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# RESEARCH ARTICLE

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# Shifting roles of the East China Sea in the phylogeography of red nanmu in East Asia

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# Abstract

**Aim:** The effect of eustasy driven by climatic oscillations on species diversification across the East China Sea (ECS) remains unclear. In this study, *Machilus thunbergii* (红楠, red nanmu in Chinese) was used as a model species to determine whether the ECS acted as either a "species pump" or "species vacuum" during the Pleistocene. **Locations:** Eastern China, and southern and central Japan.

Taxon: Lauraceae.

**Methods:** Ecological niche modelling was employed to predict the potential distribution of *M. thunbergii* during the Last Glacial Maximum (LGM) and the last interglacial period. Nuclear microsatellite and chloroplast markers were used to reveal the phylogeographic pattern and infer the population history of 33 *M. thunbergii* populations.

**Results:** The ecological niche models suggested that the ECS provided potentially suitable habitats for *M. thunbergii* during the LGM. A sharp change in cpDNA haplotypes was found along the eastern China coasts, while microsatellites revealed a clinal pattern for genetic composition from eastern China to central Japan. The divergent lineages formed an admixture on the Zhoushan Archipelago of China and Kyushu Island of Japan. The estimated divergent and admixture times were *c.* 68 kyr and *c.* 15 kyr, corresponding to the periods where there were rising sea levels after the MIS4 glaciation and falling sea levels during the LGM, respectively.

**Main conclusions:** *Machilus thunbergii* probably underwent alternating population isolation during interglacial periods and connection during glacial maxima across the ECS, but such periodicity of isolation and connection seems not to have promoted diversification as suggested by the species pump hypothesis. Incipient divergence has been periodically wiped out due to frequent coalescence, rendering the ECS more like a "species vacuum", particularly for species with relatively long generation lengths.

# KEYWORDS

East China Sea, land bridge, Last Glacial Maximum, *Machilus thunbergii*, phylogeography, Sino-Japanese disjunction, species pump, species vacuum

Kai Jiang and Xin Tong contributed equally to this work.

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# 1 | INTRODUCTION

Pleistocene climatic oscillations are widely viewed as important evolutionary engines that shape biodiversity patterns, but the impacts of those oscillations on species diversification are unclear (Gillespie & Roderick, 2014; Weigelt et al., 2016). It has been argued that alternating glaciation and deglaciation events may have caused repeated cycles of population isolation and connection, which in turn promoted or constrained species diversification in a "species pump" or "species vacuum" manner depending on the spatial and temporal scales and periodicity of isolation and connection (Brown et al., 2013; Gillespie & Roderick, 2014). This species pump hypothesis was originally proposed to describe how fragmented glacial refugia triggered tropical diversification (Haffer, 1969) and was then extended to temperate (Weir et al., 2016) and boreal regions (Arcones et al., 2021; Bringloe & Saunders, 2019). However, opposing processes should be expected in marine island systems, where exposed seabed associated with falling sea levels at glacial maxima might connect otherwise isolated populations (Ali & Aitchison, 2014), and in alpine habitats, where the lowering of vegetation belts during glacial effects might provide opportunities for connections between sky island populations (Knowles, 2000). Such Pleistocene species pump models have generally been supported by a number of phylogeographic studies of alpine taxa (Flantua et al., 2019; Nevado et al., 2018; Schoville et al., 2012; Weng et al., 2020). However, the hypothesis is still under debate for island biota (Ali & Aitchison, 2014; Brown et al., 2013; Papadopoulou & Knowles, 2015a, 2017). In contrast to the species pump hypothesis, frequent connections might periodically reduce the incipient divergence shown by some organisms in island systems, which would impede in situ diversification. that is, "species vacuum" (Papadopoulou & Knowles, 2017).

Marine islands can be classified as either oceanic or continental depending on whether they have been connected to the adjacent mainland (Triantis et al., 2012). The Pleistocene species pump hypothesis has been mostly tested on islands that have oceanic Pleistocene Aggregate Island Complex systems, such as the Galápagos, Philippines, Caribbean and Mediterranean archipelagos (Ali & Aitchison, 2014; Brown et al., 2013; Papadopoulou & Knowles, 2015a, 2015b). However, the major dynamic forces impacting the biogeographical processes of migration and diversification may be different between oceanic and continental islands (Fernandez-Palacios et al., 2016; Patino et al., 2017). On the one hand, the sea level transgression and regression driven by Pleistocene glacial cycles (100-1 kyr) could lead to fusion and fission among nearby oceanic islands (Ali & Aitchison, 2014). On the other hand, oceanic island biota may have been comparably or more affected by ancient (>1 Myr) plate tectonics and island ontogeny events, and by recent volcanic activities (<1 kyr; Fernandez-Palacios et al., 2016; Gillespie & Roderick, 2014). In contrast, eustasy during glacial cycles may have particular relevance to biogeographical dynamics on continental islands where the roles of plate tectonics, island ontogeny and volcanic activity should be relatively minor (Fernandez-Palacios et al., 2016). Furthermore, rising sea levels caused substantial island

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area losses and seamount submergence, which would enhance the extinction rate and impede diversification on oceanic islands (Fernandez-Palacios et al., 2016; Papadopoulou & Knowles, 2017; Weigelt et al., 2016), whereas the other end of the pump process on continental island systems (i.e. the mainland) is much more stable. Furthermore, the varied bathymetry suggests that the duration of separation may be different between oceanic and continental island systems. These complex processes on oceanic islands probably obscured potential species pump effects on island fusion-fission cycles. Therefore, there is a need for more biogeographic studies on continental island systems to better understand how the species pump action of sea-level oscillations affects diversification.

The temperate climate zone of East China, South Japan and the South Korean Peninsula is a hotspot of plant species richness and endemism. Almost all of the endemic genera are shared between China and Japan despite the fact that Japan and Korea are geographically closer (Xie, 1997). This phenomenon may suggest that the East China Sea (ECS) lying between East China and Japan acted as a dispersal corridor at some time(s) after the separation of Japan from the Eurasian continent (Early Miocene, c. 23 Myr; Wakita, 2013). Furthermore, sea level oscillations are hypothesized to have alternately separated and joined plant distributions between East China and Japan throughout Pleistocene glacial cycles, creating opportunities for vicariant allopatric diversification and speciation (Qian & Ricklefs, 2000). Geological evidence suggests that while the ECS isolated forests in East China and Japan for most of the time, wide areas of continental shelf and seabed emerged from the ECS when the sea level was lowered by c. 85-140 m at several glacial maxima (Siddall et al., 2003; Spratt & Lisiecki, 2016; Figure 1). The sea level fall was particularly drastic during the Last Glacial Maximum (LGM). which occurred around 21-18 kyr before the present period. Paleobiome reconstructions further indicated that during the LGM and possibly other glacial maxima, a band of temperate deciduous forest extending across the exposed ECS seabed connected the currently isolated temperate forests of East China and South Japan (Harrison et al., 2001).

The phylogeographic evidence revealing trans-ECS divergence associated with the Pleistocene species pump is limited and partial. Only a few investigations have identified high levels of genetic differentiation across the ECS, primarily in understory herbs and shrubs, including Croomia japonica (Li et al., 2008), Kirengeshoma palmata (Qiu, Sun, et al., 2009), Ligularia hodgsonii (Wang et al., 2013), Platycrater arguta (Qi et al., 2014) and Kerria japonica (Luo et al., 2021). Except for the intraspecific diversification of K. japonica, which began in the late Miocene (c. 7 Myr), all of the estimated divergence times fall into the mid-Pleistocene (c. 1 Myr). These findings may reflect the consequences of the species pump across the ECS in the early-to-mid Pleistocene, but also suggest that the ECS imposed an impenetrable barrier to the dispersal of those species during the Late Pleistocene (Qi et al., 2014). In other cases, the ECS may have acted as a dispersal corridor at the LGM, mainly for tree species, but there is little genetic differentiation evidence to support this (Qi et al., 2012; Sakaguchi et al., 2012). Therefore, whether the



FIGURE 1 The upper figure shows the bathymetric map of the East China Sea based on the global seafloor topography (Smith & Sandwell, 1997). The bottom figure shows the sea level fluctuation since the mid-Pleistocene according to the data reported by Spratt and Lisiecki (2016). The grey bands indicate the highest oxygen isotope levels, which are recognized as glacial periods according to Railsback et al. (2015)

last glacial cycle drove the species pump in the ECS, and if so, how the species pump has affected diversification remain poorly understood. These unanswered questions are crucial because the LGM probably played an important role in triggering trans-ECS dispersal according to the geological and climatological predictions (Siddall et al., 2003) and paleo-biome reconstructions (Harrison et al., 2001) and because the tempo of population isolation and coalescence will largely determine the evolutionary outcomes of species pump actions (Gillespie & Roderick, 2014; Papadopoulou & Knowles, 2015b).

The exposed ECS land bridge might have allowed certain species to disperse while filtering those unable to tolerate the cold environmental conditions across the paleo-landscape (Qi et al., 2014). Hence, we used one of the most cold-resistant evergreen broad-leaved trees, *Machilus thunbergii* Sieb. et Zucc. (Lauraceae), also called red nanmu in China as a model system to investigate the above issues. This species is long-lived and may reach to hundreds of years old. Many populations of the species are located at the periphery of the hypothesized forest band that extended across the ECS land bridge (Harrison et al., 2001), which facilitated research on the ECS species pump. Furthermore, the Zhoushan Archipelago of China and Kyushu Island of Japan are both naturally inhabited by *M. thunbergii*. These populations are likely remnants of the lineage extending throughout the exposed ECS land bridge (Cao et al., 2018), and if so, represent the consequences of dispersal and gene exchange across the ECS.

Specifically, we hypothesized that (1) the ECS acted as a barrier during the last interglacial period, but as a dispersal corridor during the LGM (i.e. as a species pump during the last glacial cycle); and (2) the Pleistocene species pump did not drive trans-ECS divergence in *M. thunbergii*, and population admixing of the divergent lineages may be found on Zhoushan and/or Kyushu. We first tested hypothesis 1 using species paleo-geographic distribution modelling, and then tested hypothesis 2 based on the phylogeographic structure revealed by chloroplast DNA sequences and nuclear microsatellite markers. We further tested the two hypotheses more rigorously by modelling the population demographic history, which allowed the divergence and admixture times to be inferred. According to the geology of the ECS and Pleistocene sea-level changes (Figure 1), we expected that the admixture time would match the LGM, and that the divergence time would coincide with some rising sea-level periods.

# 2 | MATERIALS AND METHODS

# 2.1 | Species overview and sampling

Machilus thunbergii usually grows on mountain slopes, coastal hillsides and valleys in the warmer parts of East Asia (Wei et al., 2008). It is one of the most cold-resistant evergreen broad-leaved trees and the northernmost distribution of the species is recorded at Lao Mountain, Qingdao, China (36.16°N, 120.63°E). In Japan, the species is mainly distributed around coastal areas, and is strongly affected by winter monsoons and typhoons (Hattori, 1992). This species blooms in May and June, and its heterodichogamous flowers attract a variety of insects, such as bees, wasps, flies and beetles (Yumoto, 1987).

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The mature fruits of *M. thunbergii* are dark purple and entice various frugivorous birds (Pan et al., 2017), which can promote long-distance seed dispersal. Rats and ants may also disperse *M. thunbergii* seeds.

We collected fresh leaf tissues from 640 adult trees of 33 populations from the mainland of eastern China (MEC), the Zhoushan Archipelago (ZA), Kyushu (KS) and Honshu (HS; Figure 2a; Table S1 in Appendix S1). All voucher specimens were deposited at Shanghai Chenshan Botanical Garden Herbarium.

# 2.2 | Ecological niche modelling

To predict the palaeohabitats that were suitable for *M. thunber*gii during the last inter-glacial and glacial periods, we used the maximum-entropy approach in MAXENT 3.3.1 (Phillips & Dudík, 2008) to model the current distribution of *M. thunbergii*, and

reconstructed its potential distribution at the Last Interglacial (LIG) and the LGM. First, we searched Web of Science, the China National Knowledge Infrastructure, the Global Biodiversity Information Facility and the Chinese Virtual Herbarium using the keyword "M. thunbergii", and obtained 2717 records of its occurrence. We excluded the records obviously beyond the natural distribution range. Spatial autocorrelation may blur the modelling so we removed records that were close to others (within 48 km). Finally, a total of 221 records, together with our 33 sampling sites, were used for the modelling. We extracted the data for 19 climate variables from the WorldClim database (http://www.worldclim. org/) at a 2.5 in. spatial resolution for the present, LGM (MIROC and CCSM models), and LIG. Highly correlated bio-climatic (r > 0.7) variables were excluded using ENMTOOLS 1.3 (Warren et al., 2008), and four climatic variables (Bio1, Bio3, Bio16 and Bio19) were retained. We performed 20 independent model cross-validations,



FIGURE 2 Map of the *Machilus thunbergii* sampling sites in East Asia. Population codes are shown in Table S1 in Appendix S1. (a) Inner pie charts represent the frequencies of individuals assigned to different clusters by STRUCTURE (K = 2) and outer circles denote the geographical distribution of the five chlorotypes. (b) Principal coordinates results based on nSSR genetic distances. (c) Parsimony network for cpDNA haplotypes H1–H5. Circle sizes are proportional to the haplotype frequencies. Each solid line between circles represents one mutation step. Another species (M. *balansae*) was selected as the outgroup and is connected to H3 by 15 mutation steps. (d) The best-fitting model inferred by approximate Bayesian computation. The proportions of individuals from each group that belonged to different lineages are indicated by the pie charts. HS, Honshu Island; MEC, Mainland Eastern China;  $r_a$ , admixture rate from HS to ZA-KS;  $r_b$ , admixture rate from MEC to ZA-KS; SCM, secondary contact model;  $T_{ADM}$ , secondary contact time;  $T_{DIV}$ , divergence time between MEC and HS; ZA-KS, Zhoushan Archipelago and Kyushu Island

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with 75% of the data for training and the remaining 25% for testing. The area under the receiver operating characteristic curve (AUC) was used to evaluate model performance, where 1.0 represented perfect modelling and 0.5 suggested a random prediction (Elith et al., 2011).

# 2.3 | DNA extraction, amplification, genotyping and sequencing

We extracted total DNA from silica gel-dried leaves using a plant genomic DNA kit (Tiangen). The microsatellite loci developed for *M*. *thunbergii* by Kaneko et al. (2012) were screened across 30 samples from two populations, and 10 loci showing high-level polymorphism were selected (Table S2 in Appendix S1). Detailed amplification protocols and conditions are described in Kaneko et al. (2012). The products were scanned by an ABI 3730xl automated sequencer and scored using GENEMAPPER 4.0 (Applied Biosystems). We used four samples as positive controls in each 96-cell plate to improve the accuracy and uniformity across different batches. Alleles were called independently by two persons.

We amplified and sequenced 12 samples using five cpDNA fragments (*psbA-trnH*, *rpl32-trnL*, *trnL-trnF*, *trnV-trnM* and *trnH-ycf2*) for each population (a total of 361 sample, as even across populations as possible; Table S3 in Appendix S1). The primers were redesigned according to a *M*. *thunbergii* complete chloroplast sequence (GenBank accession number: NC\_038204.1). Detailed amplification protocols and conditions are described in Fan et al. (2016) and Wu et al. (2006). The amplified products were bidirectionally sequenced with an ABI 3730xl sequencer.

# 2.4 | Population genetic diversity and structure analyses

MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004) was used to correct genotyping errors in the nSSR dataset and to test for null alleles. The neutrality test was performed using LOSITAN (Antao et al., 2008) and the Hardy-Weinberg equilibrium test was performed using GENALEX 6.51 (Peakall & Smouse, 2012) on each population and locus. Genotypic linkage disequilibrium was tested in all locus pairs within each population by randomization using FSTAT 2.9.3 (Goudet, 2001) and adjusted by the sequential Bonferroni correction (Rice, 1989). The locus MT07 showed significant signs of divergent selection, so it was excluded from all subsequent analyses. In addition, we analysed all the populations, apart from three populations (BYX, JSD and XBX) that had less than five individuals. The number of alleles  $(N_{\Delta})$ , observed  $(H_{\Omega})$  and expected heterozygosity  $(H_{\rm F})$ , allelic richness (with rarefaction to a sample size of seven;  $A_{R}$ ) and the number of private alleles ( $N_{PA}$ ) were calculated for each population and the four groups (i.e. MEC, ZA, KS and HS) using GENALEX.

Comparable genetic diversity would be expected among the groups under the species pump hypothesis. Therefore, we compared  $A_{\rm R}$  and  $H_{\rm E}$  among the MEC, ZA, KS and HS groups using 1000 permutations in FSTAT. In addition, a gradient decline in genetic diversity may not be expected under the species pump hypothesis. Therefore, Pearson's correlation analysis was performed using R 3.5.2 (R Core Team, 2018) to test the relationship between allelic richness and longitude.

Stong population structure patterns are expected under the species pump hypotheses. We therefore estimated genetic differentiation by  $F_{sT}$  using FSTAT. The genetic variation structure was estimated by a hierarchical analysis of molecular variance (AMOVA) using GENALEX with significance tested by 10,000 nonparametric permutations. We also conducted a Bayesian clustering analysis using STRUC-TURE 2.3.4 (Pritchard et al., 2000). Twenty independent replications were performed for each K (K = 1-20) with  $10^5$  burn-in steps followed by 10<sup>6</sup> MCMC steps. The admixture ancestry model with correlated allele frequencies was used. The optimal K was determined by both LnP(D), that is, the change in log-likelihood of the data for each run, and  $\Delta K$ , that is, the second-order rate of change for LnP(D) between successive values of K. The program StructureSelector was used to calculate  $\Delta K$  and produce the final graphics (Li & Liu, 2018). A principal coordinate analysis (PCoA) implemented in GENALEX was also used to infer population structure based on the genetic distance among populations.

Assignment tests in an MCMC-based Bayesian framework implemented in BAYESASS 3.04 (Wilson & Rannala, 2003) were used to calculate the contemporary (within the past few generations) migration rate ( $m_c$ ) between pairs of the four groups. We ran  $10^7$  iterations after a burn-in of  $10^6$  with a sampling frequency of  $10^3$ . Delta parameter values (a-allele frequency, f-inbreeding value and m-migrate rate) were adjusted to keep the accepted rate between 20% and 60% to maximize the log-likelihood values. The trace files for the log-probability were plotted to diagnose convergence.

We used BioEDIT 7.0.8 (Hall, 1999) to concatenate and align the five cpDNA fragments in the cpDNA dataset. DNASP 5.10.01 (Librado & Rozas, 2009) was employed to determine chlorotypes, and to estimate nucleotide diversity ( $\pi$ ) and haplotype diversity (h) according to Nei (1987) for each population. NETWORK 4.6 (Bandelt et al., 1999) was used to construct the relationship among haplotypes based on sequence variation. The G<sub>ST</sub> and N<sub>ST</sub> values were calculated by PERMUTECPSSR 1.0 (Pons & Petit, 1996), and their differences were tested using 10<sup>5</sup> random permutations. A significantly higher N<sub>ST</sub> than G<sub>ST</sub> indicates the presence of a phylogeographical structure.

We also estimated the pollen-to-seed migration ratio (r) using the Ennos (1994) method:

$$r = \left(\left(\frac{1}{F_{\mathsf{ST}(n)}} - 1\right) - 2\left(\frac{1}{F_{\mathsf{ST}(c)}} - 1\right)\right) / \left(\frac{1}{F_{\mathsf{ST}(c)}} - 1\right),$$

where  $F_{ST(n)}$  and  $F_{ST(c)}$  are the estimates of genetic differentiation based on the nSSR and cpDNA markers, respectively.

#### 2.5 Population demography analyses

Population demographic history, including population size change, and population divergence and admixture was inferred using approximate Bayesian computation (ABC; Beaumont, 2010) and microsatellite data. Although it usually has large confidence intervals, the ABC approach provides accurate estimations of divergence and admixture events, especially those during ancient periods (Excoffier et al., 2005). We divided the populations into three genetic groups based on the STRUCTURE and PCoA results, which were MEC, ZA-KS and HS. We randomly selected 100 individuals from each group following Tamaki et al. (2018) and Setsuko et al. (2020).

We first tested three single-population models of different population size changes for each group (Figure 3a). Specifically, the standard neutral model (SNM) assumed that there was no size change in the past; the population growth model assumed that the population grew exponentially at rate G from the past to the present; and the instantaneous size change model assumed that population size changed instantaneously at time T. Details about the model specifications can be found in Text S1 in Appendix S3.

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Then, using the best population size change models and the estimated effective population sizes of MEC and HS, we constructed and compared four two-population scenarios to model divergence between MEC and HS (Figure 3b). The isolation without migration model (IOM) assumed that HS diverged from MEC at time  $T_{DIV}$  and no gene flow occurred from  $T_{\text{DIV}}$  to the present; the isolation with migration model was the same as the IOM except there was bidirectional gene flow from  $T_{DIV}$  to the present; the vicariance without migration model (VOM) assumed that MEC and HS diverged from their common ancestor at time  $T_{\text{DIV}}$  and that no gene flow occurred from  $T_{DIV}$  to the present; and the vicariance with migration model (VWM) was the same as the VOM except that bidirectional gene flow occurred from  $T_{\rm DIV}$  to the present. Further details about the model specifications can be found in Text S1 in Appendix S3.



FIGURE 3 Candidate models for population size change and population divergence. (a) ICM, instantaneous size change model; PGM, population growth model; SNM, standard neutral model. (b) IOM, Isolation without migration model; IWM, Isolation with migration; VOM, Vicariances without migration model; VWM, Vicariances with migration model. (c) EFM01, eastward dispersal with subsequent fragmentation model; SCM, secondary contact model; TDM01, trifurcation divergence model. G, growth rate; HS (H), Honshu Island; MEC (M), Mainland Eastern China; ZA-KS, Zhoushan Archipelago and Kyushu Island; N<sub>ANC</sub>, ancestral effective population size; Nm<sub>ii</sub>, number of migrants per generation from *i* to *j*;  $r_a$ , the admixture rate; *T*, event time related to population size change or population divergence;  $T_{ADM}$ , event time related to the new divergence or admixture event; T<sub>DIV</sub>, event time related to the divergence time. Bold arrows denote the admixture event, and the thin arrows represent migration

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We assessed the relationships among MEC, ZA-KS and HS using the best-supported divergence scenario between MEC and HS, and the best population change model for ZA-KS. Too many similar scenarios often undermine support for the best model (Setsuko et al., 2020). Therefore, we constructed three possible three-population models after the preliminary analyses (see Text S2 in Appendix S3) to infer the isolation and admixture events (Figure 3c). The eastward dispersal with subsequent fragmentation model (EFM01) assumed that ZA-KS and HS diverged at time  $T_{ADM}$  from the ancestry population, which diverged from MEC at time  $T_{DIV}$  Bidirectional gene flow occurred between MEC and ZA-KS and between HS and ZA-KS. The secondary contact model (SCM) assumed that HS diverged from MEC at time  $T_{DIV}$  and the admixture event occurred with admixture rate  $r_a$ :  $(1 - r_a)$  to form ZA-KS at time  $T_{ADM}$ . There was no gene flow from  $T_{ADM}$  to the present. The trifurcation divergence model (TDM01) assumed that the three groups simultaneously diverged at time  $T_{\text{DIV}}$  and there were bidirectional gene flows between MEC and ZA-KS, and between HS and ZA-KS. Further details about the model specifications can be found in Text S1 in Appendix S3.

We used the *runif* function in R 3.5.2 (R Core Team, 2018) to generate priors and conducted coalescent simulations with FASTSIM-COAL 2.6.0.3 (Excoffier & Foll, 2011). Six summary statistics, that is, averages and standard deviations of three parameters (number of alleles, expected heterozygosity and allele size range), were used for the single-population models. These six summary statistics and two additional statistics (global  $F_{ST}$  and pairwise  $F_{ST}$ ) were calculated for the two- and three-population models. In total, six, 13 and 22 summary statistics were used for the single-, two- and three-population models, respectively. The simulations were repeated 10<sup>5</sup> times and summary statistics were calculated using ARLSUMSTAT 3.5.2 (Excoffier & Lischer, 2010). The parameter details and a FASTSIMCOAL model definition are given in Text S1 in Appendix S4.

The generalized stepwise mutation model (GSM) was used in the nSSR mutation model (Estoup et al., 2002). The GSM has two parameters, mutation rate per generation  $(\mu)$  and geometric parameter for GSM ( $P_{GSM}$ ).  $P_{GSM}$  ranges from 0 to 1 and represents the proportion of mutations that will change allele size by more than one step. For the nSSR, we simulated eight independent loci whose upper limit in repeat number was set to 40. The mean mutation rate was fixed at  $5.0 \times 10^{-4}$  (Bai et al., 2014; Estoup et al., 2002), and the individual  $\mu$  value was randomly drawn from the Gamma distribution (shape, rate). The prior distribution of the parameter shape was drawn from the Uniform distribution (0.5, 5) and the parameter rate was then calculated by shape/mean  $\mu$ . The prior distribution for the mean  $P_{GSM}$  value was drawn from the Uniform distribution (0, 1) and each locus value was randomly drawn from the Beta distribution (a, b). The *a* and *b* values were calculated using 0.5 + 199 × mean  $P_{\text{GSM}}$  and  $a \times (1 - \text{mean } P_{\text{GSM}})/\text{mean } P_{\text{GSM}}$ , respectively (Excoffier et al., 2005). The prior distributions and ranges of other structure parameters for the single-, two- and three-population models can be found in Table S4 in Appendix S1.

We conducted model selection in R 3.5.2 using the Random Forest approach implemented in the ABCRF package (Pudlo et al.,

2016). One thousand trees were constructed and the best model was selected by the highest vote rate. To evaluate the model selection, we calculated the confusion matrix and thereby obtained misclassification rates and the out-of-bag (OOB) error rate. We also performed and evaluated the model selection using the ABC method and package ABC 2.1 (Csilléry et al., 2012). For the best models, we used 10<sup>5</sup> simulations using the package ABC to estimate the posterior distributions of parameters using neural network regression with logit transformation. The tolerance value was set to 0.05 and the summary statistics of 5000 simulations nearest to the observed data were used for parameter estimation. The posterior mode and 95% highest posterior density (HPD) were calculated using CODA package 0.19.1 for R (Plummer et al., 2006). We used 10 years per generation to convert the time parameters from generation to year (Fan et al., 2016) and set the generation length as 15 years to obtain the upper limits of the estimated values for the time parameters.

The mutation rate assumption may have a substantial impact on the posterior values. Therefore, we set mutation rates at  $10^{-3}$  and  $10^{-4}$ , and reran the best model with  $10^5$  simulations. We compared the best models using the different mutation rates and the Random Forest and ABC methods, and evaluated the confusion matrix and posterior probabilities. The posterior mode and 95% HPD were estimated for the different mutation rates.

We used 1000 samples randomly produced from the posterior distribution to verify the model fitting for the observed data in the best two- and three-population models. To validate that the preferred model provided a good fit to the data, we superimposed the observed value on the histogram for the null distribution under the best model (Csilléry et al., 2012). We computed the *p*-value to test the fit to the best model, and an insignificant *p*-value indicated a good fit. We additionally tested whether the two other demographic models (EFM01 and TDM01 in Figure 3c) provided a good fit for the observed data. The *gfit* function in package ABC 2.1 (Csilléry et al., 2012) was used to perform the model goodness-offit analysis.

# 3 | RESULTS

# 3.1 | Ecological niche modelling

The modelling results showed high AUC values for LGM-MIROC (0.998), LGM-CCSM (0.924) and LIG (0.936), indicating that the three model predictions were accurate. *M. thunbergii* populations occupied mainland China, Japan and Korea, but were isolated by the extensive ocean during the LIG (Figure 4). In particular, mainland Eastern China, Taiwan Island, Kyushu Island and the western coast of the Honshu harboured extensive, suitable habitats for this species. Those areas were still habitable during the LGM, but the exposed ECS seabed also provided suitable habitats (Figure 4). The comparison between the LIG and LGM predictions showed that suitable habitats were uniformly scattered across most regions of East Asia during the LIG, but mainland Eastern China, the exposed ECS



FIGURE 4 Predictions made by species distribution modelling for Machilus thunbergii (a) at present; (b) during the LGM under the MIROC model; (c) during the LGM under the CCSM model; and (d) during the LIG. Predicted distribution probability is shown as a logistic value with a 2.5 arc-min pixel solution. Black dots indicate the specimen localities used for the modelling. LGM, Last Glacial Maximum; LIG, Last Interglacial

seabed, and southern coastal regions of Japan had more extensive and suitable habitats than other regions during the LGM.

#### 3.2 Genetic diversity and structure based on microsatellites

There were 145 alleles at the remaining nine loci after excluding one locus (MT07) that showed significant signs of homozygote excess and divergent selection. These loci possessed high allelic diversity, with  $N_{\Lambda}$  ranging from 6 (MT03) to 35 (MT10). No significant genotypic linkage disequilibrium was detected across all populations after correction. Detailed locus information is shown in Table S5 (Appendix S1). Across the study range, allelic richness was not correlated with longitude (p = 0.188; Figure 5a). The  $N_A$ ,  $N_{PA}$ ,  $H_O$ ,  $H_{\rm E}$ ,  $A_{\rm R}$  and  $F_{\rm IS}$  values varied widely for all populations (Table S1 in Appendix S1). There were significant differences in  $H_0$  (p = 0.002) and  $F_{1S}$  (p = 0.001) among MEC, ZA, KS and HS, but no significant differences found for  $A_{R}$  (p = 0.600) and  $H_{F}$  (p = 0.195; Figure 5b,c).

Out of the 129 alleles found in Japanese populations (including HS and KS), 39 (30.2%) were not found in MEC and 22 (17.1%) were not found in either MEC or ZA.

In the STRUCTURE analysis, the true number of clusters was difficult to determine based on LnP(D), which increased progressively as K increased. The  $\Delta K$  statistic was highest at K = 2. We identified a western lineage and an eastern lineage when K = 2. The MEC populations were dominated by the western lineage and the HS populations mainly belonged to the eastern lineage. However, the ZA and KS populations were admixtures of the two lineages, probably constituting a third cluster (Figure S1 in Appendix S2). The PCoA analysis also separated the populations into three groups: MEC, ZA-KS and HS (Figure 2b).

The overall  $F_{\rm ST}$  was 0.098, and pairwise  $F_{\rm ST}$  values ranged from -0.024 (between populations TBS and JSS) to 0.396 (between populations GPC and HGS). The hierarchical AMOVA analysis showed that most of the molecular variance could be explained by intra-population genetic variation. Only 3% of the variance was attributable to the variation between the Chinese and Japanese populations. This small



Component proportion of East China using STRUCTURE results



Proportion of Japanese haplotype

**FIGURE 5** (a) Relationship between allelic richness and longitude. (b) Quartile bar plot of allelic richness in each region. (c) Quartile bar plot of expected heterozygosity in each region. (d) Population genetic structure according to the STRUCTURE results and haplotype distributions. Red represents the Bayesian probability, and green open dots represent the proportion of Japanese haplotypes in each population. HS (green): Honshu Island; KS (yellow): Kyushu Island; MEC (red): Mainland Eastern China; ZA (pink): Zhoushan Archipelago

|                  |                     | Proportic    | on of votes     |                |       |       |       |       |       |       |       | Doctarior   | OOB error |
|------------------|---------------------|--------------|-----------------|----------------|-------|-------|-------|-------|-------|-------|-------|-------------|-----------|
| Analysis         | Lineage             | SNM          | PGM             | ICM            | MOI   | IWM   | MOV   | MWV   | EFM01 | SCM   | TDM01 | probability | rate      |
| M <sub>One</sub> | MEC                 | 0.784        | 0.125           | 0.091          |       | I     | I     | I     | I     | I     | I     | 0.787       | 0.323     |
|                  | ZA-KS               | 0.590        | 0.104           | 0.306          |       | I     | I     | I     | I     | Ι     | I     | 0.608       | 0.323     |
|                  | HS                  | 0.825        | 0.057           | 0.118          |       | I     | I     | I     | I     | I     | I     | 0.830       | 0.322     |
| $M_{Two}$        | MEC & HS            | I            | I               | I              | 0.550 | 0.014 | 0.397 | 0.039 | I     | I     | I     | 0.594       | 0.525     |
| $M_{Three}$      | AII                 | I            | I               | I              | I     | I     | I     | I     | 0.011 | 0.939 | 0.050 | 0.943       | 0.093     |
| lote: Best m     | odel selected by th | e Random Fo. | rest analysis i | is shown in bo | ld.   |       |       |       |       |       |       |             |           |

two-population divergence model; PGM, population growth model; SCM, secondary contact model; SNM, standard neutral model; TDM01, trifurcation divergence model; VOM, Vicariance without migration model; VWM, vicariance with migration model single-population size change models;  $\mathsf{M}_{\mathsf{Three}}$ , three-population divergence and admixture model;  $\mathsf{M}_{\mathsf{Two}}$ 

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value was probably caused by genetic admixtures in the ZA and KS populations. When we excluded the ZA and KS populations from the analysis, 13% of the variance could be attributed to between Chinese and Japanese populations (Table S6 in Appendix S1).

The BAYESASS analysis showed very low contemporary migration rates among the four groups. Of the 12 pairwise estimates, only one showed unambiguous evidence of contemporary migration between populations (from KS to HS with a 95% CI of 0.062–0.118 for  $m_{a}$ ), while the 95% CI for  $m_c$  overlapped or approximately overlapped zero for the other populations (Figure S2 in Appendix S2).

#### Genetic diversity and differentiation based on 3.3 cpDNA markers

We concatenated the five cpDNA fragments into one sequence of 3519 bp in length. Five haplotypes (H1-H5) with four variable sites were identified across all populations (Figure 2c; Table S7 in Appendix S1). The total nucleotide diversity ( $\pi_{T}$ ) was 5.02 × 10<sup>-3</sup>. All the Japanese populations had only one haplotype (H4), which was also shared by the ZA populations. All five haplotypes were found in China, but most populations had only one haplotype (Figure 2a). The proportion of the Japanese haplotype (H4) declined sharply from 1 in the ZA populations to 0 in most of the MEC populations (Figure 4d). The  $N_{sT}$  value was significantly higher than  $G_{ST}$  across all populations ( $N_{ST}$  = 0.861,  $G_{ST}$  = 0.817; p = 0.010), indicating a strong phylogeographical structure.

The pollen-to-seed dispersal ratio estimate was 46.42 according to the nuclear and chloroplast differentiation, indicating that pollen made a much larger contribution than seed dispersal to the genetic structure of M. thunbergii populations.

#### 3.4 Population demography history

The Random Forest analyses showed low OOB error rates when modelling population size change. For groups MEC, ZA-KS and HS, the SNM had the highest vote rates of 0.784, 0.590 and 0.825, with posterior probabilities of 0.787, 0.608 and 0.830, respectively (Table 1; Table S8 in Appendix S1), indicating that all the three groups had a relatively stable effective population size from the past to the present. The structural parameters for the SNM in the three groups showed a single peak (Figure S3 in Appendix S2), and current population sizes  $(N_{CLIP})$  were larger in ZA-KS (8168) than in MEC (4324) and HS (4616).

When considering the divergence between MEC and HS, the Random Forest analyses showed that the isolation-withoutmigration model (IOM) had the highest vote rate (0.550) followed by the vicariance-without-migration model (VOM; 0.397). Due to the relatively high classification error rate (0.525), we extracted and compared the IOM and VOM scenarios. The results showed that IOM had a higher vote rate (0.588) and the classification error rate was 0.291. Therefore, IOM was the best model. This was also supported by the ABC-based model selection analysis, which produced posterior probabilities of 0.593 and 0.407 for IOM and VOM, WILEY- Journal of Biogeography

respectively. Summary statistics for the observed data are shown in Figure S4 (Appendix S2). All posterior distributions showed a clear single peak under scenario IOM (Figure S5 in Appendix S2). The observed values of 13 summary statistics were covered by 95% HPD (Figure S6 in Appendix S2), indicating good agreement between the observed and simulated datasets under IOM. The estimate of the divergence time ( $T_{\text{DIV}}$ ) between MEC and HS was 6836 (95% HPD: 2397-107,682) generations ago.

With regards to the relationships among MEC, ZA-KS and HS, the secondary contact migration (SCM) scenario showed the highest voting rate of 0.939 (Table 1) and a low OOB error rate (0.093). The ABC model selection also supported SCM as the best model because it had the highest posterior probability (0.932) and a low misclassification rate (0.024, Texts S2 and S3 in Appendix S3). Summary statistics for the observed data are shown in Figure S7 (Appendix S2). All posterior distributions showed a single peak under scenario SCM (Figure S8 in Appendix S2). The observed values of the 22 summary statistics were covered by 95% HPD (Figure S9 in Appendix S2), indicating good agreement between the observed and simulated datasets under SCM. Furthermore, the high goodness-of-fit demonstrated that the observed data were described well by SCM (p = 0.779), but poorly by EFM01 (p = 0.065) and TDM01 (0.251); Figure S10 in Appendix S2). The estimate for the timing of the admixture between MEC and HS (T<sub>ADM</sub>) was 1458 (262–3153) generations ago (Figure 2d; Table S9 in Appendix S1). The admixture proportion was 46% from MEC and 54% from HS (Figure 2d).

The estimates for divergence and admixture time under the different mutation rate and generation length assumptions are summarized in Table S10 (Appendix S1). The highest vote rates in the Random Forest analyses (Table S10 in Appendix S1) and the highest posterior probabilities in the ABC-based model selection (0.827 and 0.843 for the two-population and three-population models, respectively) were both found when assuming a moderate mutation rate (0.0005). With generation lengths of 10 and 15 years, the admixture time would be about 15 and 22 kyr, respectively, and the divergence times were 68 and 103 kyr, respectively.

# 4 | DISCUSSION

The ECS via a species pump is widely hypothesized to have driven diversification and contributed to high diversity across East Asia (Luo et al., 2021; Qiu, Sun, et al., 2009; Wang et al., 2013). In our study, both the species paleo-distribution reconstruction and population demographic modelling supported alternating population isolation during interglacial periods and connection during glacial maxima across the ECS. However, the estimated time of the trans-ECS divergence suggested that this isolation and connection periodicity did not promote diversification as suggested by the species pump hypothesis. Instead, the tempo of coalescence with regard to the generation length for *M. thunbergii* was broadly so rapid that incipient divergence was periodically wiped out, rendering the repeated isolation-connection cycles across the ECS a "species vacuum".

# 4.1 | Late Pleistocene trans-ECS divergence

The ABC analyses indicated divergence between the Chinese mainland lineage and that of Japan during the Late Pleistocene (c. 68 kyr). This suggested that the ECS started playing a role as a barrier around that time. It is consistent with the rising sea-level after the major glacial period MIS4 glaciation (c. 70 kyr, De Deckker et al., 2019) when the sea level was about -100 m according to the KL11 age model (Siddall et al., 2003). Then, submergence of the ECS land bridge separated the Chinese and Japanese lineages. The divergence between Chinese and Japanese populations of *Neolitsea sericea* is estimated to have occurred at a similar time (65–196 kyr; Cao et al., 2018).

The ECS probably acted as a dispersal corridor before that time, otherwise the estimate of divergence time would be much earlier in light of the long history because of the separation between Japan and the Eurasian continent (Taira et al., 2016; Wakita, 2013). The species might have been present on both sides of the ECS before the formation of the corridor and then came into contact through the ECS land bridge at about 70 kyr. An alternative scenario suggested that the species might have been absent on one side until migrants from the other side colonized around that time (Luo et al., 2021). However, we have unambiguously identified two distinct lineages on the Chinese mainland and in Japan. About 30% of alleles in the Japanese populations were absent in the MEC populations, and similar levels of genetic variation, in terms of allelic richness and expected heterozygosity, were found in the HS and MEC populations (Figure 5b,c).

The Honshu populations were probably dispersed from the Chinese populations before the Late Pleistocene, followed by fragmentation due to submergence of the ECS land bridge. Sea level fluctuations during the Pleistocene provided multiple opportunities for contacts between Chinese and Japanese flora via the ECS land bridge. A mid-Pleistocene link between the Chinese and Japanese lineages has been reported for *P. arguta* (Qiu, Qi, et al., 2009), *K. pal-mata* (Qiu, Sun, et al., 2009) and *Cardiandra* (Setoguchi et al., 2006). Chinese populations of *N. sericea* are suspected to have had contact with Japanese populations in the Late Pleistocene (Cao et al., 2018). *M. thunbergii* dispersed from mainland China to Japan no later than the mid-Pleistocene because *M. thunbergii* fossils have been reported in the Yoshino formation (*c.* 370 kyr) of Japan (Iwauchi & Hase, 1992). However, the exact expansion time is not known.

# 4.2 | Lineage admixture at the LGM

Most of the previous phylogeographic studies on Sino-Japanese disjunct species, although few in absolute number, have not found any genetic signal for lineage admixing on either side of the ECS (Qiu et al., 2011) except for *N. sericea* (Cao et al., 2018). Those studies were inconsistent with the hypothesis proposed by Qian and Ricklefs (2000) that the ECS rejoined plant distributions during the LGM. In contrast, we observed in *M. thunbergii* that the cpDNA haplotype of the eastern lineage (H4) exclusively dominated the ZA populations, and we found co-occurrence of haplotypes from the two divergent

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lineages in two nearby mainland populations (LCS and TTS). These patterns, together with the clinal structure at the nuclear microsatellite loci from mainland China across the ZA and KS to HS, provided strong evidence that the ECS acted as a land bridge in mediating secondary contact after post-dispersal divergence.

The contact probably involved at least two expansion events. One was the extension of M. thunbergii from Japan through the ECS land bridge to KS and the ZA, presumably during the LGM. The ABC framework also indicated that our genetic data were best explained by the admixture of the two lineages during the LGM (around 15 kyr, Figure 2d). Despite a unique H5 haplotype derived from the Japanese haplotype H4 in TTS, a possible extension from the TTS-LCS-Zhoushan region to Japan can be ruled out because the divergence between the two lineages and the fossil records long predate the LGM, and the Japanese populations had higher allelic richness and more private alleles than the Zhoushan populations (Figure 5b; Table S1 in Appendix S1). The presence of H5 might be the outcome of fast post-colonization mutation or from sampling biases. The second expansion event was the eastward expansion of the Chinese lineage. However, the cpDNA and nDNA markers showed different leading edges. The leading edge of the chloroplast DNA range expansion of the most common haplotype on mainland China (H3) contacted with the Japanese lineage in the TTS-LCS-Zhoushan zone. Nuclear DNA genomes dispersed to populations on islands in the ECS and KS, and similar proportions of Chinese and Japanese lineages admixed and formed the ZA-KS group (Figure 2d).

Differences in the expansions of the cpDNA and nDNA genomes caused a cytoplasmic-nuclear discordance in the genetic structure within the contact zone, particularly on the ZA where a large part of the microsatellite polymorphism was shared with the western lineage, but none of the cpDNA haplotypes from the western lineage were detected (Figure 2a). The discordance was likely to be the result of asymmetrically large M. thunbergii pollen flow compared to its seed flow. This large pollen flow allowed nuclear gene interchange among island and mainland populations leading to the observed clinal pattern for microsatellites. In contrast, the very limited seed dispersal meant that haplotype H3 was unable to invade the ZA. This explanation was supported by our estimate of the pollen-to-seed dispersal ratio, which was much larger than the median value across 93 species from Petit et al. (2005; 46 vs. 17). It is surprising that small insects instead of large birds play a more critical role in dispersing genes in the insectpollinated ornithochores, given the apparently short dispersal distances of insects and that a pollen grain carries one copy of genes while a seed carries two copies (Chen et al., 2008). Such underestimations of pollen dispersal by direct observation compared to those indirectly inferred from genetic markers are common in insect-pollinated plants, that is, Slatkin's paradox (Liu et al., 2015; Slatkin, 1987).

# 4.3 | Impacts of Pleistocene climatic oscillations on divergence

Pleistocene climatic oscillations provide opportunities for individuals from different distribution areas to swap (Shi et al., 2014; Tong

et al., 2021). They also lead to repeated isolation and connection within species (Hewitt, 2000, 2004, 2011). When individuals from separated populations have a large genetic difference, full or partial reproductive isolation will hinder the hybridization between those individuals (Baack et al., 2015; Satokangas et al., 2020; Servedio & Hermisson, 2020). In such cases, repeated isolation and connection will facilitate speciation (Brown et al., 2013; Gillespie & Roderick, 2014). These separated populations evolve to a stage where fully reproductive isolation is crucial because the secondary contact populations are less able to produce fertile offspring after this stage. Different tempos of isolation and connection cycles may lead to a continuum from none to partially and then to fully reproductive isolation in different areas (Papadopoulou & Knowles, 2015b; Wang et al., 2014). Furthermore, the same periodicity of isolation and connection may mold different evolutionary outcomes for species with different generation lengths (Miyatake & Shimizu, 1999). It has also been argued that other taxon-specific traits, such as dispersal ability and habitat association, may have affected divergence under the Pleistocene climatic oscillations (Papadopoulou & Knowles, 2015a).

The impacts of generation length on the role of Pleistocene climatic oscillations, whether as a "species pump" or "species vacuum", could be substantial, at least for the ECS. In plants, it has been suggested that species with relatively long generation length (e.g. trees and shrubs) show low rate of molecular evolution (Smith & Donoghue, 2008). In the ECS, large genetic divergence has been reported in some herbal species with short generation length (e.g. C. japonica, K. palmata, L. hodgsonii and K. japonica), indicating that the repeated cycles of isolation and connection promoted species diversification (Luo et al., 2021; Oiu, Sun, et al., 2009; Wang et al., 2013). However, in some long-lived woody species with relatively long generation length (e.g. N. sericea, Kalopanax septemlobus and Quercus variabilis), only small amounts of genetic divergence have been found between Chinese and Japanese populations, reflecting the effects of genetic homogenization through population connection during glacial periods (Chen et al., 2012; Sakaguchi et al., 2012; Zhai et al., 2012). M. thunbergii is a long-lived tree with a relatively long generation length. Thus, the reproductive isolation process was probably repeatedly broken up by secondary contact through the emergence of the ECS land bridge while the speciation time is longer than the periodicity of isolation and coalescence.

# 5 | CONCLUDING REMARKS

The ECS has long been postulated to have separated and rejoined plant distributions as a consequence of sea level fluctuations in the Milankovitch cycles (Qian & Ricklefs, 2000), but there is only strong evidence of isolation for the role, whereas the corridor function has rarely been proved (Qi et al., 2012; Qiu et al., 2011). Our study showed that the Chinese and Japanese *M. thunbergii* populations experienced post-dispersal divergence in the Late ILEY- Journal of Biogeography

Pleistocene, but secondarily contacted through the ECS land bridge during the LGM, which generated a genetic admixture within the relict populations on the ZA and KS. The isolation and connection cycles seemed to have reduced rather than enhanced the divergence of M. thunbergii populations between western and eastern populations of the ECS. Thus, it remains an open question whether Pleistocene climatic oscillations actually contributed to the formation of high biodiversity in the Sino-Japanese regions in a species pump manner. This issue requires further investigation into speciation processes, such as adaptive divergence and reinforcement, but our results highlighted that the ECS played a role in shaping biogeographic patterns because it acted as a dispersal corridor during glacial periods, including the LGM. This corridor might have more widespread effects than previously suggested according to a palaeovegetation reconstruction, which indicated a temperate and deciduous forest band extending across the ECS land bridge (Harrison et al., 2001), and the significance of the land bridge to the biogeography and evolution of evergreen broadleaved forests may have been overlooked.

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# DATA AVAILABILITY STATEMENT

All of the gene sequences have been deposited in GenBank under the accession numbers MT152303 to MT52312. Microsatellite data and distribution information for ecological niche modelling are available on Dryad (https://doi.org/10.5061/dryad.9zw3r22f5).

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# BIOSKETCH

Kai Jiang is a researcher at the Shanghai Chenshan Botanical Garden and a PhD candidate of the East China Normal University. This collaborative work reflects the research interests of the authors in understanding the evolutionary history and phylogeography of endemic, dominant and widely distributed evergreen broad-leaved tree species in East Asia.

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Author contributions: K.J. and X.-Y.C. conceived the ideas; K.J. and Z.-W.W. conducted the fieldwork and collected the samples; K.J. and L.-Y.M. conducted the laboratory work; K.J., X.T., Y.-E.X., W.-C.H., Y.-H.H. and X.-Y.C. analysed the genetic data; K.J. and Y.-Q.D. performed the ABC analyses; K.J. performed the ENM analyses; K.J., T.X. and X.-Y.C. led the writing with contributions from Y.-E.X., W.-C.H. and Y.-H.H. All authors gave final approval for publication.

# SUPPORTING INFORMATION

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