The phylogeography of *Fagus hayatae* (Fagaceae): genetic isolation among populations

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Abstract

The beech species *Fagus hayatae* is an important relict tree species in subtropical China, whose biogeographical patterns may reflect floral responses to climate change in this region during the Quaternary. Previous studies have revealed phylogeography for three of the four *Fagus* species in China, but study on *F. hayatae*, the most sparsely distributed of these species, is still lacking. Here, molecular methods based on eight simple sequence repeat (SSR) loci of nuclear DNA (nDNA) and three chloroplast DNA (cpDNA) sequences were applied for analyses of genetic diversity and structure in 375 samples from 14 *F. hayatae* populations across its whole range. Both nDNA and cpDNA indicated a high level of genetic diversity in this species. Significant fixation indexes and departures from the Hardy–Weinberg equilibrium, with a genetic differentiation parameter of *Rst* of 0.233, were detected in nDNA SSR loci among populations, especially those on Taiwan Island, indicating strong geographic partitioning. The populations were classified into two clusters, without a prominent signal of isolation-by-distance. For the 15 haplotypes detected in the cpDNA sequence fragments, there was a high genetic differentiation parameter (*Gat* = 0.712) among populations. A high *Gat* of 0.829 was also detected outside but not within the Sichuan Basin. Consistent with other *Fagus* species in China, no recent population expansion was detected from tests of neutrality and mismatch distribution analysis. Overall, genetic isolation with limited gene flow was prominent for this species and significant phylogeographic structures existed across its range except for those inside the Sichuan Basin. Our study suggested long-term geographic isolation in *F. hayatae* with limited population admixture and the existence of multiple refugia in the mountainous regions of the Sichuan Basin and southeast China during the Quaternary. These results may provide useful information critical for the conservation of *F. hayatae* and other Chinese beech species.

Introduction

*Fagus* L. had a widespread distribution across the continents of the Northern Hemisphere during the Tertiary (Denk et al. 2005; Denk and Grimm 2009), and the 10 species of *Fagus* are still among the dominant components in northern temperate forests (Shen 1992; Denk 2003). Unlike Europe and North America, where *Fagus* species dominate the climax forests in temperate regions (Forcier 1975; Peters 1997), the generally recognized four *Fagus* species endemic to China are restricted to the subtropical mountains, with a northern limit near 34°N (Cao et al. 1995; Fang and Lechowicz 2006). Among them, *Fagus hayatae* Palib. has the narrowest species range and is the only one disjunctly distributed from mainland subtropical mountains to Taiwan Island (Shen et al. 2015).
According to the contemporary patterns of genetic diversity and spatial population genetic structure, *Fagus* species were found to shrink southward with refugia distribution in the Quaternary Ice Ages in Europe (Demeasure et al. 1996; Vettori et al. 2004; Vornam et al. 2004; Shanjani et al. 2010), North America (Kitamura and Kawano 2001), and Japan (Tomaru et al. 1998; Okaura and Harada 2002; Kobashi et al. 2006; Hiraoka and Tomaru 2009a), and then expanded northwards in the postglacial stage. The impacts of climate change on species distributions have also been revealed for *Fagus* species in China. Phylogeographical analysis of *Fagus engleriana* detected the effects of glacial periods and postglacial population dynamics (Lei et al. 2012), as also inferred by Zhang et al. (2013) from comparison of the demographic histories of *Fagus lucida* and *Fagus longipetiolarata*.

*Fagus hayatae* is monoeocious with unisexual flowers. Normally 4–6 male flowers constitute an umbrella capitulum and only 1–2 female flowers connect in a pedicel of *F. hayatae*. The cupule generally encloses two triangle-shaped nuts with three shallow ridges. While *Fagus* species are wind-pollinated (Faegri and van der Pijl 1979), *F. hayatae* is monoecious with unisexual flowers.

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out at each locus. In view of any departure from Hardy–Weinberg equilibrium probably due to the presence of null alleles (Soulsbury et al. 2007), the null allele frequency at each locus was calculated. As the presence of microsatellite null alleles was common in genetics studies, its effects could be moderate and negligible when its frequency was lower than 0.2, otherwise the large effects should be considered (Dakin and Avise 2004; Chapuis and Estoup 2007). Among populations, genetic differentiation was analyzed with the differentiation parameter ($R_{st}$) (Michalakis and Excoffier 1996), and the isolation-by-distance (IBD) effect (Rousset 1997) was examined using the Mantel test (Mantel 1967; Smouse et al. 1986). These analyses here were all performed using Arlequin v3.1 (Excoffier et al. 2005), except the Hardy–Weinberg equilibrium tests and null allele frequency calculations with GENEPOP v4.0 (Rousset 2008).

In addition, Bayesian clustering analysis with a Markov Chain Monte Carlo (MCMC) approach over all loci was performed for individual clustering under a model assuming admixture and noncorrelated allele frequencies using Structure v2.1 (Pritchard et al. 2009). A series of 10 independent runs were performed for each of 1–15 clusters ($K$) with a burn-in period of $10^5$ steps followed by $10^6$ replicates. The optimum was detected by calculating the parameter $\Delta K$ (Evanno et al. 2005), which considers the value of $K$ corresponding to the maximum $\Delta K$ appropriate.

*P < 0.05.

Table 1. Sample locations, sizes, genetic diversity, and Fi based on nDNA SSR loci in 14 populations of $Fagus hayatae$.

| Province (Abbreviation) | Code | Mountain     | Lat. (N)    | Long. (E) | Altitude (m) | Size | $A$ | $H_o$ | $H_e$ | $F_i$ | Fi  |
|------------------------|------|--------------|-------------|-----------|--------------|------|-----|-------|-------|------|-----|
| Shaanxi (S)            | S1   | Liping       | 32°48'     | 106°38'   | 1317         | 30   | 9.75| 0.621 | 0.692 | 0.229*|     |
|                        | S2   | Micang       | 32°38'     | 106°32'   | 1794         | 30   | 8.38| 0.565 | 0.642 | 0.029 |     |
| Sichuan (C)            | C1   | Gucheng      | 32°39'     | 106°31'   | 1672         | 30   | 8.62| 0.573 | 0.694 | 0.240*|     |
|                        | C2   | Guangwu      | 32°37'     | 106°49'   | 1803         | 30   | 10.12| 0.569 | 0.705 | 0.565*|     |
|                        | C3   | Tiantai      | 30°16'     | 103°06'   | 1302         | 30   | 6.75| 0.690 | 0.690 | 0.356*|     |
| Chongqing (Y)          | Y1   | Baizhi       | 30°55'     | 108°25'   | 1648         | 30   | 8.50| 0.508 | 0.606 | 0.256*|     |
|                        | E1   | Shennongjia  | 31°29'     | 110°21'   | 1884         | 30   | 11.75| 0.767 | 0.809 | 0.446*|     |
|                        | E2   | Xiaozhai     | 30°07'     | 110°07'   | 1591         | 30   | 9.75| 0.696 | 0.769 | 0.386*|     |
|                        | E3   | Qizimei      | 30°02'     | 109°43'   | 1661         | 30   | 11.50| 0.710 | 0.804 | 0.343*|     |
| Zhejiang (Z)           | Z1   | Qingliangfeng| 30°06'     | 118°53'   | 975          | 25   | 6.25| 0.535 | 0.621 | 0.167*|     |
|                        | Z2   | Sihai        | 28°30'     | 120°43'   | 909          | 15   | 6.12| 0.600 | 0.649 | 0.161 |     |
| Taiwan (T)             | T1   | Chatian      | 24°47'     | 121°27'   | 1667         | 20   | 8.25| 0.467 | 0.731 | 0.757*|     |
|                        | T2   | Niaozui      | 24°44'     | 121°18'   | 1720         | 15   | 6.25| 0.425 | 0.667 | 0.600*|     |
|                        | T3   | Tong         | 24°31'     | 121°39'   | 1587         | 30   | 9.12| 0.411 | 0.798 | 0.899*|     |
| Total                  |      |              |            |           |              | 14   | 375 | 21.75| 0.598 | 0.834 | 0.545*|

*P < 0.05.

Figure 1. (A) Distribution of 15 haplotypes of $Fagus hayatae$ and (B) their statistical parsimony network with "OutGroup" ($Fagus lucida$). The size of each colored circle is proportional to the relative haplotype frequency and the haplotype colors correspond to those shown in (A), while solid lines represent nucleotide site differences between haplotypes.
PCR amplification, sequencing, and analysis of cpDNA sequence fragments

For amplification and sequencing of cpDNA, three polymorphic primers (3'\textit{trnL}–\textit{trnF}, \textit{trnH}–\textit{psbA}, and \textit{rpl}20–5'\textit{rps}12) were selected after a screening of 20 primers described by Taberlet et al. (1991), Hamilton (1999), and Shaw et al. (2007). Amplification reactions were performed in a volume of 20 μL containing 10 μL of 2× Taq PCR StarMix (GenStar, Beijing, China), 1 μL each of forward and reverse primers (10 μmol/L), 1 μL of template DNA (about 100 ng), and 7 μL of ddH$_2$O. Amplifications were carried out using the same PCR cycler as those for SSR amplification in section PCR amplification, genotyping and analysis of microsatellite markers of nDNA, with the following conditions: initial 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 53°C and 2 min at 72°C, with a final 10 min at 72°C. Amplification products were checked by electrophoresis on 2% agarose gels stained with ethidium bromide and sequenced on a 3730xl DNA Analyzer (ABI).

DNA sequences were edited using Sequencher v5.0 (Gene Codes, Ann Arbor, USA) and aligned using Clustal X v1.81 (Thompson et al. 1997) with manual refining.

The distribution of haplotype frequency was mapped using ArcGIS 9.3 (http://www.esri.com). A statistical parsimony network of haplotypes was generated using TCS v1.21 with a median-joining algorithm using a maximum-likelihood network of haplotypes was generated using TCS v1.21 with a median-joining algorithm using a maximum-likelihood (ML) approach with Akaike information criterion and among all 14 populations (Table 1). The same significant FI and FI values with heterozygosity deficiency and significant departure from Hardy–Weinberg equilibrium among populations were detected (Table S1). Over eight loci, genetic differentiation with \textit{A} and FI for each population varied within 6.12–11.75 and 0.029–0.899, respectively, with high values of \textit{A}, but also statistically significant FI values detected in populations in Taiwan (Table 1). The same significant FI and heterozygosity deficiency occurred in most populations and among all 14 populations (Table 1). At each locus, with relatively low null allele frequency (<0.2), high FI values with heterozygosity deficiency and significant departure from Hardy–Weinberg equilibrium among populations were detected (Table S1). Over eight loci, genetic differentiation with \textit{R}$_{st}$ = 0.233 was detected. The Mantel test applied for all 14 populations over eight loci did not show a significant IBD effect ($R^2 = 0.0920$, $P = 0.21$) (Fig. S1).

Bayesian clustering analysis

Over eight loci, all 14 populations were most likely clustered into two groups, corresponding to the largest Δ$K$ value (Fig. S2). Populations S1, S2, C1, E1, E3, Z1, and Z2 were clustered into one group and the others into another group, but there was mismatching between populations.
and genetic groups for some individuals (Fig. 2). For example, some individuals of population C2 were classified into genetic groups S1, S2, C1, E1, E3, Z1, and Z2.

**cpDNA haplotypes and their distributions**

With a total of 1249 bp spliced, the aligned sequences of 3′trnL–trnF, trnH–psbA, and rpl20–5′rps12 from 14 populations of *F. hayatae* were 169, 343, and 737 bp, respectively, and a total of 22 variable sites, including 11 substitutions and 11 insertion/deletions, were detected in defining 15 haplotypes (Table S2). Haplotype H1 was fixed in the populations sampled in the Sichuan Basin, and the haplotypes fixed in the populations in Taiwan Island were more diverse. Additionally, a major specific haplotype was fixed with high frequency in most populations (Fig. 1A).

The statistical parsimony network with “OutGroup” (*F. lucida*) indicated not only many interior nodes but also many missing intermediate haplotypes (Fig. 1B). In the Bayesian phylogenetic tree, the “OutGroup” branch was no older than that of H1 or H7, whereas its low posterior probability was detected (Fig. 3). In the other haplotypes recovered, H6 in population E3, H14, and H15 in populations Z1 and Z2 were older than others (Fig. 3). In addition, several haplotypes fixed in populations in Taiwan (H8 in T1; H10 in T2; and H11, H12 and H13 in T3) were clustered in a clade with two sub-branches, while H4 and H5 were clustered into another clade, as shown by high posterior probabilities of Bayesian inference for these clades, which were also highly supported by the MP and NJ, as well as ML with two procedures (Fig. 3).

**Haplotype diversity and differentiation**

*Hd* and *π* values varied within 0–0.385 and 0–7.86 × 10⁻⁴, respectively, with the first and second highest values found in Taiwan populations (Table 2). The among-population values in the Sichuan Basin (*Hd* = 0.0342, *π* = 7.50 × 10⁻⁵) were lower than those outside (*Hd* = 0.834, *π* = 7.77 × 10⁻³); and values among all 14 populations (*Hd* = 0.714, *π* = 4.87 × 10⁻³) were higher than the mean (*Hd* = 0.0864, *π* = 1.45 × 10⁻⁴) (Table 2).

The results showed significantly high genetic differentiation among all 14 populations with *Gst* = 0.712. Very high genetic differentiation was also detected for populations outside the Sichuan Basin (*Gst* = 0.829) but little for those inside. The relative rate of pollen over seed flows (*r*) was 10.6 among the 14 populations.

Phylogeographic structures were detected among all 14 populations, and populations outside the Sichuan Basin, with correspondingly significantly higher *Nst* values (0.977 and 0.980, respectively) than *Gst* values (0.712 and 0.829, respectively) (*P < 0.001*). However, there was no significant difference between *Nst* (0.009) and *Gst* (0.005) among populations in the Sichuan Basin (*P > 0.1*).

**Test of neutrality and mismatch distribution**

The results of Tajima’s *D* and Fu’s *F*_ values found in Taiwan populations, while there were conflicting results between the two tests among populations within the Sichuan Basin (Table S3). However, all the mismatch distributions were roughly multimodal (Fig. S3), suggesting no signals of population expansion in the analysis.

**Discussion**

**Genetic diversity**

Based on SSR of nDNA, the average number of alleles (*A*) was 21.75 among the 14 *F. hayatae* populations (Table 1), which was much higher than for any other *Fagus* species studied, such as *F. longipetiolata* (*A* = 4.9, Liu 2008), *Fagus crenata* (*A* = 9.9, Tanaka et al. 1999), *Fagus japonica* (*A* = 8.64, Hiraoka and Tomaru 2009a), *Fagus sylvatica* (*A* = 10.75, Vornam et al. 2004), *Fagus orientalis* (*A* = 10.33, Shanjani et al. 2010), and *Fagus grandifolia* (*A* = 13.8, Koch et al. 2009). Our study was in accord with the results of Kung (2012), which also showed an average of 24.33 alleles for populations mainly from Taiwan.

By sequencing 1249-bp cpDNA fragments, we detected 15 haplotypes over 345 individuals in 14 *F. hayatae* populations. This was generally more than that detected in other *Fagus* species using the same analysis, such as...
F. lucida (14 haplotypes, 1918 bp, 276 individuals, and 21 populations, Zhang et al. 2013), F. longipetiolata (13 haplotypes, 1664 bp, 201 individuals, and 26 populations, Liu 2008; and 14 haplotypes, 1918 bp, 276 individuals, and 28 populations, Zhang et al. 2013), F. engleriana (six haplotypes, 2149 bp, 350 individuals, and 25 populations, Lei et al. 2012), F. crenata (13 haplotypes, 3229 bp, 109 individuals, and 45 populations, Fujii et al. 2002; and seven haplotypes, 1515 bp, 351 individuals, and 21 populations, Okaura and Harada 2002), and F. grandifolia (20 haplotypes, 1920 bp, 232 individuals, and 137 populations, Morris et al. 2010). Studies of F. sylvatica with chloroplast cleaved amplification polymorphism sequence-tagged sites detected 11 haplotypes from 399 individuals in 85 populations (Demesure et al. 1996) and 14 haplotypes from 335 individuals in 67 populations (Vettori et al. 2004). A similar study of F. orientalis with chloroplast microsatellites detected eight haplotypes from 562 individuals in 13 populations (Shanjani et al. 2010).

Ellegren (2009) suggested that high genetic diversity generally resulted from large populations and high mutation rates, while the latter may make more contribution. Considering the very sparsely scattered distributions of

**Table 2.** $H_d$ and $\pi$ based on cpDNA sequence fragments within and among populations.

| Population     | $H_d$   | $\pi$     |
|----------------|---------|-----------|
| In Sichuan Basin |         |           |
| S1             | 0.0690  | 0.000170  |
| S2             | 0       | 0         |
| C1             | 0.0667  | 0.000163  |
| C2             | 0.0667  | 0.000109  |
| C3             | 0       | 0         |
| Y1             | 0       | 0         |
| Mean           | 0.0337  | 0.0000737 |
| Among populations | 0.0342  | 0.0000750 |
| Outside Sichuan Basin |         |           |
| E1             | 0.142   | 0.000116  |
| E2             | 0.133   | 0.000108  |
| E3             | 0       | 0         |
| Z1             | 0       | 0         |
| Z2             | 0.143   | 0.000234  |
| T1             | 0.385   | 0.000786  |
| T2             | 0       | 0         |
| T3             | 0.205   | 0.000345  |
| Mean           | 0.126   | 0.000199  |
| Among populations | 0.834   | 0.00777   |
| Over 14 populations |         |           |
| Mean           | 0.0864  | 0.000145  |
| Among populations | 0.714   | 0.00487   |

Figure 3. Bayesian phylogenetic tree of 15 haplotypes with “OutGroup” (Fagus lucida). Values above branches corresponding to posterior probabilities (%) and only those over 50% are shown. Values below branches correspond to bootstrap supports (%) of the nodes resulting from ML with Akaike information criterion, ML with Bayesian information criterion, MP and NJ phylogenetic inferences, and only those over 50% are shown.
**F. hayatae** (Zhang et al. 2013; Shen et al. 2015) populations across the rather wide range, genetic mutation in the mutually isolated habitats might play a primary role in forming the relatively high genetic diversity of *F. hayatae*, compared with the *Fagus* species mentioned above. Zhang et al. (2013) suggested that generally low numbers of missing intermediate haplotypes in the statistical parsimony network indicated low extinction rates. In contrast, many missing intermediate haplotypes in this study (Fig. 1B) may also be related to the relatively high mutation rate of *F. hayatae*. Thus, the lower genetic diversity of populations in the Sichuan Basin compared to that outside (Table 2) indicated that the corresponding mutation rate in the Sichuan Basin was relatively low.

### Genetic structure

Differences between biparentally inherited nDNA SSR markers and maternally inherited cpDNA sequence fragments have been used to detect genetic differentiation among populations (Petit et al. 2005; Cormoran and Arnold 2007), and it is important to combine the two approaches to reveal genetic structure and the historical reasons underlying it (Hodgins and Barrett 2007; Bai et al. 2010).

Over eight SSR loci, our results indicated inbreeding in *F. hayatae* among the 14 populations, and significant FI with heterozygosity deficiency in most populations (Table 1). It is suggested that inbreeding in this wind-pollinated species is mainly due to habitat fragmentation with limited pollen flow (Knapp et al. 2001; Jump and Peñuelas 2006). Thus, with little influence of null alleles, significant FI and departures from the Hardy–Weinberg equilibrium among populations of *F. hayatae* at each locus (Table S1) must also result from isolation and fragmentation by mountains in subtropical China. It is noteworthy that *F. hayatae* had \( R_{st} = 0.233 \), comparable to other *Fagus* species distributed in China (e.g., *F. longipetiolata*, \( R_{st} = 0.232 \), Liu 2008; and *F. engleriana*, \( R_{st} = 0.263 \), Zhou and Fang 2012), but much higher than those *Fagus* species distributed in other regions (e.g., *F. japonica*, \( R_{st} = 0.025 \), Hiraoka and Tomaru 2009a; *F. crenata*, \( R_{st} = 0.041 \), Hiraoka and Tomaru 2009b; *F. sylvatica*, \( R_{st} = 0.054 \), Comps et al. 1990; *F. orientalis*, \( R_{st} = 0.048 \), Shanjani et al. 2010; and *F. grandiifolia*, \( R_{st} = 0.168 \), Kitamura and Kawano 2001) and that of the mean of 77 angiosperm species (\( R_{st} = 0.184 \), Petit et al. 2005).

Based on SSR analysis, the Mantel test showed no significant IBD effect among populations over eight loci (Fig. S1). Two clusters each with mixed populations in different locations and many mismatched individuals also indicated that genetic distance was not correlated with geographic distance (Fig. 2). It was that broadly and randomly occurring mutation of nDNA played an important role in shaping population genetic structure, as SSR genetic mutation could be of relatively great historical importance for the lack of IBD effect (Hutchison and Templeton 1999).

The chloroplast genetic differentiation of *F. hayatae* (\( G_{st} = 0.712 \)), as well as of other *Fagus* species (*F. lucida*, \( G_{st} = 0.705 \), Zhang et al. 2013; *F. longipetiolata*, \( G_{st} = 0.900 \) and 0.936, Liu 2008 and Zhang et al. 2013; *F. engleriana*, \( G_{st} = 0.831 \), Lei et al. 2012; *F. sylvatica*, \( G_{st} = 0.902 \), Demesure et al. 1996; and *F. orientalis*, \( G_{st} = 0.800 \), Shanjani et al. 2010), was also much higher than that of the mean for 124 angiosperm species (\( G_{st} = 0.637 \), Petit et al. 2005). The low seed flow mainly responsible for the high differentiation detected in other *Fagus* species (Tomaru et al. 1998; Kitamura and Kawano 2001; Rowden et al. 2004; Liu 2008; Lei et al. 2012; Zhang et al. 2013) may also be the case for *F. hayatae*. Therefore, the inefficient seed dispersal strategy with predation of vertebrates and habitat fragmentation may have jointly resulted in the genetic isolation among populations.

In addition, Petit et al. (2005) showed that the median of \( r \) is about 17 in seed plants, suggesting a general asymmetry between gene flows, as gene exchange through pollen is much more important than through seeds. However, the value of \( r = 10.6 \) in *F. hayatae* was much lower compared with other *Fagus* species (e.g., *F. longipetiolata*, \( r = 34.2 \), Liu 2008; *F. sylvatica*, \( r = 84 \), Demesure et al. 1996; and *F. orientalis*, \( r = 44 \), Shanjani et al. 2004), suggesting a stronger genetic isolation with also a limitation to pollen flow, possibly due to the more spatially isolated distribution compared to other *Fagus* species in China (Shen et al. 2015).

The results of tests between \( N_{st} \) and \( G_{st} \) showed that significant phylogeographic structure among all 14 populations was mainly from the contribution of high genetic differentiation among populations outside the Sichuan Basin. In contrast, within the Sichuan Basin, no significant phylogeographic structure (with \( G_{st} \) close to 0 and almost 100% variation within populations) suggested that these populations may have had a single origin and long existed within an enclosed environment, that is the Sichuan Basin.

### Demographic history and glacial refugia

The phylogenetic tree showed that H1 and H7 were older than the other haplotypes (Fig. 3). The fact that it is very common for beech species to share haplotypes (Zhang et al. 2013) indicates that H1 and H7 may be shared by *F. hayatae* and other *Fagus* species and that population T1 and populations in the Sichuan Basin were more
ancient elements of *F. hayatae*. Zhang et al. (2013) also suggested that Chinese beeches started to diversify on entering the Pliocene and that this diversification intensified during the Pleistocene, thus more haplotypes in the phylogenetic tree (Fig. 3) were young and branched during the Quaternary. The lack of evidence for population expansion, according to the tests of neutrality and mismatch distribution (Table S3, Fig. S3), suggested that *F. hayatae* may have experienced a long isolation with limited admixture and independent evolution among populations.

The genetic structure in *F. hayatae* could be due to its demographic dynamics during the Quaternary. Pollen records indicate that the ancestor of *F. hayatae* migrated to southern China possibly from continental northeast Asia during the Pliocene (Denk and Grimm 2009), suggesting that the species was probably widespread in north China during the Tertiary. Migration of *F. hayatae* southwards to mountainous areas of subtropical China may have occurred in the early Pleistocene when the climate became colder (Liu et al. 2003). Possible migration and admixture among populations of the ancestors of *F. hayatae* could also have occurred (Denk and Grimm 2009), especially for populations with close locations, such as E1 and E2 in Hubei with haplotypes H4 and H5 clustered in one clade in the phylogenetic tree (Fig. 3). After the admixture disappeared, outside the Sichuan Basin the populations evolved independently with high mutation rate (see section Genetic diversity). In the Sichuan Basin, however, relatively low mutation rate (see section Genetic diversity) and the decline in gene flow or exchange with outside populations allowed the single ancient haplotype H1 to spread widely among populations in this enclosed environment.

Thus, the existence of multiple refugia for *F. hayatae* in mountains of subtropical China during the glaciations was critical for the contemporary genetic spatial structure of the species. Mountains around the Sichuan Basin could be glacial refugia of *F. hayatae*, with the fixed haplotype H1 firstly derived from the ancestor. To-and-fro migrations in *F. hayatae* between the Sichuan Basin and surrounding mountains occurred during the glacial and interglacial periods. However, the range of its distribution and migration gradually reduced and was circumscribed to low-altitude areas close to mountains, and the species rarely entering lowlands during the Quaternary due to moisture limitation (Liu et al. 2003). This was also a critical factor that drove the upward migration in mountains surrounding the Sichuan Basin rather than northward expansion, facing climate warming after the last glacial period (Cao et al. 1995; Fang and Lechowicz 2006; Shen et al. 2015).

Mountains and hills in southeast China, including Taiwan Island, could also be glacial refugia where abundant haplotypes were fixed. Similar to the case in the Sichuan Basin, the distribution range of *F. hayatae* gradually reduced in each isolated mountain during the Pleistocene, consistent with the pollen evidence indicating that it was either more extensive or lower in altitude than it is today (Zheng and Li 2000). The isolated pattern was maintained or even enhanced by the monsoon climate in the post-glacial period (Fang and Lechowicz 2006; Guo and Wenger 2010; Shen et al. 2015). The fact that *F. hayatae* is now absent from the Dabie or Luoxiao Mountains located at the same latitude and between the sampled populations in Hubei and Zhejiang, respectively, is an unresolved issue, requiring further information from pollen and perhaps also field exploration in these two mountains.

Hewitt (2000) suggested that during the Quaternary, climate change and the varied topography in southern temperate regions and the tropics tended to isolate species into populations that evolved independently with only occasional gene flow, as confirmed for *F. hayatae* in subtropical China. Postglacial expansion has not been detected in any *Fagus* species studied in China (Liu 2008; Lei et al. 2012; Zhang et al. 2013), including *F. hayatae* in the present study, in contrast to the rapid northward range expansion for *Fagus* species in Japan (Okaura and Harada 2002; Hiraoka and Tomaru 2009b), Europe (Demesure et al. 1996; Magri et al. 2006), and North America (Kitamura and Kawano 2001). The potential environmental or ecological barriers may have acted as refugia for *Fagus* in the Quaternary Ice Ages.

**Conservation implications**

Our results could provide information useful for the conservation of not only *F. hayatae*, a species on the China Species Red List, but also other endemic (especially paleo-endemic) plant species assembled in the mountains of subtropical China. Ying (2001) localized three hot spots of plant diversity and endemism in China: the Hengduan Mountain Ranges, the Central China region, and the Southern Mountain Ranges. The populations of *F. hayatae* in Sichuan and Hubei were located in the Central China region and may be better protected than those in Zhejiang and Taiwan which are on the east border of the species range. The populations Z1 and Z2 in Zhejiang were small, with nearly only a single fixed haplotype, and so should be given more attention. Despite the high haplotype diversity of *F. hayatae* in Taiwan, isolation with inbreeding could leave populations at risk. Although a high genetic diversity remains among all populations of *F. hayatae*, genetic isolation with high genetic differentiation resulting from habitat fragmentation caused by natural and anthropogenic factors needs to be taken into account in conservation of this relict species.
Conclusions

Molecular evidence – nDNA SSR loci and cpDNA sequence fragments – showed that *F. hayatae*, the *Fagus* species endemic to China with the most dispersed distribution, had a demographic history with multiple refugia during glacial periods and an isolated population structure with high genetic differentiation, in which climate change during the Quaternary and genetic mutation may have played important roles. Despite some differences between the populations in and outside the Sichuan Basin, long-term genetic isolation among populations with habitat fragmentation was the main characteristic of *F. hayatae*.

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Conflict of Interest

None declared.

References

Asuka, Y., N. Tani, Y. Tsumura, and N. Tomaru. 2004. Development and characterization of microsatellite markers for *Fagus crenata* Blume. Mol. Ecol. Notes 4:101–103.

Bai, W., W. Liao, and D. Zhang. 2010. Nuclear and chloroplast DNA phylogeography reveal two refuge areas with asymmetrical gene flow in a temperate walnut tree from East Asia. New Phytol. 188:892–901.

Burban, C., R. J. Petit, E. Carcreff, and H. Jactel. 1999. Range-wide variation of the maritime pine best scale *Matsucoccus feytaudi* Duc. (Homoptera: Matsucoccidae) in relation to the genetic structure of its host. Mol. Ecol. 8:1593–1602.

Cao, K., R. Peters, and R. A. Oldeman. 1995. Climatic range and distribution of Chinese *Fagus* species. J. Veg. Sci. 6:317–324.

Chapuis, M. P., and A. Estoup. 2007. Microsatellite null alleles and estimation of 485 population differentiation. Mol. Biol. Evol. 24:621–631.

Chen, T.-Y., C.-F. Hsieh, J.-J. Mao, Y.-C. Lai, S.-T. Lin, J.-M. Hu, et al. 2011. Taiwan beech: a relict summer-green forest (Chinese edition). Forestry Bureau, Taipei, China.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9:1657–1659.

Comps, B., B. Thièbaut, L. Paule, D. Merzeau, and J. Letouzey. 1990. Allozymic variability in beechwoods (*Fagus sylvatica* L.) over central Europe: spatial differentiation among and within populations. Heredity 65:407–417.

Cornman, R. S., and M. L. Arnold. 2007. Phylogeography of *Iris missouriensis* (Iridaceae) based on nuclear and chloroplast markers. Mol. Ecol. 16:4585–4598.

Dakin, E. E., and J. C. Avise. 2004. Microsatellite null alleles in parentage analysis. Heredity 93:504–509.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9:772–772.

Demesure, B., B. Comps, and R. J. Petit. 1996. Chloroplast DNA phylogeography of the common beech (*Fagus sylvatica* L.) in Europe. Evolution 50:2515–2519.

Denk, T. 2003. Phylogeny of *Fagus* L. (Fagaceae) based on morphological data. Plant Syst. Evol. 240:55–81.

Denk, T., and G. W. Grimm. 2009. The biogeographic history of beech trees. Rev. Palaeobot. Palynol. 158:83–100.

Denk, T., G. W. Grimm, and V. Hemleben. 2005. Patterns of molecular and morphological differentiation in *Fagus* (Fagaceae): phylogenetic implications. Am. J. Bot. 92:1006–1016.

Doyle, J. J. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11–15.

El Mousadik, A., and R. J. Petit. 1996. Chloroplast DNA phylogeography of the argan tree of Morocco. Mol. Ecol. 5:547–555.

Ellegren, H. 2009. Is genetic diversity really higher in large populations? J. Biol. 8:41.

Ennos, R. A. 1994. Estimating the relative rates of pollen and seed migration among plant populations. Heredity 72:250–259.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14:2611–2620.

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol. Bioinf. Online 1:47–50.

Faegri, K., and L. van der Pijl. 1979. The principles of pollination ecology. Pergamon Press, New York, USA.

Fang, J., and M. J. Lechowicz. 2006. Climatic limits for the range and distribution of Chinese *Fagus species*. J. Veg. Sci. 17:155–160.

Forcier, L. K. 1975. Reproductive strategies and the co-occurrence of climax tree species. Science 189:808–810.

Fu, Y. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925.
The phylogeography of *Fagus hayatae*

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Fujii, N., N. Tomaru, K. Okuyama, T. Koike, T. Mikami, and K. Ueda. 2002. Chloroplast DNA phylogeography of *Fagus crenata* (Fagaceae) in Japan. Plant Syst. Evol. 232:21–33.

Goudet, J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. J. Hered. 86:485–486.

Guo, S. W., and E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361–372.

Guo, K., and M. J. A. Werger. 2010. Effect of prevailing monsoons on the distribution of beeches in continental East Asia. For. Ecol. Manage. 259:2197–2203.

Hamilton, M. B. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol. Ecol. 8:521–523.

Hewitt, G. 2000. The genetic legacy of the Quaternary ice ages. Nature 405:907–913.

Hiraoka, K., and N. Tomaru. 2009a. Population genetic structure of *Fagus japonica* revealed by nuclear microsatellite markers. Int. J. Plant Sci. 170:748–758.

Hiraoka, K., and N. Tomaru. 2009b. Genetic divergence in nuclear genomes between populations of *Fagus crenata* along the Japan Sea and Pacific sides of Japan. J. Plant. Res. 122:269–282.

Hodgins, K. A., and S. C. H. Barrett. 2007. Population structure and genetic diversity in tristylous *Narcissus triandrus*: insights from microsatellite and chloroplast DNA variation. Mol. Ecol. 16:2317–2332.

Hsieh, C. 1989. Structure and floristic composition of the beech forest in Taiwan. Taiwania 34:28–44.

Hutchison, D. W., and A. R. Templeton. 1999. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 53:1898–1914.

Ida, H., M. Hotta, and Y. Ezaki. 2004. Predispersal predation by rodents to beechnuts (*Fagus crenata* Blume). Ecol. Res. 19:503–509.

Ji, C.-J., H.-H. Shen, J.-Y. Fang, C.-F. Xie, and Y.-J. Fan. 2002. RAPD analysis of *Fagus* species in China. Beijing da xue xue bao. Zi ran ke xue bao 38:817–822.

Ju, L., H. Shih, and Y. Chiang. 2012. Microsatellite primers for the endangered beech tree, *Fagus hayatae* (Fagaceae). Am. J. Bot. 99:e453–e456.

Jump, A. S., and J. Peñuelas. 2006. Genetic effects of chronic habitat fragmentation in a wind-pollinated tree. PNAS 103:8096–8100.

Kato, S., T. Koike, T. T. Lei, C.-F. Hsieh, K. Ueda, and T. Mikami. 2000. Analysis of mitochondrial DNA of an endangered beech species, *Fagus hayatae* Palibin ex Hayata. New Forest. 19:109–114.

Kitamura, K., and S. Kawano. 2001. Regional differentiation in genetic components for the American beech, *Fagus grandifolia* Ehrh., in relation to geological history and mode of reproduction. J. Plant. Res. 114:353–368.

Knapp, E. E., M. A. Goedde, and K. J. Rice. 2001. Pollen-limited reproduction in blue oak: implications for wind pollination in fragmented populations. Oecologia 128:48–55.

Kobashi, S., N. Fujii, A. Nojima, and N. Horii. 2006. Distribution of chloroplast DNA haplotypes in the contact zone of *Fagus crenata* in the southwest of Kanto District, Japan. J. Plant. Res. 119:265–269.

Koch, J. L., D. W. Carey, and M. E. Mason. 2009. Use of microsatellite markers in an American beech (*Fagus grandifolia*) population and paternity testing. Silvae Genet. 9:62–68.

Kung, H.-H. 2012. Population genetic structure of *Fagus hayatae* Palib. based on chloroplast and nuclear microsatellite markers. [Master Thesis] (Chinese Edition), National Taiwan University, Taipei, China.

Lei, M., Q. Wang, Z. Wu, J. López-Pujol, D. Li, and Z. Zhang. 2012. Molecular phylogeography of *Fagus engeliana* (Fagaceae) in subtropical China: limited admixture among multiple refugia. Tree Genet. Genomes 8:1203–1212.

Li, T.-F., and J.-Q. Li. 2008. The origination, distribution, regeneration and genetic diversity of Chinese beech. Chin. Agric. Sci. Bull. 24:185–191.

Liu, M.-H. 2008. Phylogeography of *Fagus longipetiolata*: insights from nuclear DNA microsatellites and chloroplast DNA variation. [Ph.D. Thesis] (Chinese Edition), East China Normal University, Shanghai, China.

Liu, H., Q. Xing, Z. Ji, L. Xu, and Y. Tian. 2003. An outline of Quaternary development of *Fagus* forest in China: palynological and ecological perspectives. Flora 198: 249–259.

Magri, D., G. G. Vendramin, B. Comps, I. Dupanloup, T. Geburek, D. Gömöry, et al. 2006. A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. New Phytol. 171:199–221.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209–220.

Michalakis, Y., and L. Excoffier. 1996. A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics 142:1061–1064.

Morris, A. B., C. H. Graham, D. E. Soltis, and P. S. Soltis. 2010. reassessment of phylogeographical structure in an eastern North American tree using Monmonier’s algorithm and ecological niche modelling. J. Biogeogr. 37:1657–1667.

Nei, M. 1977. F-statistics and analysis of gene diversity in subdivided populations. Ann. Hum. Genet. 41:225–233.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590.

Nei, M., and W. H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. PNAS 76:5269–5273.
Okaura, T., and K. Harada. 2002. Phylogeographical structure revealed by chloroplast DNA variation in Japanese beech (Fagus crenata Blume). Heredity 88:322–329.
Peters, R. 1997. Beech forests. Kluwer Academic Publishers, Dordrecht, the Netherlands.
Pettit, R. J., J. Duminiil, S. Fineschi, A. Hampe, D. Salvinii, and G. G. Vendramini. 2005. Invited review: comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Mol. Ecol. 14:689–701.
Pons, O., and R. J. Petit. 1996. Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144:1237–1245.
Posada, D. 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25:1253–1256.
Pritchard, J. K., W. Wen, and D. Falush. 2009. Documentation for STRUCTURE software: version 2. Available at http://computing.bio.cam.ac.uk/local/doc/structure.pdf.
Rogers, A. R., and H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9:552–569.
Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, et al. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61:539–542.
Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. Genetics 145:1219–1228.
Rousset, F. 2008. Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Resour. 8:103–106.
Rowden, A., A. Robertson, T. Allnutt, S. Heredia, G. Williams-Linera, and A. C. Newton. 2004. Conservation genetics of Mexican beech, Fagus grandifolia var. mexicana. Conserv. Genet. 5:475–484.
Schneider, S., and L. Excoffier. 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. Genetics 152:1079–1089.
Shanjani, P. S., C. Vettori, R. Giannini, and R. A. Khavari-Nejad. 2004. Intraspecific variation and geographic patterns of Fagus orientalis Lipsky chloroplast DNA. Silvae Genet. 53:5–6.
Shanjani, P. S., G. G. Vendramin, and M. Calagari. 2010. Genetic diversity and differentiation of Fagus orientalis Lipsky in Hycrangan forests revealed by nuclear and chloroplast microsatellite markers. Conserv. Genet. 11:2321–2331.
Shen, C. 1992. A monograph of the genus Fagus Tourn. ex L. (Fagaceae). [Ph.D. Thesis], City University of New York, New York.
Shen, Z., J. Fang, C. Chiu, and T. Chen. 2015. The geographical distribution and differentiation of Chinese beech forests and the association with Quercus. Appl. Veg. Sci. 18:23–33.
Slatkin, M., and R. R. Hudson. 1991. Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. Genetics 129:555–562.
Smouse, P. E., J. C. Long, and R. R. Sokal. 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Syst. Zool. 35:627–632.
Soulsbury, C. D., G. Iossa, K. J. Edwards, P. J. Baker, and S. Harris. 2007. Allelic dropout from a high-quality DNA source. Conserv. Genet. 8:733–738.
Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, MA.
Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17:1105–1109.
Tajima, F. 1983. Evolutionary relationship of DNA sequences and the closely related species, F. japonica. Theor. Appl. Genet. 99:11–15.
Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.
Tajima, F. 1993. Measurement of DNA polymorphism. Pp. 37–59 in N. Takahata and A. G. Clark, eds. Mechanisms of Molecular Evolution. Sinaur Associates, Sunderland, MA.
Tanaka, K., Y. Tsumura, and T. Nakamura. 1999. Development and polymorphism of microsatellite markers for Fagus crenata and the closely related species, F. japonica. Theor. Appl. Genet. 99:11–15.
Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876–4882.
Tomaru, N., M. Takahashi, Y. Tsumura, M. Takahashi, and K. Ohba. 1998. Intraspecific variation and phylogeographic patterns of Fagus crenata (Fagaceae) mitochondrial DNA. Am. J. Bot. 85:629–629.
Vettori, C., G. G. Vendramin, M. Anzidei, R. Pastorelli, D. Paffetti, and R. Giannini. 2004. Geographic distribution of chloroplast variation in Italian populations of beech (Fagus sylvatica L.). Theor. Appl. Genet. 109:1–9.
Vornam, B., N. Decarli, and O. Gailing. 2004. Spatial distribution of genetic variation in a natural beech stand (Fagus sylvatica L.) based on microsatellite markers. Conserv. Genet. 5:561–570.
The phylogeography of *Fagus hayatae* L.-X. Ying et al.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38: 1358–1370.
Ying, J.-S. 2001. Species diversity and distribution pattern of seed plants in China. Biodivers. Sci. 9:393–398.
Zhang, Z.-Y., R. Wu, Q. Wang, Z.-R. Zhang, J. López-Pujol, D.-M. Fan, et al. 2013. Comparative phylogeography of two sympatric beeches in subtropical China: species-specific geographic mosaic of lineages. Ecol. Evol. 3:4461–4472.
Zheng, Z., and Q. Li. 2000. Vegetation, climate, and sea level in the past 55,000 years, Hanjiang Delta, Southeastern China. Quatern. Res. 53:330–340.
Zhou, Z.-G., and Y.-M. Fang. 2012. Study on genetic diversity of *Fagus engleriana*. J. Southwest Forestry Univ. 32:1–5.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Genetic diversity, Fi, P-values of Hardy–Weinberg equilibrium tests and null allele frequency at each SSR locus among populations of *F. hayatae*.
**Table S2.** Variable sites of 1249-bp spliced cpDNA sequence fragments in 15 haplotypes in 14 populations of *F. hayatae*.
**Table S3.** Results of neutrality tests.
**Figure S1.** Mantel tests based on nDNA SSR loci between genetic differentiation (\(F_{st}\)) and geographical distance (\(Dist\)).
**Figure S2.** Relationship of \(\Delta K\) with the number of clusters (\(K\)).
**Figure S3.** Mismatch distributions for populations (a) inside and (b) outside the Sichuan Basin, and (c) over the 14 populations, with black lines representing the distributions expected for expanding populations and red lines representing the observed mismatch distance.