality wood. However, parentage analysis indicated that its pollen-mediated gene flows were not severely affected within the disturbed suburban areas, and genetic diversity was stably maintained across different generations. A previous simulation study of *E. fordii* also indicated that its longevity with iteroparity provided the potential to maintain genetic diversity in small isolated populations (Zhu et al., 2013), and our present study supports this conclusion. However, for most of the *Feng Shui*  woodlands, the major threat to the long-term adaptation and evolution of *E. fordii* is the lack of suitable regeneration habitats. The maintenance of large and continuous populations to guarantee high gene flow is also required for long-term species sustainability.

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#### CONFLICT OF INTEREST

The authors declare no competing interests.

#### AUTHOR CONTRIBUTIONS

WZF conceived and designed the project and carried out the laboratory procedures and data analyses. WZF and LHL carried out the field collections. All authors contributed to writing the manuscript.

#### DATA AVAILABILITY STATEMENT

The RAD sequences data are available in the NCBI SRA database with accession numbers SRX5010692 and SRX5010693. The microsatellites are available in the NCBI database with accession numbers shown in Table S2. Sample information and full microsatellite dataset are deposited in ResearchGate (https://www.researchgate.net/publication/333131062\_Table\_S7, https://doi.org/10.13140/RG.2.2.17309.56807).

## ORCID

Zheng-Feng Wang b https://orcid.org/0000-0002-7599-7891 Se-Ping Dai b https://orcid.org/0000-0003-1021-5083

## ENDNOTE

<sup>1</sup> www.iucnredlist.org

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