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Fengshui forests conserve genetic diversity: a case study of *Phoebe bournei* (Hemsl.) Yang in southern China

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ABSTRACT. Fengshui forests (sacred groves) are important in traditional Chinese culture and home to many endangered species. These forests may provide protection for some endangered plant species outside the nature reserves, but little is known about their role in genetic conservation. Using inter-simple sequence repeat (ISSR) markers, we compared the genetic diversity of 6 populations of *Phoebe bournei* (Hemsl.) Yang, a commercially important woody species, which is under second-class national protection and endemic to China. Samples were collected from the nature reserves and Fengshui forests in southern China. Herein, we show that Fengshui forest populations are capable of maintaining some level of genetic diversity. For nature reserve populations, the average N_A and N_E were 1.58 and 1.39, respectively; and for Fengshui forests, they were 1.39 and 1.12, respectively. For nature reserve populations, Nei's gene diversity (*H*) and Shannon's index (*I*) were

0.32 and 0.11, respectively; and for Fengshui forests, they were 0.22 and 0.07, respectively. We discuss the reasons for the genetic differences between populations of the Fengshui forests and nature reserves and propose conservation strategies for the Fengshui forest.

Key words: Fengshui forest; *Phoebe bournei* (Hemsl.) Yang; Nanmu; Genetic diversity; Nature reserves

INTRODUCTION

The negative impacts of forest degradation and industrialization are complex and broad, including species extinctions and the loss of genetic diversity (Chapin et al., 2000; Feyissa et al., 2007). Increasing numbers of species are becoming endangered, with some populations restricted to nature reserves. Therefore, nature reserves play an important role in the conservation of the genetic diversity for such species (Sanderson et al., 2002; Chen et al., 2009).

In China, the Fengshui forests (sacred groves) are home to many endangered species and, alongside nature reserves, are important for the protection of endangered species (Jan et al., 2007). Fengshui forests are man-made forests, usually located near a village, backyard, or temple, that have been sown from seeds or are an extension of a natural forest. They are important in traditional Chinese culture and are strictly managed (e.g., the felling of large trees is forbidden) (Yuan and Liu, 2009). Fengshui forests have spiritual symbolic meanings and can be traced back to the Shang (1700-1100 BC) and Zhou (1066-256 BC) Dynasties; they are very popular in China and other East Asian countries that were historically influenced by the Chinese culture (Kim, 2005).

Many studies have shown that the Fengshui forests have great value to ecology, biodiversity, and human livelihood on a daily basis (Zhong and Boris, 2007). However, there are few reports on the role of Fengshui forests in conserving genetic diversity and comparisons of genetic diversity between nature reserves and Fengshui forests.

To understand the potential of Fengshui forests for genetic conservation, we chose an endemic Chinese plant, *Phoebe bournei* (Hemsl.) Yang. Also known as Nanmu, it is a commercially important woody species and considered an excellent material by Chinese ancient civilizations because of its brilliant color and remarkable durability. Nanmu has been used intensively over the centuries, especially during the Ming and Qing Dynasties, for high-grade furniture manufacturing, shipbuilding, temple decoration, and Palaces such as the Forbidden City. *P. bournei* is under second-class national protection in China (IUCN, 2012). Currently, populations can be found only in several nature reserves and Fengshui forests.

We sequenced inter-simple sequence repeats (ISSRs) to compare the genetic diversity of *P. bournei* populations in Fengshui forests and nature reserves (Aga et al., 2005). We addressed the following: 1) whether Fengshui forests can somehow conserve genetic diversity; 2) comparisons of the genetic diversity of *P. bournei* populations located in the nature reserves and Fengshui forests; and 3) protection of populations in the Fengshui forests.

MATERIAL AND METHODS

Plant material and DNA isolation

P. bournei was once abundant in southern China, but many populations have been reduced to isolated stands containing very few individuals, and relatively large populations are only found in Zhejiang, Fujian, Guangdong, Hunan, and Jiangxi Provinces (Ge et al., 2012; Zhang et al., 2012). Based on this distribution, we chose 6 populations (3 from the Fengshui forests and 3 from the nature reserves) throughout these 5 provinces (Figure 1; Table 1). We made sure that the leaf sample sizes from each population were similar in order to avoid effects of population size on genetic diversity. Total genomic DNA was extracted from each sample using a modified ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The DNA was diluted to a working concentration of 20 ng/μL.

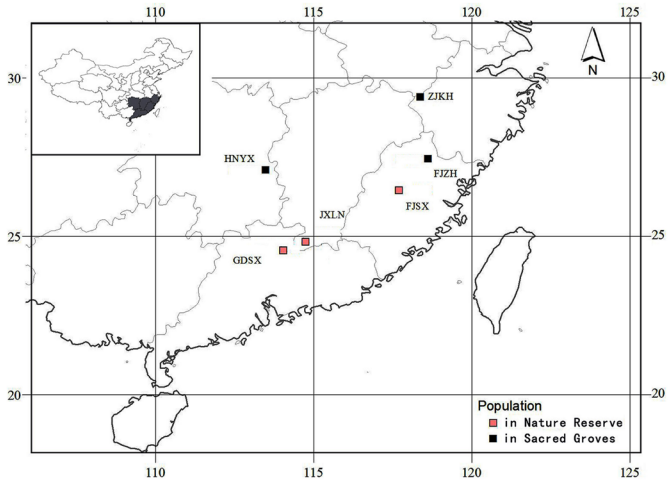

Figure 1. Geographical distribution of the sample populations used in the present study.

Table 1. Ecological and geographical parameters of the 6 populations sampled.

Population code	Zone/Province	Latitude(N)/ longitude(E)	Elevation (m)	Annual rainfall (mm)	Annual mean temperature (°C)	Population area/ha
FJSX	Shaxian/Fujian	26°444/117°705	540	1662	19.2	17.35
FJZH	Zhenghe/Fujian	27°443/118°616	520	1636	18.5	4.13
GDSX	Shixing/Guangdong	24°723/114°256	580	1468	19.6	5.33
HNYX	Youxian/Hunan	27°098/113°492	100	1410	17.8	1.05
JXLN	Longnan/Jiangxi	24°582/114°44	280	1981	17.4	13.33
ZJKH	Kaihua/Zhejiang	29°396/118°379	440	1901	16.0	0.78

ISSR analysis

ISSR primers (Table 2) were synthesized by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. based on the sequences from the Biotechnology Laboratory, University of British Columbia, Canada (UBC Primers). The polymerase chain

reaction (PCR) amplification was conducted in a total volume of 20 µL; the reaction mixture contained 60 ng genomic DNA, 500 nM primers, 0.2 mM dNTPs, 1.0 unit *Taq* polymerase, 1.8 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, and 2% formamide (deionized). The amplification was performed on the thermocycler PCR 9700 (Applied Biosystems, Foster City, CA, USA) by initial denaturing at 94°C for 3 min; followed by 35 cycles each of 94°C for 30 s, 53°C for 45 s, and 72°C for 90 s; and a final elongation at 72°C for 7 min. PCR products were separated by electrophoresis on a 1.8% agarose gel in 1X TAE buffer, stained with ethidium bromide for visualization, and later photographed under UV light (Lin et al., 2009).

Table 2. Primers used in ISSR analysis, numbers of reproducible bands, number and percent of polymorphic bands.

Primer code	Sequences from the 5' to 3' ends	Reproducible bands	Polymorphic bands	
			No.	Percent (%)
1	ACACACACACACACACT	7	4	57
4	ACACACACACACACACAG	5	3	60
17	GACAGACAGACAGACA	6	4	67
23	ACACACACACACACACTA	6	6	100
25	ACACACACACACACACCA	5	3	60
32	AGAGAGAGAGAGAGAGAC	7	7	100
33	AGAGAGAGAGAGAGAGAT	5	5	100
35	AGAGAGAGAGAGAGAGTA	6	6	100
36	AGAGAGAGAGAGAGAGTC	5	4	80
42	ACACACACACACACACCG	6	5	83
44	ACACACACACACACACGA	6	6	100
46	ACACACACACACACACGG	6	5	83
47	ACACACACACACACACGT	6	5	83
56	AGAGAGAGAGAGAGAGTT	6	6	100
74	ACTGACTGACTGACTG	6	6	100
818	CACACACACACACACAG	4	4	100
835	AGAGAGAGAGAGAGAGYC	5	3	60
846	CACACACACACACACART	7	6	86
Mean		5.7	4.8	84
Total		104	88	85

Data scoring and analysis

For each type of marker, only clear and intense bands in the gel were scored as present (1); all others were scored as absent (0). Genetic similarity was analyzed using the software package NTSYS-PC Version 2.10.E (Rohlf, 2000) based on Dice's coefficient (Dice, 1945) with the ISSR data. We used the POPGENE software v3.2 (Yeh et al., 1999) to compute gene diversity and measures of population differentiation. The total genetic diversity (H_T), within population genetic diversity (H_S), among population genetic diversity (D_{ST}), and coefficient of genetic differentiation (G_{ST}) were related by the following expression according to Nei (1977):

$$H_T = H_S + D_{ST}, \text{ and } G_{ST} = D_{ST} / H_T \quad \text{(Equation 1)}$$

Analysis of molecular variance (AMOVA) was performed using GenALEx6 to describe genetic structure and variability within and between populations (Peakall and Smouse, 2006).

RESULTS

The 18 ISSR primers produced a total of 104 scorable bands; 88 bands (84.61%) were

polymorphic across 60 *P. bournei* leaf samples collected from 6 populations. Approximately 3-7 bands were obtained from each primer, with an average of 4.8 per primer (Table 2).

The H_T, H_S, D_{ST}, and G_{ST} of all 6 populations were 0.33, 0.13, 0.20, and 0.39, respectively. The results of the AMOVA (Table 3) also showed significant genetic differences ($P < 0.001$) among the populations (57.21%).

The proportion of polymorphic bands within populations varied from 16.35 to 61.54% for the HNYX and FJSX populations, respectively, with a mean of 36.86% (Table 4). *H* and Shannon's index (*I*) estimates are given in Table 4. The HNYX population exhibited the lowest genetic diversity in accordance with the polymorphic fragment percentage. The N_A of populations located in the nature reserves ranged from 1.54 to 1.62, and varied from 1.06 to 1.12 for populations located in the Fengshui forests. The effective number of alleles (N_E) varied from 1.38 to 1.40 in populations located in the nature reserves and ranged from 1.11 to 1.14 for populations located in the Fengshui forests. The *H* value of all 6 populations varied from 0.06 to 0.24, with an average of 0.15. The *I* value ranged from 0.09 to 0.35, with an average of 0.22. *H* and *I* inferred a very significant difference between populations in the Fengshui forests and those in the nature reserves but not among the populations of the 3 Fengshui forests or the 3 nature reserves.

Populations in the nature reserves had higher genetic diversity than those in the Fengshui forests (Table 4). Within-population genetic diversity was the lowest in the 3 Fengshui forest populations (i.e., HNYX followed by ZJKH and FJZH). Table 5 shows Nei's (1978) unbiased genetic distances between these populations. The largest genetic distance (0.37) was found between the ZJKH and JXLN populations, and the smallest genetic distance (0.18) was observed between the JXNL and GDSX populations.

Table 3. Summary of the analysis of molecular variance (AMOVA).

	Source of variation	df	SSD	MSD	Var.	Total (%)	P
Populations	Among populations	2	195.48	97.74	7.55	43.84	<0.001
in nature reserve	Within populations	33	319.10	9.67	9.67	56.16	<0.001
Populations	Among populations	2	218.56	109.28	11.91	78.28	<0.001
in Fengshui forest	Within populations	26	85.93	3.31	3.31	21.72	<0.001
Total populations	Among populations	5	540.01	108.00	9.38	57.21	<0.001
	Within populations	60	420.80	7.01	7.01	42.79	<0.001

SSD, sum of squared deviation; MSD, mean squared deviation; P, significance of the variance components after 999 random permutations. Both "among population" and "within population" values are considered to be highly significant at $P < 0.001$

Table 4. The genetic diversity of the different provenances of *Phoebe bournei*.

	Population	Polymorphic loci number	Polymorphic loci percent	N_A	N_E	Nei's *H*	Shannon's *I*
Populations in	FJSX	64	61.54	1.62 ± 0.15^{aA}	1.40 ± 0.14^{aA}	0.24 ± 0.02^{aA}	0.35 ± 0.03^{aA}
nature reserve	GDSX	62	59.61	1.54 ± 0.15^{aA}	1.39 ± 0.14^{aA}	0.21 ± 0.02^{aA}	0.29 ± 0.03^{aA}
	JXLN	60	57.69	1.58 ± 0.15^{aA}	1.38 ± 0.14^{aA}	0.22 ± 0.02^{aA}	0.32 ± 0.03^{aA}
	Mean	62 ± 2^{aA}	59.61 ± 1.93^{aA}	1.58 ± 0.14^{aA}	1.39 ± 0.11^{aA}	0.22 ± 0.02^{aA}	0.32 ± 0.03^{aA}
Populations in	HNYX	17	16.35	1.06 ± 0.14^{bB}	1.11 ± 0.12^{bB}	0.06 ± 0.01^{bB}	0.09 ± 0.011^{bB}
Fengshui forest	ZJKH	20	19.23	1.09 ± 0.14^{bB}	1.12 ± 0.13^{bB}	0.08 ± 0.01^{bB}	0.11 ± 0.02^{bB}
	FJZH	23	22.12	1.12 ± 0.15^{bB}	1.14 ± 0.12^{bB}	0.08 ± 0.01^{bB}	0.12 ± 0.02^{bB}
	Mean	20 ± 3^{bB}	19.23 ± 2.89^{bB}	1.09 ± 0.13^{bB}	1.12 ± 0.12^{bB}	0.07 ± 0.01^{bB}	0.11 ± 0.02^{bB}

Lowercase and capital letters indicate statistically significant differences between populations at $P < 0.05$ and $P < 0.01$, respectively.

Table 5. Nei's (1978) unbiased genetic distance for 6 *Phoebe bournei* populations.

Population	FJSX	FJZH	GDSX	HNYX	JXLN	ZJKH
FJSX	-					
FJZH	0.25	-				
GDSX	0.22	0.29	-			
HNYX	0.30	0.26	0.30	-		
JXLN	0.23	0.32	0.18	0.28	-	
ZJKH	0.22	0.34	0.35	0.36	0.37	-

DISCUSSION

Fengshui forests can conserve some genetic diversity

Genetic diversity is essential for effective genetic conservation of species (Millar and Marshall, 1991), and the value of conserving genetic diversity has received significant attention (Joshi et al., 2007; Cao et al., 2009). Fengshui forest population N_A and N_E values reached 1.09 and 1.12, respectively; and those for the nature reserves reached 1.58 and 1.39, respectively (Table 4). Despite very significant differences between forest and reserve populations, a certain amount of genetic diversity was preserved in the Fengshui forests. Because Fengshui forests have been strictly protected, most *P. bournei* individuals in these populations are very large and hundreds of years old, and they may have maintained many excellent genes adapted to the environment that could be useful for molecular breeding. We found that most *P. bournei* populations were distributed throughout remote areas at the junctions of provinces (Figure 1) where there is little interference from human activities, which is probably one of the reasons that these populations have survived over the years.

Reasons for lower genetic diversity than that of the nature reserves

There may be a number of reasons why *P. bournei* genetic diversity was lower in the Fengshui forest populations than those in the nature reserves. First, Fengshui forest populations are smaller, and we expect more genetic diversity in large populations than small populations (Savolainen and Pyhäjärvi, 2007). Second, we know that almost all Fengshui forests are located nearby villages where human interference is greater than that in the nature reserves. Pollination and seed dispersal behaviors will certainly be different when compared to those in the nature reserves, thus affecting the breeding system and leading to a lower level of genetic diversity (Xu et al., 2012).

The most significant reason for the lower genetic diversity in Fengshui forest populations is likely because they did not originate from natural forests but were planted by village founders. Zhuang and Gorlett (1997) also suggest that Fengshui forests in Hong Kong were afforestated. The low genetic diversity of Fengshui forests could be a result of seedling collection from a limited number of parent plants and a combination of selective harvesting for timber, firewood, and other products under village management. Consistent with our results, the difference in genetic diversity between natural populations and planted stands of *Inga edulis* populations was statistically significant (Hollingsworth et al., 2005).

Conservation suggestions

Many Fengshui forests have been established since the settlement of villages, and they are not the same as the home-garden systems, which can be maintained by multiple collections from diverse wild populations over several years (Shrinidhi and Sathish, 2009; Gao et al., 2012). Forest trees in general have long generation times and life spans that can last up to hundreds of years (Savolainen and Pyhäjärvi, 2007); the regrowth of forests has not be an ongoing process, and the reintroduction of material form multiple wild populations may have been difficult because ancient human populations lacked the modern technical advances of seed orchard establishment. The matter of protecting and improving genetic diversity of populations located in the Fengshui forests is an urgent problem.

First of all, Fengshui forests should be protected not only for their genetic diversity but also for their cultural contributions. Thus, an appeal should be proposed to protect these populations *in situ*. We should seek to control human interference and protect pollination and seed dispersal systems. Seedlings in these populations must be protected and prevented from harvestation for firewood.

Second, low genetic diversity and small communities cause instability in Fengshui forest populations. We can introduce seedlings from other populations that have large genetic distances to increase the number of individuals and genetic diversity in these Fengshui forest populations. We should apply modern seed orchard techniques to reconstruct populations located within the Fengshui forests to promote genetic diversity (Chaisurisri and Kassaby, 1994). We hope our findings are taken into account with regard to the development of conservation management policies for Fengshui forests.

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