

1 Allopatric divergence, local adaptation, and multiple 2 Quaternary refugia in a long-lived tree (*Quercus spinosa*) from 3 subtropical China

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5 Li Feng¹, Yan-Ping Zhang¹, Xiao-Dan Chen¹, Jia Yang¹, Tao Zhou¹, Guo-Qing Bai^{1,2}, Jiao
6 Yang¹, Zhong-Hu Li¹, Ching-I Peng³ and Gui-Fang Zhao^{1*}

7
8 ¹Key Laboratory of Resource Biology and Biotechnology in Western China (Ministry of
9 Education), College of Life Sciences, Northwest University, Xi'an 710069, China;

10 ²Xi'an Botanical Garden, Xi'an 710061, China;

11 ³Biodiversity Research Center, Academia Sinica, Taipei Nangang 115, Taiwan

12
13 *Author for correspondence: E-mail: gfhao@nwwu.edu.cn Tel: +86-29-88305264

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25 Summary

- 26 ● The complex geography and climatic changes occurring in subtropical China
27 during the Tertiary and Quaternary might have provided substantial opportunities
28 for allopatric speciation. To gain further insight into these processes, we
29 reconstruct the evolutionary history of *Quercus spinosa*, a common evergreen tree
30 species mainly distributed in this area.
- 31 ● Forty-six populations were genotyped using four chloroplast DNA regions and 12
32 nuclear microsatellite loci to assess genetic structure and diversity, which was
33 supplemented by divergence time and diversification rate analyses, environmental
34 factor analysis, and ecological niche modeling of the species distributions in the
35 past and at present.
- 36 ● The genetic data consistently identified two lineages: the western Eastern
37 Himalaya-Hengduan Mountains lineage and the eastern Central-Eastern China
38 lineage, mostly maintained by populations' environmental adaptation. These
39 lineages diverged through climate/orogeny-induced vicariance during the
40 Neogene and remained separated thereafter. Genetic data strongly supported the
41 multiple refugia (per se, interglacial refugia) or refugia within refugia hypotheses
42 to explain *Q. spinosa* phylogeography in subtropical China.
- 43 ● *Q. spinosa* population structure highlighted the importance of complex geography
44 and climatic changes occurring in subtropical China during the Neogene in
45 providing substantial opportunities for allopatric divergence.

46

47 Keywords: allopatric divergence, environmental adaptation, evolutionary history,
48 multiple refugia, *Quercus spinosa*, subtropical China.

49 Introduction

50 Historical processes such as geographic and climatic changes have profoundly shaped
 51 the population genetic structure and demographic history of extant species (Hewitt,
 52 2000, 2004). Climatic changes and heterogeneous environments could also provide
 53 opportunities for genetic divergence and diversification through adaptation to local or
 54 regional environments (Rainey & Travisano, 1998). When populations are adapted to
 55 dissimilar habitats, gene flow among them could be limited by selection and this
 56 might indirectly influence the whole genome, promoting neutral divergence through
 57 increased genetic drift (Wright, 1931; Nosil *et al.*, 2005).

58 Numerous studies have considered the effects of climatic changes since the
 59 Tertiary (e.g., Li *et al.*, 2013; Liu *et al.*, 2013; Wang *et al.*, 2015), but only a few have
 60 disentangled the roles of isolation by environment (IBE) and isolation by distance
 61 (IBD) in the population genetic divergence of temperate species (Mayol *et al.*, 2015;
 62 Zhang *et al.*, 2016). However, lack of exploring the roles of geographic and
 63 environmental forces in driving genetic structure and for inferring species' past
 64 demography, may hinder the accurate and precise inference of the distinct roles of
 65 IBD and IBE in population genetic structure and in species' demographic scenarios
 66 (Mayol *et al.*, 2015). Until recently, multiple matrix regression with randomization
 67 (MMRR) provided a robust framework, allowing powerful inferences of the different
 68 effects of IBD and IBE (Wang, 2013), and approximate Bayesian computation (ABC),
 69 allowing us to evaluate the most plausible demographic scenario and estimating the
 70 divergence and/or admixture time of the inferred demographic processes with a
 71 relatively low computation effort (Beaumont, 2010).

72 Wu & Wu (1998) suggested most of the Chinese flora could be divided into three
 73 subkingdoms (Sino-Japanese Forest, Sino-Himalayan Forest, and Qinghai-Xizang
 74 Plateau), all of are which included in subtropical China (21–34° N in South China).
 75 This region has a mild monsoon climate, complex topography, and high species
 76 diversity (Myers *et al.*, 2000; Qian & Ricklefs, 2000). Several hypotheses have been
 77 proposed to explain high species diversity here, among which the best known are
 78 those of Qian & Ricklefs (2000) and Harrison *et al.* (2001). Qian & Ricklefs (2000)

suggested that the numerous episodes of evolutionary radiation of temperate forests, through allopatric divergence and speciation driven by mid-to-late Neogene and Quaternary environmental changes, promoted species diversity; species would have spread to lower elevations and formed a continuous band of vegetation during glacial periods and retreated to “interglacial refugia” at higher elevations during warmer periods. However, according to palaeovegetation reconstructions based on fossil and pollen data, Harrison *et al.* (2001) suggested that temperate forests were considerably less extensive than today and would have retreated southward to *ca.* 30° N during the Last Glacial Maximum (LGM), being replaced by non-forest biomes or by boreal and temperate-boreal forests.

In general, phylogeographic studies in subtropical China have reinforced the allopatric speciation hypothesis for species diversity, indicating a general pattern of multiple refugia and little admixture among refugial populations throughout glacial-interglacial cycles (Qiu *et al.*, 2011; Liu *et al.*, 2012). However, most of these studies focused on endangered species or temperate deciduous species with limited distribution, and only a few (Shi *et al.*, 2014; Xu *et al.*, 2014; Wang *et al.*, 2015) considered temperate evergreen species. Thus, further investigations are necessary to verify if this pattern of multiple refugia and allopatric speciation are applicable to the typical and dominant evergreen species inhabiting the temperate zone.

Quercus spinosa David ex Franch, belonging to the group *Ilex* (syn. *Quercus* subgenus *Heterobalanus*) within family Fagaceae, is a long-lived, slow-growing tree inhabiting East Asian temperate evergreen forests. This tree species could offer additional advantages to investigate the impacts of IBE and IBD compared to short-lived trees and buffer the effects of changes in population genetic structure due to its life history traits (e.g., longevity, overlapping generations, prolonged juvenile phase) (Austerlitz *et al.*, 2000). Its distribution range extends from eastern Himalaya to Taiwan, and is commonly found in subtropical China (Fig. 1a), growing on slopes and cliffs, in low- to mid-elevation (900–3800 m above sea level) (Wu *et al.*, 1999; Menitskii & Fedorov, 2005). Based on recent molecular phylogenetic evidence (Denk & Grimm, 2009, 2010; Hubert *et al.*, 2014; Simeone *et al.*, 2016), the Group *Ilex* (*c.*

109 30 spp.) is monophyletic and diversified rapidly during the late Oligocene/early
 110 Miocene, suggesting *Q. spinosa* originated during the Miocene. In addition, recent
 111 studies suggested a significant role for environmental adaptation in the origin and
 112 maintenance of genetic divergence among forest lineages (e.g., Mayol *et al.*, 2015;
 113 Ortego *et al.*, 2015; Sexton *et al.*, 2016) and Petit *et al.* (2013) suggested Fagaceae as
 114 ideal models for integrating ecology and evolution. Hence, this species provides an
 115 ideal model for investigating the intraspecific divergence and evolutionary dynamics
 116 of an evergreen forest species in subtropical China subjected to geologic and climatic
 117 changes since the Tertiary.

118 In this study, we employed an integrative approach to determine the evolutionary
 119 history and genetic divergence of *Q. spinosa* and its response to climatic changes. The
 120 specific aims were to (i) characterize the range-wide phylogeographical patterns and
 121 genetic structure; (ii) determine the divergence times of intraspecific lineages and any
 122 underlying environmental and geographical causes; (iii) evaluate how climatic and
 123 geographical variation impact the genetic structure and divergence of *Q. spinosa*; and
 124 (iiii) reveal whether multiple refugia existed for *Q. spinosa*. We believe that
 125 knowledge of the population structure and evolutionary history of the evergreen oak
 126 species would be important to understand the complicated evolutionary history of
 127 species in subtropical China.

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129

130 **Materials and methods**

131

132 **Sampling and genotyping**

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134 Leaf samples were collected from 776 adult belonging to 46 natural populations of
 135 *Quercus spinosa*, covering most of its distribution range in China. All sampled
 136 individuals distanced at least 100 m from each other, and sample size varied from four
 137 to 20, depending on population size (**Supporting Information Table S1**). After DNA
 138 extraction, 12 nSSR loci and four cpDNA fragments were amplified (see details in
 139 **Note S1**).

140

141 **DNA sequence analysis**

142 **Genetic diversity, phylogenetic analyses, and divergence time estimation**

143

144 Relationships among the haplotypes obtained for *Q. spinosa* were evaluated using
 145 NETWORK v4.6 (Bandelt *et al.*, 1999). Haplotype (H_e) and nucleotide (π) diversities,
 146 and Tajima's D (Tajima, 1989) and Fu's (F_s) (Fu & Li, 1993) neutrality tests to assess
 147 possible expansions and their associated significance values were calculated in
 148 DNASP v5.00.04 (Librado & Rozas, 2009), at the population, region, and species
 149 levels. In addition, for specified clades (see results section), the average gene diversity
 150 within populations (H_S), total gene diversity (H_T), and the differentiation of unordered
 151 (G_{ST}) and ordered (N_{ST}) alleles based on 1,000 random permutations were estimated
 152 in PERMUT v1.2.1 (Pons & Petit, 1996).

153 Congruence among sequences of different fragments was examined with the
 154 partition homogeneity test (Farris *et al.*, 1995) as implemented in PAUP* v4.0b10
 155 (Swofford, 2003). The HKY + G nucleotide substitution model, which was
 156 determined in JMODELTEST v1.0 (Posada, 2008), and an uncorrelated lognormal
 157 relaxed clock (Drummond *et al.*, 2005) were used to estimate the phylogenetic
 158 relationships and divergence times between lineages according to the Bayesian
 159 inference methods implemented in BEAST v1.7.5 (Drummond *et al.*, 2012). *Castanea*

160 *mollissima* and *Trigonobalanus doichangensis* were used as outgroups, and a Yule
161 process tree prior was specified. Based on fossil evidence, we set the divergence time
162 between *T. doichangensis* and other two species (F1 in Fig. 2) at 44.8 million years
163 ago (Ma) (\pm SD = 3.0 Ma), providing a 95% confidence interval (CI) of 37.2–52.3 Ma.
164 The divergence time between *C. mollissima* and *Q. spinosa* (F2 in Fig. 2) was set 28.4
165 Ma (\pm SD = 2.2 Ma), providing a 95% CI of 23.0–33.9 Ma. The detailed calibration
166 for each point is described in Sauquet *et al.* (2012). Three independent runs of 5×10^7
167 Markov chain Monte Carlo (MCMC) steps were carried out, sampling at every 5,000
168 generations, following a burn-in of the initial 10% cycles. To confirm sampling
169 adequacy and convergence of the chains to a stationary distribution, MCMC samples
170 were inspected in TRACER v1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>). Trees were
171 visualized using FIGTREE v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

172 Furthermore, haplotype phylogenetic relationships were inferred using maximum
173 likelihood (ML), treating gaps (indels) as missing data. The ML analysis based on the
174 HKY + G substitution model, as selected by JMODELTEST, was performed on
175 RAXML v7.2.8 (Stamatakis *et al.*, 2008). Node support was assessed using 1,000
176 ‘fast bootstrap’ replicates.

177

178 **Demographic history and diversification analysis**

179

180 The demographic patterns of all *Q. spinosa* populations and of each of the two groups
181 identified in BEAST and in STRUCTURE v2.3.3 (Pritchard *et al.*, 2000) analyses
182 (see results section) were examined through mismatch distribution analysis (MDA) in
183 ARLEQUIN v3.5 (Excoffier & Lischer, 2010). For clades identified (see results
184 section), we also tested the null hypothesis of spatial expansion using mismatch
185 distribution analysis (MDA) in ARLEQUIN (see details and results in Note S2).
186 Population demographic history was also evaluated by estimating the changes in the
187 effective population size over time using a Bayesian skyline plot (BSP, Drummond *et al.*, 2005), as implemented in BEAST, and selecting the piecewise-linear model for
188 tree priors. This approach incorporates uncertainty in the genealogy as it uses MCMC
189

190 integration under a coalescent model. Chains were run for 100 million generations,
191 sampling at every 10,000 generations, and their convergence and output were checked
192 and analyzed in TRACER.

193 The temporal dynamics of *Q. spinosa* diversification were measured using
194 lineages through time (LTT) plots in the APE package (Paradis *et al.*, 2004) of R v3.3.
195 0 (<https://www.r-project.org>). Plots were produced based on 100 random trees that
196 resulted from BEAST analysis. In addition, BAMM v2.2.0 (Rabosky, 2014) was used
197 to explore the diversification rate heterogeneity between different *Q. spinosa* groups
198 (see results section). Analysis run for 1×10^7 generations, sampling every 5,000
199 generations, and convergence was tested using the CODA package (Plummer *et al.*,
200 2006) in R, the first 10% as burn-in. Effective sample sizes were above 1,000 for all
201 estimated parameters. The results were used to calculate diversification rates with the
202 R package BAMMTOOLS v2.0.2 (Rabosky *et al.*, 2014).

203 In order to quantify genetic variations among populations and genetic clusters (as
204 identified by STRUCTURE, NETWORK, and BEAST analyses, see below), we
205 performed analyses of molecular variance (AMOVA) in ARLEQUIN using the Φ - and
206 *R*-statistics, respectively. The significance of fixation indices was tested using 10,000
207 permutations (Excoffier *et al.*, 1992).

208

209 **Microsatellite data analysis**

210

211 **Population genetic analysis**

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213 We used MICROCHECKER v2.2.3 (Van Oosterhout *et al.*, 2004) to test the presence
214 of null alleles in all loci. POPGENE v1.31 (Yeh *et al.*, 1999) was used to estimate the
215 total number of alleles (A_0), observed heterozygosity (H_0), expected heterozygosity
216 over all populations (H_E), gene diversity within populations (H_S), and total genetic
217 diversity (H_T). Linkage disequilibrium (LD) and departure from Hardy-Weinberg
218 equilibrium (HWE) were evaluated using FSTAT v2.9.3 (Goudet, 2001). Significance
219 levels were corrected by the sequential Bonferroni method (Rice, 1989).

Genetic differentiation among populations was evaluated using θ (F_{ST}) (Weir & Cockerham, 1984) and G'_{ST} (Hedrick, 2005) across loci in SMOGD (Crawford, 2010). Compared to traditional measures, G'_{ST} is a more suitable measure for highly polymorphic markers such as microsatellites.

Genetic groups were inferred using the Bayesian clustering approach implemented in STRUCTURE, based on the admixture model with independent allele frequencies. Two alternative methods were utilized to estimate the most likely number of genetic clusters (K) in STRUCTURE HARVESTER (Earl & vonHoldt, 2012), i.e., by tracing changes in the average of log-likelihood ($L(K)$, Pritchard *et al.*, 2000) and by calculating delta K (ΔK , Evanno *et al.*, 2005). Twenty independent simulations ($1 \leq K \leq 20$) with 5.0×10^5 burn-in steps followed by 5.0×10^5 MCMC steps were run. These long burn-in and run lengths, along with the large number of replicates, ensured the reproducibility of the STRUCTURE results (Gilbert *et al.*, 2012). These parameters were used in the STRUCTURE analysis conducted for each *Q. spinosa* group, except for $1 \leq K \leq 18$ in the EH-HM group (see results section). The estimated admixture coefficients (Q matrix) over the 20 runs were averaged using CLUMPP v1.1 (Jakobsson & Rosenberg, 2007). Graphics were produced using DISTRUCT v1.1 (Rosenberg, 2004).

As Meirmans (2012) noted, tests of isolation by distance (IBD) may be strongly biased by the hierarchical population structure as revealed in STRUCTURE analysis. A false positive relationship between genetic distance and geographical distance among populations might be produced when population structure is not properly accounted for. In order to avoid this problem, we used a stratified Mantel test in which the locations of the populations within each putative cluster identified by STRUCTURE were permuted: 10,000 random permutations were performed between the matrix of pairwise genetic distances calculated as $F_{ST}/(1 - F_{ST})$, and that of geographic distances, using the package Vegan (Oksanen *et al.*, 2013) in R.

To estimate historical and contemporary gene flow between the clusters revealed in STRUCTURE (EH-HM and CEC clusters) and between the sub-clusters within each of this clusters, we used the programs MIGRATE-N v3.6 (Beerli, 2006) and

250 BAYESASS v3.0 (Wilson & Rannala, 2003), respectively (see details and results in
251 Note S2).

252 To predict putative barriers to gene flow, we used BARRIER v2.2 (Manni *et al.*,
253 2004) to find the limits associated with the highest rate of genetic change according to
254 Monmonier's maximum difference algorithm. We obtained 1,000 Nei's genetic
255 distance matrices from microsatellite data using MICROSATELLITE ANALYZER
256 v4.05 (Dieringer & Schlötterer, 2003). In addition, the program 2MOD v0.2 (Ciofi *et*
257 *al.*, 1999) was used to evaluate the relative likelihood of migration-drift equilibrium,
258 i.e., the relative contribution of gene flow vs. genetic drift to the current population
259 structure. After 100,000 iterations and discarding the first 10% as burn-in, the Bayes
260 factor was obtained as P (gene flow) / P (drift).

261

262 Tests of dynamic history by ABC modeling

263

264 Based on the STRUCTURE results for the EH-HM and the CEC clusters, five
265 lineages were identified (see Supporting Information Fig. S1): pop1, pop2, and pop3
266 within the CEC cluster and pop4 and pop5 within the EH-HM cluster (see results
267 section). As 120 different scenarios can be tested for five populations, we narrowed
268 this number by defining nested subsets of competing scenarios that were analyzed
269 sequentially (Table S9).

270 Firstly, the relationships among the three CEC populations (i.e., pop1, pop2, and
271 pop3) were investigated. Ten possible scenarios were tested (Step 1 in Fig. 5) and the
272 most plausible scenario (i.e., scenario 2) was chosen, then to the five populations'
273 analysis. In total, 15 alternative scenarios of population history were summarized for
274 the lineages and tested using the ABC procedure (Beaumont *et al.*, 2002) in DIYABC
275 v2.0.3 (Cornuet *et al.*, 2008; 2014).

276 In all ABC-related analyses, uniform priors were assumed for all parameters
277 (Table S10) and a goodness-of-fit test was used to check the priors of all parameters
278 before implementing the simulation. Following Cavender-Bares *et al.* (2011), we
279 assumed an average generation time of 150 years for *Q. spinosa*. To select the model

that best explains the evolutionary history of this species, 10 and 5 million simulations were run for all scenarios in steps 1 and 2 of the ABC analyses, respectively. The 1% simulated data closest to the observed data was used to estimate the relative posterior probabilities of each scenario via a logistic regression approach and parameters' posterior distributions based on the most likely scenario (Cornuet *et al.*, 2008, 2014). Each simulation was summarized by the following statistics: mean number of alleles and mean genic diversity for each lineage, F_{ST} , mean classification index, and shared allele distance between pairs of lineages.

288

289 **Ecological niche modeling**

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To assess the distribution shift of *Q. spinosa* during different periods, the potential habitats present at the last interglacial (LIG, 120,000 – 140,000 years BP), the LGM (21,000 years BP), mid-Holocene (MH, 6,000 BP), and under the current climate conditions, were estimated using the maximum entropy approach (MAXENT, Elith *et al.*, 2006; Phillips & Dudík, 2008) and a genetic algorithm for rule set production (GARP, Anderson *et al.*, 2003) (see details in **Note S2**).

297

298 **Dissimilar climate conditions and the impact of environmental factors on genetic structure (isolation by environment)**

299

In order to evaluate differences in present climatic conditions between EH-HM and CEC, 20 bioclimatic variables (i.e., altitude plus the 19 environmental variables) were obtained from the 46 sampling points. Also, in order to determine the contribution of present environmental conditions to the genetic structure of *Q. spinosa*, we tested the pairwise relationships between F_{ST} and climatic distances while controlling for the geographic distance among the 46 populations, using partial Mantel tests ('mantel.partial' function, R Core Team, 2015) and multiple matrix regressions (MMRR script in R; Wang, 2013). Significance was tested based on 10,000 permutations (see details in **Note S2**).

310 Results

311

312 CpDNA diversity and population structure

313

314 Analysis of the multiple alignment of the four cpDNA regions surveyed across 397 *Q.*
315 *spinosa* individuals from 46 populations (2,091 bp total length) revealed that 82 sites
316 were variable, corresponding to 72 substitutions and 10 indels (Supporting
317 Information **Tables S3-S6**). These polymorphisms defined 37 haplotypes (C1-C37),
318 with most of the 46 surveyed populations showing a single haplotype (**Fig. 1a, Table**
319 **S1**). At the species level, the cpDNA data revealed high haplotype diversity ($H_T =$
320 0.978) and nucleotide diversity ($\pi = 0.00538$) (**Table S7**). The parsimony network (**Fig.**
321 **1b**) grouped the 37 cpDNA haplotypes into two major clades (EH-HM and CEC)
322 separated by seven mutational steps, and suggested that C9, C14, and C25 could be
323 the ancestral haplotypes of *Q. spinosa*.

324 The nonhierarchical AMOVA revealed a strong population structure at the species
325 level ($\Phi_{ST} = 0.90$, $P < 0.001$). The hierarchical AMOVA revealed that 42.35% of the
326 genetic variation was partitioned among groups (EH-HM and CEC), 49.70% among
327 populations within groups, and 7.95% within populations (**Table 1**). There were no
328 significant phylogeographic structures at the species and region levels.

329

330 Molecular dating, diversification rate and demography based on cpDNA data

331

332 The BEAST-derived cpDNA tree suggested *Q. spinosa* diverged from outgroup
333 species *c.* 28.77 Ma (node F2 in **Fig. 2**; 95% CI: 24.43 – 32.78 Ma, PP = 1.00),
334 indicating *Q. spinosa* and *C. mollissima* diverged during the Mid to Late Oligocene.
335 The coalescence time estimated between the two cpDNA clades, i.e., EH-HM and
336 CEC (25.67 Ma, node A in **Fig. 2**, 95% CI: 18.35 – 31.99 Ma; PP = 1.00), suggested a
337 Late Oligocene/Early Miocene split between the two clades. Divergence times within
338 the EH-HM (node B in **Fig. 2**) and CEC clades (node C in **Fig. 2**) were 21.19 Ma (95%
339 CI: 12.52 – 29.63 Ma; PP = 0.93) and 18.70 Ma (95% HPD: 10.69 – 27.26 Ma; PP =

0.93), respectively. For this chronogram, BEAST provided an average substitution rate of 1.88×10^{-10} s/s/y, which was slower than the mean rates in other plants (e.g., 3.18×10^{-10} s/s/y in *Cercidiphyllum*, Qi *et al.*, 2012; and 9.6×10^{-10} s/s/y in *Quercus glauca*, Xu *et al.*, 2014).

Our LTT analysis revealed an increase of the diversification rate of *Q. spinosa* through time (Fig. 3a). The BAMM analysis suggested a high heterogeneity in the diversification rate of the two haplotype lineages across time, with CEC presenting higher diversification rate than EH-HM (Fig. 3b).

The two clades of *Q. spinosa* generally presented non-significant Tajima's *D* and Fu's *F_s* (Supporting Information Table S7). The BSP analyses indicated population sizes declined at the species and cluster levels during the Pleistocene (c. 0.5 Ma, 0.8 Ma, and 0.3 Ma for whole populations, EH-HM, and CEC, respectively; Fig. 3c).

Nuclear microsatellite diversity and population structure

The null alleles test indicated a lower frequency of null alleles at each of the 12 loci than the threshold frequency ($\alpha = 0.15$) across the 46 populations, and there was no evidence for LD. After the Bonferroni corrections, significant deviation from HWE induced by homozygote excess was detected in two loci (ZAG30 and ZAG20) when all samples were treated as a single population. However, there were no HWE deviations within each population after Bonferroni correction for all nSSRs.

Screening the 776 *Q. spinosa* individuals at the 12 nSSRs revealed 160 alleles with a highly variable diversity: *A_o* ranged from 7 to 27, *H_o* from 0.091 to 0.538, *H_s* from 0.125 to 0.581, and *H_T* from 0.219 to 0.868 (Table S2). Population differentiation was significant at the 12 loci ($P < 0.05$; Table S2), with average *F_{ST}* and *G'_{ST}* reaching 0.377 and 0.573, respectively. The values of *A_R*, *H_o*, *H_E*, and *F_{IS}* of each population ranged from 1.250 to 4.667, 0.137 to 0.423, 0.097 to 0.534, and -0.290 to 0.450, respectively (Table S1).

According to the STRUCTURE analysis performed for all populations (species level), *K* = 2 was optimal, although the log-likelihood of the data, log_e P(*K*), increased

with increasing K (Fig. 4). Thus, this genetic structure was highly congruent with the two lineages obtained in the cpDNA analysis (EH-HM and CEC, Fig. 2). The STRUCTURE analysis performed for the CEC cluster revealed $\log_e P(K)$ reached a plateau when $K > 3$ and ΔK was highest for $K = 3$ (Figs. S1 and S2). Therefore, we examined the proportional membership of each individual at $K = 3$. This showed that populations located in eastern China (DH, STW, THR, and XJ) clustered into one group (pop3), while populations located in the northern part of CEC (AK, LB, LY, NWT, QL, GY, SHS, and SY) clustered into another group (pop1); the remaining populations clustered into a third group (pop2) (Fig. S1). Two populations (SQS and LS) and a few individuals showed signs of genetic admixture (Fig. 3c, $Q < 0.8$) and therefore were excluded from ABC and gene flow analyses. The STRUCTURE analysis performed for the EH-HM cluster revealed $K = 2$ as optimal, according to ΔK (Fig. S2). For $K = 2$, populations located in the western part of EH-HM (DL, CY, JL, MJS, ML, SBM, SJL, SLJ, SM, and ZL) clustered into one group (pop4) whereas populations located in the eastern part of EH-HM (CK, FYS, HFY, RFY, SMN, Yb, and YB) clustered into another group (pop5). Population YS was excluded from ABC and gene flow analyses because it had a close genetic relationship to the CEC lineage, as revealed in the STRUCTURE analysis conducted at the species level.

The AMOVA results indicated significant genetic differentiation ($R_{ST} = 0.40$, $P < 0.001$), with only 9.87% of the variation partitioned among groups, 29.85% of the variation partitioned among populations within groups, and 68.28% of the variation partitioned among individuals within populations (Table 1). The F_{ST} was significant among populations within the two groups ($P < 0.001$), and slightly higher within CEC than within EH-HM ($F_{ST} = 0.34$ and 0.32 , respectively; Table S8). There was significant IBD for *Q. spinosa* when all populations were included ($r_M = 0.198$, $P < 0.001$), and the same was found when each region was analyzed separately ($r_{MEH-HM} = 0.088$, $P < 0.001$; $r_{MCEC} = 0.412$, $P < 0.001$).

Weak genetic barriers were detected between the EH-HM and CEC lineages (bootstrap value = 14%) and within the EH-HM cluster (bootstrap values ranging from 19% to 30%) (Fig. S3). According to the 2MOD analysis, the model that most

likely explains the observed population structure is the gene flow-drift model ($P = 1.0$).

Demographic history based on nSSRs

The scenario testing results obtained in step 1 of the ABC analysis among the three eastern populations (Fig. 5) suggested scenario 2 was the most plausible, as it presented a posterior probability of 0.6913 (95% CI: 0.6399–0.7428), which was much higher than that of the other nine scenarios. In step 2, the highest posterior probability was obtained for scenario 3 (0.7564, 95% CI: 0.7102–0.8030), and this was much higher than that of the other four scenarios. According to scenario 3, the median values of the effective population sizes of pop1, pop2, pop3, pop4, pop5, and NA were 3.42×10^5 , 2.51×10^5 , 3.36×10^4 , 6.60×10^5 , 3.56×10^5 , and 1.19×10^5 , respectively. The estimated median divergence time between the EH-HM and CEC lineages (t_4), within the EH-HM lineage (t_3), and within the CEC lineage (t_2 and t_1) were 1.70×10^5 , 1.26×10^5 , 8.17×10^4 , and 9.22×10^3 generations ago, respectively. Assuming *Q. spinosa* has a generation time of 150 years, t_4 , t_3 , t_2 , and t_1 corresponded to 2.55×10^7 , 1.89×10^7 , 1.23×10^7 , and 1.38×10^6 years ago, respectively. The estimated median mutation rate and proportion of multiple step mutations, based on the generalized stepwise model of microsatellites, were 2.26×10^{-6} and 0.485, respectively (Table 3).

Ecological niche modeling

There were similar change tendencies of suitable distributions of *Q. spinosa* obtained by MAXENT and GARP (Fig. 6). All models had high predictive ability ($AUC > 0.9$). In addition, the present-day distribution obtained for *Q. spinosa* was consistent with collection records (Fig. 6), with a potentially continuous range in the EH-HM region and western part of the CEC and a patchy distribution in eastern China. Based on our results, distribution areas during the LGM presenting moderately high suitability

scores (> 0.57) significantly decreased in CCSM and MIROC compared to Holocene and present distributions, indicating a possible habitat loss during the LGM. Both procedures inferred an overall southward range shift and shrinkage of the potential distribution range during the LIG (Fig. 6e), as areas with moderately high suitability scores (> 0.57) were compressed below 30 °N and significantly decreased compared to current and MH's distributions.

Impact of the environment on *Q. spinosa* genetic structure

Climatic analyses showed that the two lineages occupied different environments, with most environmental variables significantly contributing to this divergence (Table S13 and Fig. S6). The first two PCs explained 79.21% of the variance. Whereas PC1 was mainly correlated with precipitation, PC2 was mainly correlated with temperature (Table S11). The DFA analysis suggested that 97.8% of the populations were correctly assigned to their groups (Table S12). Thus, PCA and DFA analysis clearly showed that EH-HM and CEC lineages experienced contrasting environmental conditions, presumably paving the way for divergent selection.

After controlling for geographic distance, there was a significant positive association between pairwise F_{ST} and PC1 at the species level and under current climatic conditions ($bEnv-PRE = 0.166$, $rEnv-PRE = 0.148$, $P < 0.05$); no significant relationships were obtained between pairwise F_{ST} and PC2 (Table 2). When analyzed separately, BIO4 ($bEnv-PRE = 0.184$, $P < 0.001$; $rEnv-PRE = 0.198$, $P < 0.01$), BIO7 ($bEnv-PRE = 0.189$, $rEnv-PRE = 0.186$, $P < 0.05$), and BIO18 ($bEnv-PRE = 0.165$, $rEnv-PRE = 0.158$, $P < 0.05$) explained most of the genetic structure. Within the CEC lineage, a significant correlation was found between genetic differentiation and BIO4 ($bEnv-PRE = 0.244$, $rEnv-PRE = 0.233$, $P < 0.05$) and BIO7 ($bEnv-PRE = 0.210$, $rEnv-PRE = 0.206$, $P < 0.05$). However, there were no significant associations between genetic distance and PC1 or PC2 for the EH-HM and CEC lineages.

460 Discussion

461

462 *Demographic history of Q. spinosa*

463

464 Our genetic data clearly evidenced two distinct lineages within *Q. spinosa* in
465 subtropical China: one lineage was distributed in CEC region and the other in EH-HM
466 region. Ancient events seemed to be retained in *Q. spinosa*, as suggested by the
467 divergence times estimated for the inferred demographic processes. According to
468 ABC simulations, the most likely demographic scenario for *Q. spinosa* involved an
469 initially ancient isolation of two gene pools (EH-HM and CEC) followed by several
470 divergence events within each lineage.

471 The intraspecific divergence of *Q. spinosa* dated back to 25.50 Ma (95% HPD:
472 10.83 – 41.70 Ma) or 25.67 Ma (95% HPD: 18.35 – 31.99 Ma) based on ABC
473 simulations or BEAST-derived estimations of divergence time, respectively. The deep
474 split between EH-HM and CEC, and within EH-HM (Table 3; Fig. 5), might have
475 been triggered by the rapid uplift of the Himalayan – Tibetan plateau during the
476 Oligocene (*c.* 30 Ma; Sun *et al.*, 2005; Wang *et al.*, 2012) and the early/mid Miocene
477 (21–13 Ma; Searle, 2011). Together with the intensification of the central Asian
478 aridity from the late Oligocene to the early Miocene (Guo *et al.*, 2002), both events
479 lead to climatic changes, promoting the differentiation and diversification within *Q.*
480 *spinosa*. Within the CEC lineage, pop1 and pop3 diverged 3.26 Ma (95% HPD: 1.16 –
481 10.89 Ma), coinciding with an increase in seasonality and aridity across Southeast
482 Asia and with the intensification of Asian monsoons 3.6 Ma (An *et al.*, 2001). Such
483 climatic changes might have contributed to the fragmentation of endemic populations
484 and for their ultimate isolation. In addition, LTT and diversification analysis (Fig. 3a
485 and 3b) suggested that diversification possibly started close to the Oligocene-Miocene
486 boundary, with a rapid diversification occurring during the mid to late Miocene.
487 Events occurring in the Late Miocene, like the rapid uplift of the Tibet plateau *c.* 10–7
488 Ma (Harrison *et al.*, 1992; Royden *et al.*, 2008) and the development of East Asian
489 monsoons since the late Oligocene with several intensification periods during the

490 Miocene (*c.* 15 Ma and 8 Ma; Wan *et al.*, 2007; Jacques *et al.*, 2011), might have
491 altered habitats, enhancing the geographic isolation between and within EH-HM and
492 CEC lineages and significantly influencing their diversification.

493 Divergence and diversification of *Q. spinosa* appear to have occurred earlier than
494 in other woody species in subtropical China. Whereas *Fagus* (*c.* 6.36 Ma; Zhang *et al.*
495 2013), *Cercidiphyllum* (*c.* 6.52 Ma; Qi *et al.* 2012), Asian white pine (*Pinus armandii*)
496 (*c.* 7.41 Ma; Liu *et al.* 2014), and *Tetracentron sinense* (*c.* 7.41 Ma, Fig. 4; Sun *et al.*
497 2014) diverged during the late Pliocene. *Cyclocarya paliurus* diverged in the mid
498 Miocene (*c.* 16.69 Ma; Kou *et al.* 2015), which is closer to the time estimated for *Q.*
499 *spinosa* in the present study. Notwithstanding the differences in lineages divergence
500 and diversification times evidenced above, pre-Quaternary climatic and/or geological
501 events influenced the evolutionary history of Neogene taxa in subtropical China,
502 including *Q. spinosa*.

503 The divergence time presented for *Q. spinosa* should be treated with caution as
504 our molecular dating was influenced by the large variation associated with fossil
505 calibration points, limited cpDNA variation (82 variable sites), and microsatellite data
506 characteristic such as uncertain mutation models and homoplasy (Selkoe & Toonen,
507 2006). Takezaki & Nei (1996) suggested that homoplasy at microsatellite loci tended
508 to underestimate divergence time over large time-scales. However, it does not
509 represent a significant problem as it can be compensated using numerous loci (Estoup
510 *et al.*, 2002). In addition, the assumption of no gene flow in DIYABC leads to the
511 underestimation of the divergence time between species (Leaché *et al.*, 2013),
512 although STRUCTURE analyses indicated little admixture between EH-HM and CEC
513 lineages. Thus, the reliability of dating divergence events needs to be further studied
514 using more loci. Nevertheless, the divergence time estimated from cpDNA and nSSRs
515 was almost congruent, supporting our confidence that it reflects the real divergence
516 time between EH-HM and CEC lineages. Additionally, considering the most ancient
517 closely-related fossils to *Q. spinosa* were reported from the Miocene (Zhou, 1993),
518 and recent phylogenetic studies established the origin of the major oak lineages by the
519 end of the Eocene (*c.* 35 Ma) (Zhou, 1993; Hubert *et al.*, 2014; Grímsson *et al.*, 2015;

520 Simeone *et al.*, 2016). Hence, it is plausible that the split between and within the two
521 *Q. spinosa* lineages started in the Oligocene-Miocene boundary.

522 Our ENMs analysis suggested *Q. spinosa* continued to expand its distribution
523 range since the LIG, in line with the tests of spatial expansion for the two clades of *Q.*
524 *spinosa* (Table S7). whereas the potential distribution areas of cold-tolerant species
525 inhabiting subtropical China, such as spruce and yews, stabilized or decreased slightly
526 from the LGM to present days (Li *et al.*, 2013; Liu *et al.*, 2013). In addition, given the
527 scattered mountain ridges that characterize subtropical China, especially in the CEC
528 region, it is likely that *Q. spinosa* remained both sparsely populated and spatially
529 fragmented throughout the Quaternary. This distribution was in fact evidenced from
530 the past and present modeling (Fig. 6). A higher level of fragmentation would be
531 expected to result in low gene flow among populations, which, in turn, would lead to
532 higher F_{ST} . Accordingly, population fragmentation in *Q. spinosa* was much severer in
533 the CEC than in the EH-HM lineage, the F_{ST} value of CEC (0.343) was slightly
534 higher than that of EH-HM (0.321) (Table S8), and gene flow within EH-HM was
535 significantly higher than within CEC (Fig. S5). In contrast, BSP results showed a
536 recent decrease in the effective population size of *Q. spinosa* (Fig. 3c). Despite this
537 disagreement with ENMs, MDA and cpDNA haplotype network revealed a recent
538 expansion of *Q. spinosa* in the CEC region. Recent simulation studies have found that
539 the recent population declines revealed by BSP are sensitive to the hierarchical
540 population structure and may distort the true scenario (Grant *et al.*, 2012; Heller *et al.*,
541 2013). Moreover, it should be noted that our population size-change estimations were
542 based solely on the variability of the four cpDNA fragments; more accurate
543 estimations should be inferred using more loci (Felsenstein, 2006).

544

545 *Allopatric divergence and the impact of environmental and topographical factors* 546 *on population structure*

547

548 The major phylogeographic break detected in the present study based on the
549 BEAST-derived cpDNA chronogram and on Bayesian clustering analysis is shared

550 with other widespread temperate plants, such as *Ginkgo biloba* (Gong *et al.*, 2008),
 551 *Dysosma versipellis* (Qiu *et al.*, 2009), and *Quercus glauca* (Xu *et al.*, 2014), which
 552 clearly supported long-term isolation and allopatric divergence in subtropical China.
 553 Although the EH-HM and CEC lineages of *Q. spinosa* appear to have diverged earlier
 554 than other temperate species, its time scale was from the late Oligocene to the early
 555 Miocene, when the Tibetan plateau uplifted and central Asian aridity began to
 556 intensify. Thus, ancestral *Q. spinosa* might have been distributed in eastern and
 557 western China, allopatrically diverging in response to geographical/ecological
 558 isolation during the periods of intense climatic changes and active orogeny. A similar
 559 situation might also be responsible for the subsequent lineage divergence of pop4 and
 560 pop5 in the EH-HM region and pop1, pop2, and pop3 in the CEC region (Fig. S1).
 561 However, no significant genetic barrier was detected between the two lineages or
 562 within the CEC lineage, based on the nSSR loci (Fig. S3). The poor dispersal ability
 563 of seeds might explain the maintenance of this phylogeographic break. Most *Fagus*
 564 spp. seeds drop to the ground near parent trees and only a few may roll down on steep
 565 terrain or be dispersed by animals (e.g., jays or squirrels) over short distances (Gómez,
 566 2003; Xiao *et al.*, 2009). This might also be the case for *Q. spinosa*, although its seed
 567 dispersal mode still needs to be studied in detail.

568 Recent studies have demonstrated the impacts of environmental and geographic
 569 factors on population structure (e.g., Sexton *et al.*, 2014; Wu *et al.*, 2015; Zhang *et al.*,
 570 2016). Our analyses evidenced the significant roles of geography and climate in
 571 shaping *Q. spinosa* genetic structure (Table 2). Similar to that revealed in previous
 572 studies highlighting the importance of water availability and temperature on oak
 573 species demography (Sardans & Peñuelas, 2005; Yang *et al.*, 2009; Xu *et al.*, 2013),
 574 the present study showed the effect of precipitation (PC1) on *Q. spinosa* genetic
 575 divergence but failed to uncover the effect of temperature (PC2). However, at the
 576 lineage level, both temperature (BIO 4) and precipitation (BIO 18) influenced the
 577 divergence of CEC lineage (Table 2). However, adaptation to local environments
 578 might be biased by numerous factors (Meirmans, 2012; 2015) and it wasn't possible
 579 to explicitly test selection based on our current data. Overall, *Q. spinosa* seems to

580 have adapted to local environments that reinforce population genetic divergence
581 between the two lineages, but this hypothesis requires further examination using more
582 environment-related loci.

583

584 ***Multiple refugia or refugia within refugia and long-term isolation***

585

586 Previous studies suggested that many temperate plant species of subtropical China
587 had multiple refugia during the climatic changes of Quaternary (e.g., Wang *et al.*,
588 2009; Shi *et al.* 2014; Wang *et al.* 2015). The major phylogeographic break found
589 between the EH-HM and CEC regions during the pre-Quaternary, which was
590 suggested by both cpDNA and nSSRs data analyzed in the present study, also supports
591 the existence of multiple refugia in subtropical China, although this scenario is more
592 plausible during interglacial than during glacial periods. The high population
593 differentiation of cpDNA in the two lineages with most populations showing a
594 dominant haplotype, and the subdivision of EH-HM and CEC lineages into two and
595 three gene pools, respectively, revealed in nSSRs analysis, suggested a patterns of
596 “refugia within refugia” for the two lineages. However, the ENMs predicted few
597 suitable habitats for *Q. spinosa* in the CEC region during the LIG (Fig. 6). Because
598 ENMs assume the species’ current large-scale geographical distribution is in
599 equilibrium with the environment, as well as niche conservation over time, these
600 models may fail to capture climatic variance and the effects of topography on
601 microclimate (Peterson, 2003; Gavin *et al.*, 2014). Thus, ENMs might have been
602 unable to reveal potential microrefugia for *Q. spinosa*. In addition, given the island-
603 like genetic structure and ancient divergence between or within the two lineages
604 revealed by cpDNA data, pointing out a long-term isolation for *Q. spinosa*.
605 Furthermore , CEC haplotypes had a star-like distribution that was compact and with
606 few missing haplotypes, while the EH-HM lineage had many mutational steps and
607 sparse missing haplotypes, also indicating the long-term isolation of these two
608 lineages. Similar results were obtained for other species occurring in subtropical
609 China (Sun *et al.*, 2014; Xu *et al.*, 2014). Hence, *Q. spinosa* might have experienced

610 long-term isolation among multiple refugia throughout the Quaternary, with little
611 admixture among populations from isolated refugia in the EH-HM and CEC regions.
612 Therefore, our results support the widely proposed hypothesis that temperate forests
613 in subtropical China experienced long-term isolation among multiple refugia
614 throughout the late Neogene and Quaternary (Qian & Ricklefs, 2000).

615

616 **Conclusions**

617

618 The analyses of *Q. spinosa* chloroplast and nuclear DNA combined with
619 environmental analysis and ecological niche modeling, showed that the current
620 distribution range of this species comprises two major lineages (EH-HM and CEC)
621 that most likely diverged through climate/tectonic-induced vicariance in the
622 pre-Quaternary, remaining in multiple long-term refugia with little admixture during
623 the Quaternary. Thus, pre-Quaternary environmental changes profoundly influenced
624 the evolutionary and population demographic history of *Q. spinosa* as well as its
625 modern genetic structure. These results support the widely accepted concept that the
626 complex topography and climatic changes occurring in East Asia since the Neogene
627 have provided great opportunity for allopatric divergence and speciation among
628 temperate evergreen forest species in subtropical China. Our study also pointed out
629 that combining phylogeography, ENMs, and bioclimatic analyses allows deep insight
630 into the diversification and evolutionary history of species.

631

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633 [to be completed]

634

635 **Author contributions**

636 [to be completed]

637

638

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963 **Supporting Information**

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

965 **Table S1** Geographic and genetic characteristics of the 46 *Quercus spinosa*
966 populations sampled

967 **Table S2** Characteristics of the 12 microsatellite loci surveyed across 46 *Quercus*
968 *spinosa* populations

969 **Tables S3-S6** Polymorphisms detected in the four chloroplast DNA fragments

970 **Table S7** Results of the mismatch distribution analysis and neutrality tests based on
971 the chloroplast DNA sequences of *Quercus spinosa*

972 **Table S8** Genetic diversity and genetic differentiation of the 46 *Quercus spinosa*
973 populations

974 **Table S9** The 15 scenarios used for inferring the demographic history of *Quercus*
975 *spinosa* in the DIY ABC analysis

976 **Table S10** Prior distributions for model parameters used in DIY ABC

977 **Table S11** Principal component analysis (PCA) of the 20 environmental variables

978 **Table S12** Contributions of the 20 environmental variables in the discriminant
979 function analysis (DFA) of *Quercus spinosa*

980 **Table S13** ANOVA results for each of the environmental variables

981 **Fig. S1** Results of the genetic assignment of the EH-HM and CEC *Quercus spinosa*
982 lineages performed on STRUCTURE

983 **Fig. S2** Bayesian inference of the number of clusters (*K*) of *Quercus spinosa*

984 **Fig. S3** BARRIER analyses and the geographic distribution of the main genetic
985 barriers

986 **Fig. S4** Estimates of gene flow and migration rates

987 **Fig. S5** Principal component analysis (PCA) of the 20 environmental variables at
988 present

989 **Fig. S6** Kernel density plots of the 20 environmental variables in the EH-HM and
990 CEC lineages.

991 **Note S1** molecular methods and details of ecological niche modeling and
992 environmental factors analysis

993 **Note S2** Details and results of demographic analysis for chloroplast DNA, gene flow
994 for microsatellite data, and detail methods for ecological niche modeling and
995 Environmental variables analysis and isolation by environment.
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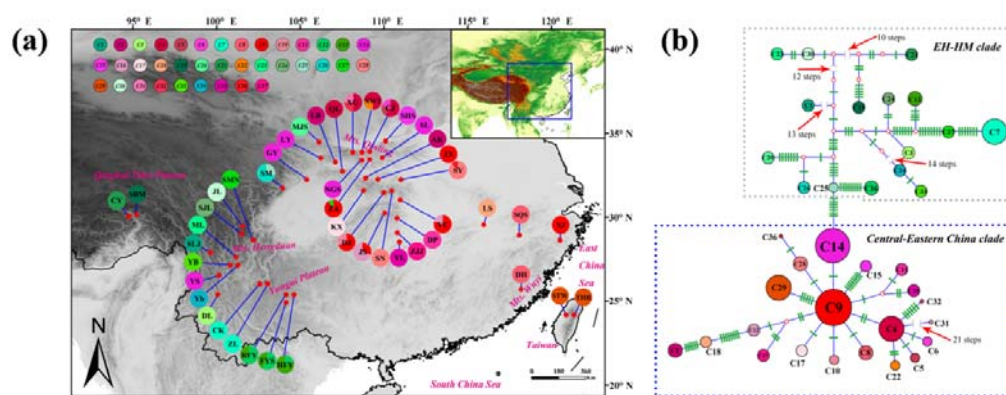
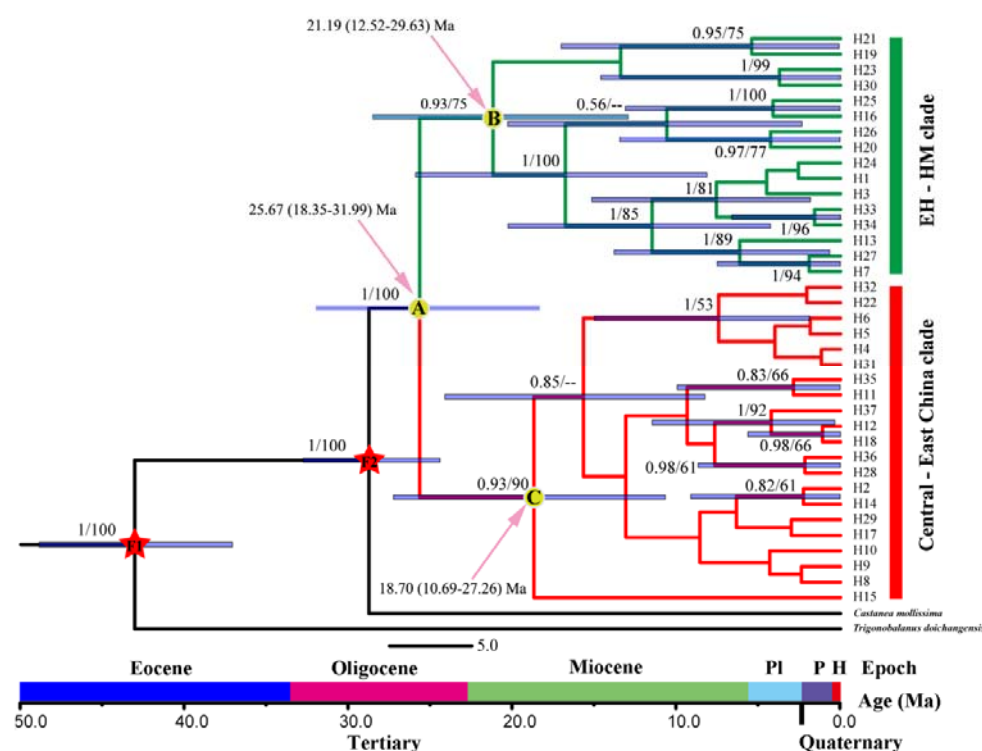


Fig. 1 (a) Geographic distribution of the chloroplast (cp) DNA haplotypes in the 46 *Quercus spinosa* populations from subtropical China. (a) Geographic distribution of haplotypes. Haplotype frequencies for each population are denoted in the pie charts and population codes are presented in the center of the pie chart (see Table S1 for population codes). (b) Genealogical relationships between the 37 cpDNA haplotypes. Each circle sector is proportional to the frequency of each chlorotype. Small open circles represent missing haplotypes. Green bars represent nucleotide variation.

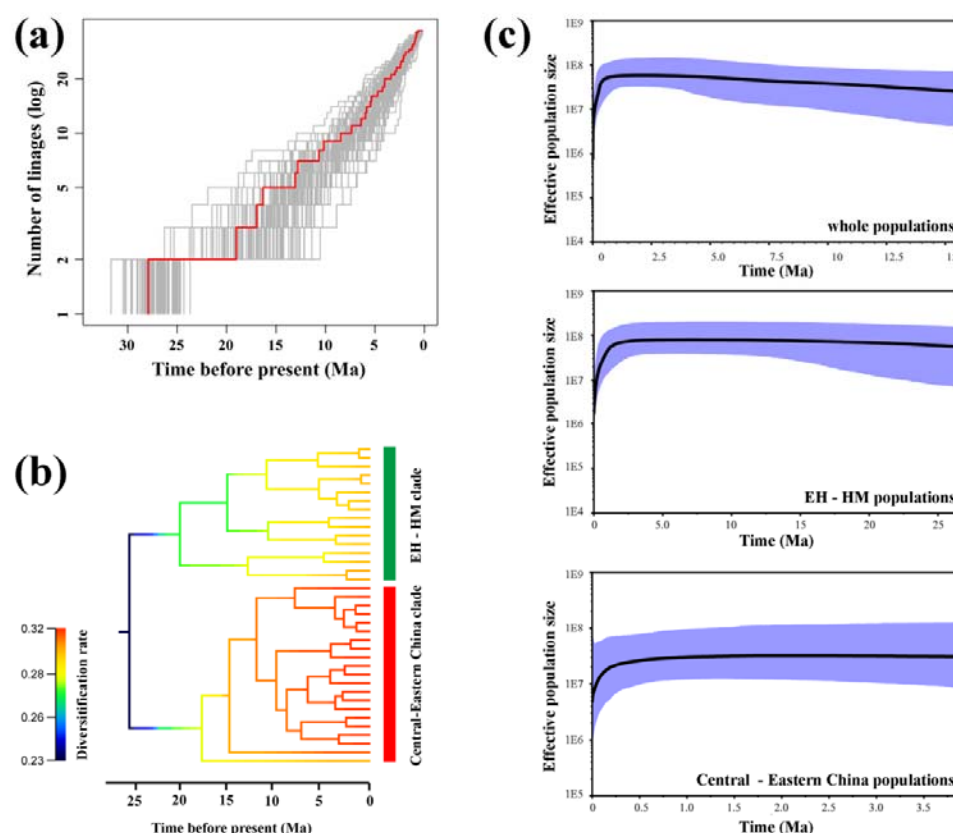
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1010 **Fig. 2** BEAST-derived chronograms of *Quercus spinosa* based on chloroplast DNA
1011 sequences (*psbA-trnH*, *psbB-psbF*, *matK*, and *YcfI*). Red stars indicate fossil
1012 calibration points. Pink arrows indicate a recent common ancestor of *Quercus spinosa*
1013 lineages. Light blue bars indicate the 95% highest posterior density (HPD) credibility
1014 intervals for node ages (in million years ago, Ma). Bootstrap values (> 50%) based on
1015 maximum likelihood (ML) analysis and posterior probabilities are indicated above
1016 nodes.

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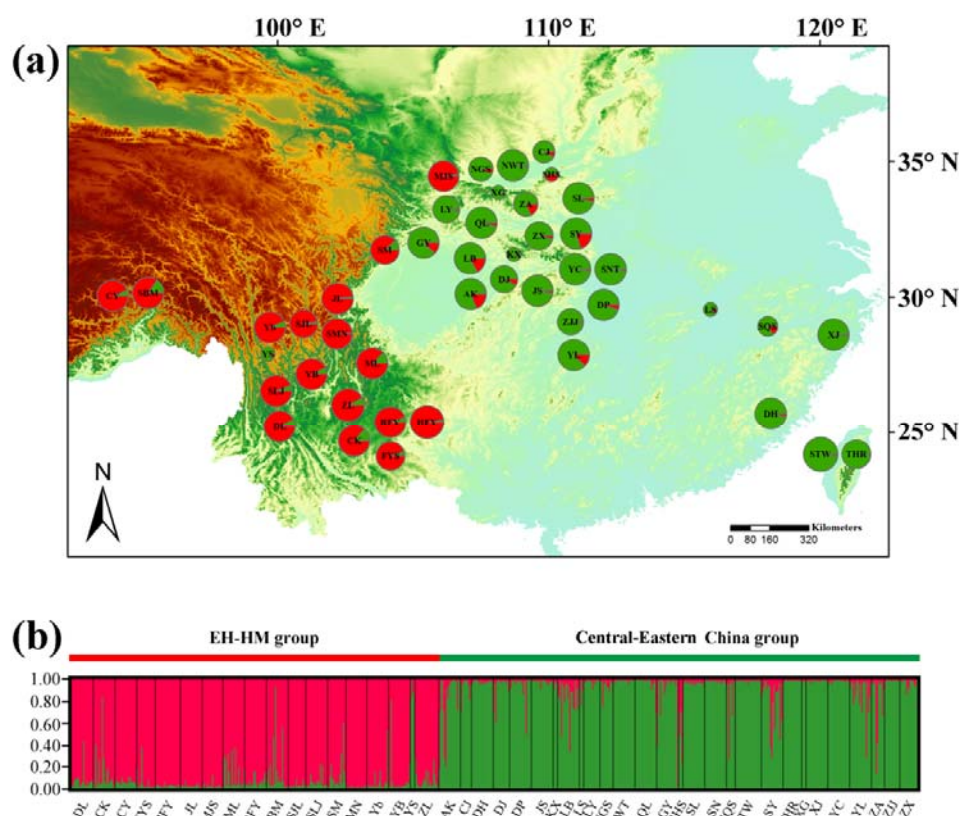


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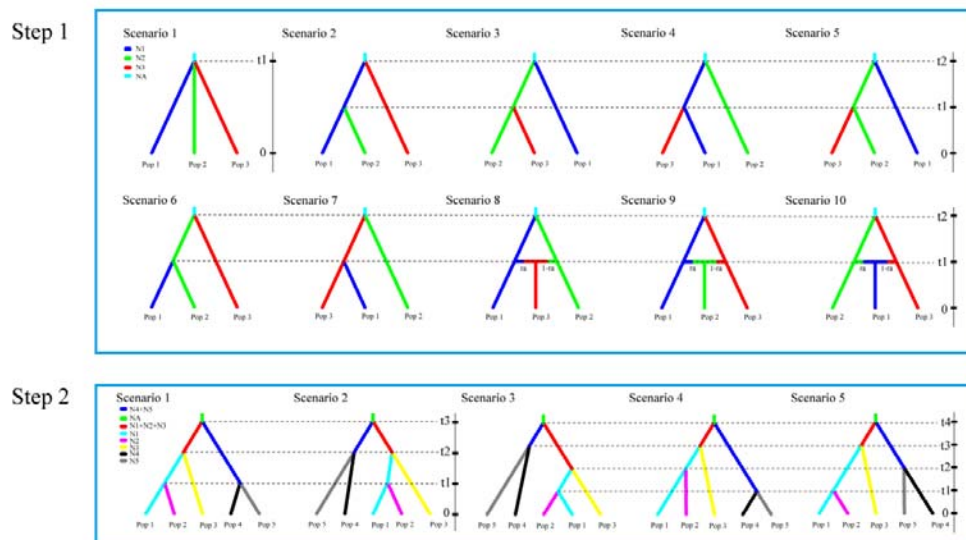
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1020 **Fig. 3** Divergence and diversification of *Quercus spinosa* through time. (a)
 1021 Multiple-lineages-through-time plots based on 100 randomly sampled trees from the
 1022 BEAST analysis. The red line indicates the lineages-through-time plot for the
 1023 consensus chronogram. (b) Diversification rates based on chloroplast DNA haplotypes,
 1024 as estimated in BAMM. (c) Bayesian skyline plot inferred from chloroplast DNA data
 1025 for all *Q. spinosa* and for the two lineages, respectively. The black lines are the
 1026 median effective population sizes through time and the blue areas are the limits of the
 1027 95% highest posterior densities confidence intervals.

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1030 **Fig. 4** STRUCTURE analysis performed for the 46 populations of *Quercus spinosa*.
1031 (a) Geographic origin of the 46 populations and their color-coded grouping at the
1032 most likely $K = 2$. (b) Histogram of the STRUCTURE assignment test for the 46
1033 populations based on genetic variation at 12 nuclear microsatellite loci.
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1037 **Fig. 5** Scenarios used in the DIYABC analyses to infer the demographic history of
1038 *Quercus spinosa* based on 12 nuclear microsatellite loci. Step 1: 10 scenarios used to
1039 analyze the relationships among the three populations belonging to the
1040 Central-Eastern China lineage. Step 2: the five scenarios used to analyze the
1041 relationships among the five populations of *Q. spinosa*.

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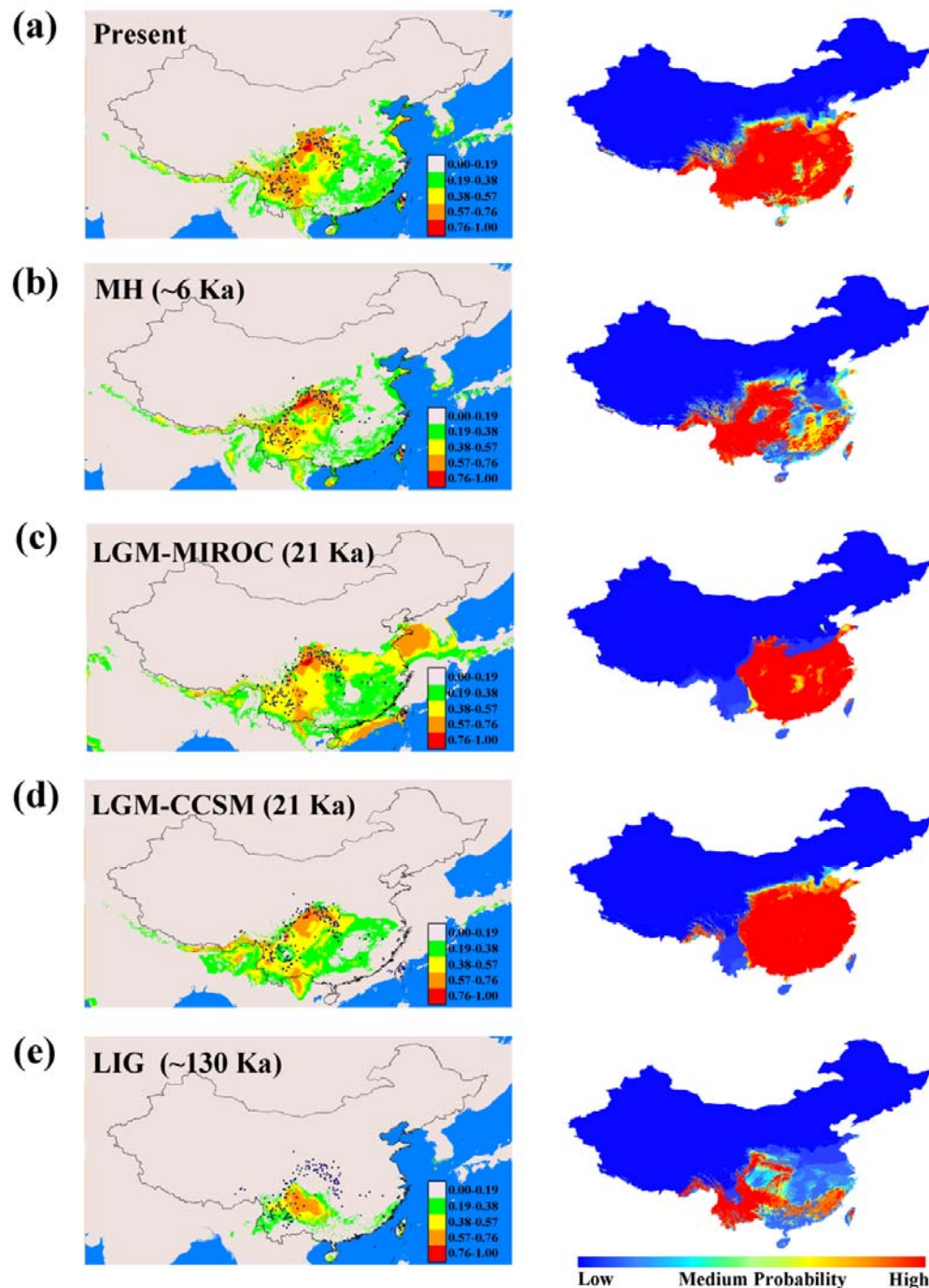


Fig. 6 Climatically suitable areas predicted for *Quercus spinosa* using MAXENT (left) and GARP (right) in subtropical China at different times. (a) Present time; (b) Mid-Holocene (MH, c. 6 Ka before present (BP)); (c, d) Last glacial maximum (LGM, c. 21 Ka BP) under the MIROC (c) and CCSM (d) models; and (e) Last interglacial (LIG, c. 120–140 Ka BP). The logistic value of habitat suitability is indicated in the colored scale-bars. Black dots indicate extant occurrence points.

Table 1 The analysis of molecular variance (AMOVA) for cpDNA data and nSSR data among two geographic regions (EH-HM and CEC) and all populations of *Quercus spinosa*

Source of variation	cpDNA					nSSRs				
	d.f.	Sum of squares	Variance components	Percentage of variation (%)	Φ -statistics	d.f.	Sum of squares	Variance components	Percentage of variation (%)	R -statistics
Two geographic regions										
Among regions	1	1060.254	5.24	42.35	$\Phi_{CT} = 0.42^{**}$	1	322.060	0.36	9.87	$R_{CT} = 0.10^{**}$
Among populations within regions	44	2373.603	6.15	49.70	$\Phi_{SC} = 0.86^{**}$	44	1728.731	1.10	29.85	$R_{SC} = 0.33^{**}$
Within populations	351	345.234	0.98	7.95	$\Phi_{ST} = 0.93^{**}$	1506	3356.416	2.23	60.28	$R_{ST} = 0.40^{**}$
Total populations										
Among populations	45	3433.857	8.74	89.88	$\Phi_{ST} = 0.90^{**}$	45	2050.790	1.29	36.62	$R_{ST} = 0.37^{**}$
Within populations	351	345.234	0.98	10.12		1506	3356.416	2.23	63.38	

Estimators for cpDNA were calculated based on the infinite alleles model (Φ -statistics) and those for nSSRs on the stepwise mutation model (R -statistics). All levels of variation were significant. See Table 1 for regional grouping of populations.

Table 2 Partial Mantel (PM) correlation (r) and multiple matrix regression (MMRR) coefficients (b) between genetic distance (F_{ST}) and environmental variables for the present time.

	PRE		
	MMRR		PM
	$b_{Geo-PRE}$	$b_{Env-PRE}$	$r_{Env-PRE}$
Whole range			
$F_{ST-PC1/Geo}$	0.360***	0.166*	0.148*
$F_{ST-PC2/Geo}$	0.486***	-0.074 ns	-0.078 ns
$F_{ST-BIO4/Geo}$	0.352***	0.198**	0.184***
$F_{ST-BIO7/Geo}$	0.370***	0.189*	0.186*
$F_{ST-BIO18/Geo}$	0.374***	0.165*	0.158*
EH-HM lineage			
$F_{ST-PC2/Geo}$	0.318*	-0.010 ns	-0.010 ns
$F_{ST-BIO4/Geo}$	-0.007 ns	0.430 ns	0.299*
$F_{ST-BIO18/Geo}$	0.135 ns	0.268 ns	0.209*
Central-Eastern China lineage			
$F_{ST-PC1/Geo}$	0.498*	0.177 ns	0.113 ns
$F_{ST-PC2/Geo}$	0.664***	-0.051 ns	-0.067 ns
$F_{ST-BIO4/Geo}$	0.485***	0.244*	0.233*
$F_{ST-BIO7/Geo}$	0.512***	0.210*	0.206*

BIO4, Temperature seasonality ($SD \times 100$); BIO7, Temperature Annual Range (BIO5-BIO6); BIO18, Precipitation of warmest quarter. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant. Positive significant tests for both MMRR and PM tests are in bold.

Table 3 Posterior median estimate and 95% highest posterior density interval (HPDI) for demographic parameters in scenarios 1 and 2 based on the nuclear multilocus microsatellite data for whole populations of *Quercus spinosa*

	Parameter	N1	N2	N3	N4	N5	NA	t1 ^a	t2 ^a	t3 ^a	t4 ^a	μ	P
Scenario 3	Median	3.42×10 ⁵	2.51×10 ⁵	3.36×10 ⁴	6.60×10 ⁵	3.56×10 ⁵	1.19×10 ⁵	9.22×10 ³	8.17×10 ⁴	1.26×10 ⁵	1.70×10 ⁵	2.26×10 ⁻⁶	0.485
	Lower_bound	1.20×10 ⁵	6.80×10 ⁴	9.16×10 ³	3.50×10 ⁵	1.32×10 ⁵	1.09×10 ⁴	4.87×10 ³	7.73×10 ³	3.46×10 ⁴	7.22×10 ⁴	1.24×10 ⁻⁶	0.166
	Upper_bound	8.04×10 ⁵	8.14×10 ⁵	2.35×10 ⁵	9.32×10 ⁵	8.09×10 ⁵	6.60×10 ⁵	9.94×10 ³	1.26×10 ⁵	1.79×10 ⁵	2.78×10 ⁵	5.26×10 ⁻⁶	0.843

^a The unit of timing is generation.

μ: mutation rate (per generation per locus).

P represents the proportion of multiple step mutations in the generalized stepwise model, GSM.