


CONTRIBUTED PAPER

Genetic origins and climate-induced erosion in economically important Asian walnuts

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Abstract

The global climate is undergoing unprecedented changes, posing significant threats to species persistence. However, the spatiotemporal impacts on genetic diversity remain poorly understood, hindering species conservation and management. Walnuts, generally referred to as *Juglans regia* and *J. sigillata*, are economically vital in Asia, but little is known about their genetic origins and how the species will be affected by future climate change. Using 31 microsatellites, we genotyped 5282 individuals from 233 populations of walnuts in Asia. We assessed genetic diversity patterns and demographic history and investigated potential future genetic erosion risks. Genetic diversity of walnuts was high in the Himalaya and Hengduan Mountains. The 2 species diverged during the Pleistocene (around 1.41 Ma BP), and *J. regia* contained 2 genetic groups (JR1 and JR2). The JR2 group had the lowest diversity and likely arrived in northern China around 9.77 ka BP, perhaps via human transport. The Western Himalaya likely served both as a glacial refugium and the center of origin for *J. regia*, and the Eastern Himalaya appears to have been the refugium for *J. sigillata*. The 2 species appear to have hybridized in the Central Himalaya and the Sichuan basin and surroundings, forming two distinct hybrid zones. Our results indicate that genetic diversity

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will be reduced by up to 9.03% due to range loss under future climate change and dramatic genetic structure turnover in the Himalaya and Hengduan Mountains. In situ conservation in the Himalaya is essential for safeguarding genetic diversity and adaptive potential in Asian walnuts, while ex situ preservation of genetically unique wild germplasm, coupled with its integration into breeding programs, will enhance climate resilience. The findings advance our understanding of the origin of Asian walnuts and how future climatic change may affect their genetic diversity, offering a model for conservation and breeding strategies in other tree species facing similar threats.

KEYWORDS

climate change, ecological niche modeling, genetic diversity, genetic erosion, *Juglans regia*, landscape connectivity

INTRODUCTION

Human activity is driving rapid changes in climate, which will have profound ecological consequences that may ultimately affect the evolutionary potential of some species (Lovell et al., 2023; Scheffers et al., 2016; Urban et al., 2016). Climate change has already affected biota and ecosystems, altering species distributions, genetic diversity, and population structure (Legge et al., 2023; Pecl et al., 2017; Vaccarelli et al., 2023; Wambulwa et al., 2025). Global temperatures have risen by about 1°C above preindustrial levels, with many regions experiencing extreme climate events (Kogan, 2023). Further substantial temperature increases (1.5–2°C) are expected by 2100 (Pörtner et al., 2022; Smith et al., 2015). Given the threat posed by these environmental upheavals to biodiversity, there is an urgent need to accurately predict biological responses to forthcoming climate change (Urban et al., 2016). Consequently, understanding how the distribution and genetic diversity of species will respond to future climate change is a matter of priority.

Genetic diversity is a fundamental aspect of biodiversity. It represents the evolutionary potential of species and is an essential requirement for the species' long-term survival (Leroy et al., 2020; Pauls et al., 2013). When genetic diversity is decreased, reduced adaptability to climatic (or other) changes can render species or populations more vulnerable to local extinction (Abreu-Jardim et al., 2021; Chung et al., 2023). From a conservation perspective, it is important to identify populations that have high genetic diversity and are expected to persist despite the projected climate change (Abreu-Jardim et al., 2021; Foden et al., 2018). A first step toward this goal is to map patterns of extant genetic diversity and to reconstruct the population demographic history across divergent populations (Gaut et al., 2018; Martin, Sheppard, et al., 2023; Quinn et al., 2023). In addition, genetic diversity depends on landscape connectivity, that is, the degree to which individuals or propagules disperse throughout the landscape (Baguette et al., 2013; Mony et al., 2022). Reduced landscape connectivity can lead to increased population isolation and thus increased inbreeding and genetic drift, which erode genetic diversity and elevate extinction risks (Miles et al., 2019). Knowledge of the landscape connectivity of species allows for the identification of populations that may

be evolutionarily discrete, functionally isolated, or vulnerable to extirpation.

Ecological niche modeling (ENM), a class of tools that predict species' potential suitable areas, is widely used to explore species vulnerability under future climate change (Smith et al., 2019; Thuiller, 2024). Traditionally, ENM is based on the assumptions that all populations share the same niche and that local adaptation has no effect (Razgour et al., 2019; Smith et al., 2019). Hence, analyses that harness information on the genetic structure to subdivide species into evolutionarily distinct units before ENM can improve model accuracy and enhance understanding of the risk faced by species under global change (Bothwell et al., 2021; Chardon et al., 2019; Ikeda et al., 2017). Quantifying connectivity is fundamental to predicting how populations will respond to future climate change. Traditionally, most methods for quantifying connectivity relied on landscape resistance; however, they fail to differentiate landscape resistance from the risk of mortality (Zeller et al., 2012). Conversely, the spatial absorbing Markov chain (SAMC) framework addresses this limitation by incorporating movement behavior and mortality risk with random walk theory (Fletcher et al., 2019; Marx et al., 2020). Furthermore, ancestry distribution models can forecast the potential impact of future climate change on genetic structure with spatially explicit simulations based on correlations between environmental variables and individual ancestry (Jay et al., 2012, 2015; Liu, 2022). Combining multiple approaches can more effectively identify populations of conservation value. Coupling multiple spatial ecological analyses with population genetics can provide novel insights into the impact of climate change on genetic diversity and population structure (Bernatchez et al., 2023; Bothwell et al., 2021; Jeon et al., 2024; Leites & Benito Garzón, 2023).

The sessile nature of plants makes them especially susceptible to climate change, and artificial selection may exacerbate crop vulnerability and thus affect yield quantity and quality (Meza et al., 2023). Indeed, climate change has affected crop distribution and productivity in the past decades, posing substantial threats to global food security. Most studies on the impacts of climate change on crops have focused on annuals (Hasegawa et al., 2022; White et al., 2011). However, annual crops can be more rapidly adjusted by shifting sowing times or switching

among cultivars at a given site. In contrast, perennial species, particularly trees, face more severe challenges due to their long life cycles and the need for long-term management planning. However, such crops have received little attention (Meza et al., 2023).

Juglans regia (common, English or Persian walnut) and *J. sigillata* (iron walnut) are economically important trees, producing the most valuable nuts in the walnut family. The global in-shell walnut production in 2022 was 3.87 million t, and Asia is the largest producer (FAOSTAT, 2022). These walnut trees hold great economic and cultural significance in many Asian countries (Lu et al., 1999; McGranahan & Leslie, 2012; Xi & Zhang, 1996), where they are highly valued for their oil-rich nuts and high-quality timber (Bernard et al., 2018; McGranahan & Leslie, 2012). *Juglans regia* occurs naturally in mountainous regions of central Asia and has been extensively cultivated in temperate zones worldwide. In contrast, *J. sigillata* exhibits a more restricted range and is endemic to southwestern China, the Eastern Himalaya, and the Naga Hills (Figure 1) (Lu et al., 1999; McGranahan & Leslie, 2012), where it hybridizes naturally with *J. regia* (Liu et al., 2023; Wang et al., 2015; Yan et al., 2024). These species are widely cultivated and distributed in climate change-sensitive areas such as the Himalaya and Hengduan Mountains, thus providing an ideal model for understanding the impact of rapid climate change on tree crops. Recent investigations have concentrated on genetic diversity, domestication, and germplasm evaluation of walnuts (e.g., Ding et al., 2022; Ji et al., 2021; Pollegioni et al., 2017; Yan et al., 2024), whereas the exact genetic origins and potential effects of future climate change remain unexplored.

By integrating population genetic and spatial ecological approaches, we aimed to characterize the genetic diversity, population structure, demographic history, and landscape connectivity of walnuts in Asia; predict their present and future potential suitable areas; and quantify the potential genetic diversity erosion and population structure alterations under future climate scenarios. We sought to provide a spatiotemporal perspective of walnut genetic diversity in Asia that can guide the formulation of conservation strategies that account for the adaptive capacity of populations and serve as a framework for assessing climate-driven genetic loss in other tree crops.

METHODS

Sampling, DNA extraction, and microsatellite genotyping

A total of 5282 individuals from 233 populations of *J. regia* and *J. sigillata* covering their entire distribution range in Asia were collected (Figure 1a; Appendix S1). These included 138 populations composed of 3123 individuals reported in a range of previous studies (Magige et al., 2022; Qi et al., 2023; Shahi Shavvon et al., 2023; Yan et al., 2024; Ye et al., 2024). To avoid sampling clones, we ensured that any 2 sampled trees were at least 100 m apart in the field. Because cultivation and grafting are common in southwestern China (Liu et al., 2023), we tried to

sample old trees with a DBH (diameter at breast height) >50 cm and estimated age >100 years to avoid recently transplanted plants and grafted individuals. Three to 5 young and disease-free leaves were collected per individual, preserved in silica gel, and stored at -20°C pending DNA extraction. Voucher specimens were deposited at the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences, and at the Calcutta University Herbarium.

Genomic DNA was extracted from about 0.02 g of dried leaves with a modified CTAB protocol (Doyle & Doyle, 1987). The DNA concentration and quality were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A multiplex of 31 nuclear microsatellite primer pairs was used for genotyping the 5282 samples (Wambulwa et al., 2022; Xiahou et al., 2023) (Appendix S2). Polymerase chain reaction (PCR) amplification was carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems) following the protocol described by Xiahou et al. (2023). The PCR products were separated according to fragment sizes with an ABI 3730XL DNA Analyzer (Applied Biosystems), and GeneMarker 2.2.0 (Soft-Genetics) was used to score alleles. The data from different batches were standardized using allelic ladders (Xiahou et al., 2023). The amount of missing rate was assessed utilizing the R package poppr 2.9.3 (Kamvar et al., 2014; R Core Team, 2021). Scoring errors and large allele dropout were checked using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004).

Estimation of genetic diversity

Pairwise gametic disequilibrium between loci was assessed using FSTAT 2.9.4 (Goudet, 1995). The number of distinct multilocus genotypes (MLGs) in the data set was identified using the R package poppr. To mitigate potential issues caused by kinship, we used the program COLONY 2.0.6.5 (Jones & Wang, 2010) to infer kinship among individuals based on MLGs. Only one individual per full-sibling cluster was retained for subsequent analyses. Then, null allele frequencies were estimated using the expectation maximization algorithm in FreeNA (Chapuis & Estoup, 2007). We employed GenAlEx 6.51b2 (Peakall & Smouse, 2006) to calculate key population genetic parameters, including the mean number of alleles per locus (N_A), the mean number of effective alleles per locus (N_E), observed and expected heterozygosity (H_O and H_E , respectively), the Shannon diversity index (H), and the number of private alleles (N_P) for each population. Additionally, allelic richness (A_R) and the inbreeding coefficient (F_{IS}) were calculated using the R package HIERFSTAT 0.5-10 (Goudet, 2005).

Population genetic structure analyses

To determine population structure, Bayesian clustering analysis was carried out in STRUCTURE 2.3.4 (Pritchard et al., 2000). Markov chain Monte Carlo (MCMC) iterations were set to 12,000,000 with a burn-in period of 1,000,000 in the admixture model with correlated allele frequencies (Falush et al.,

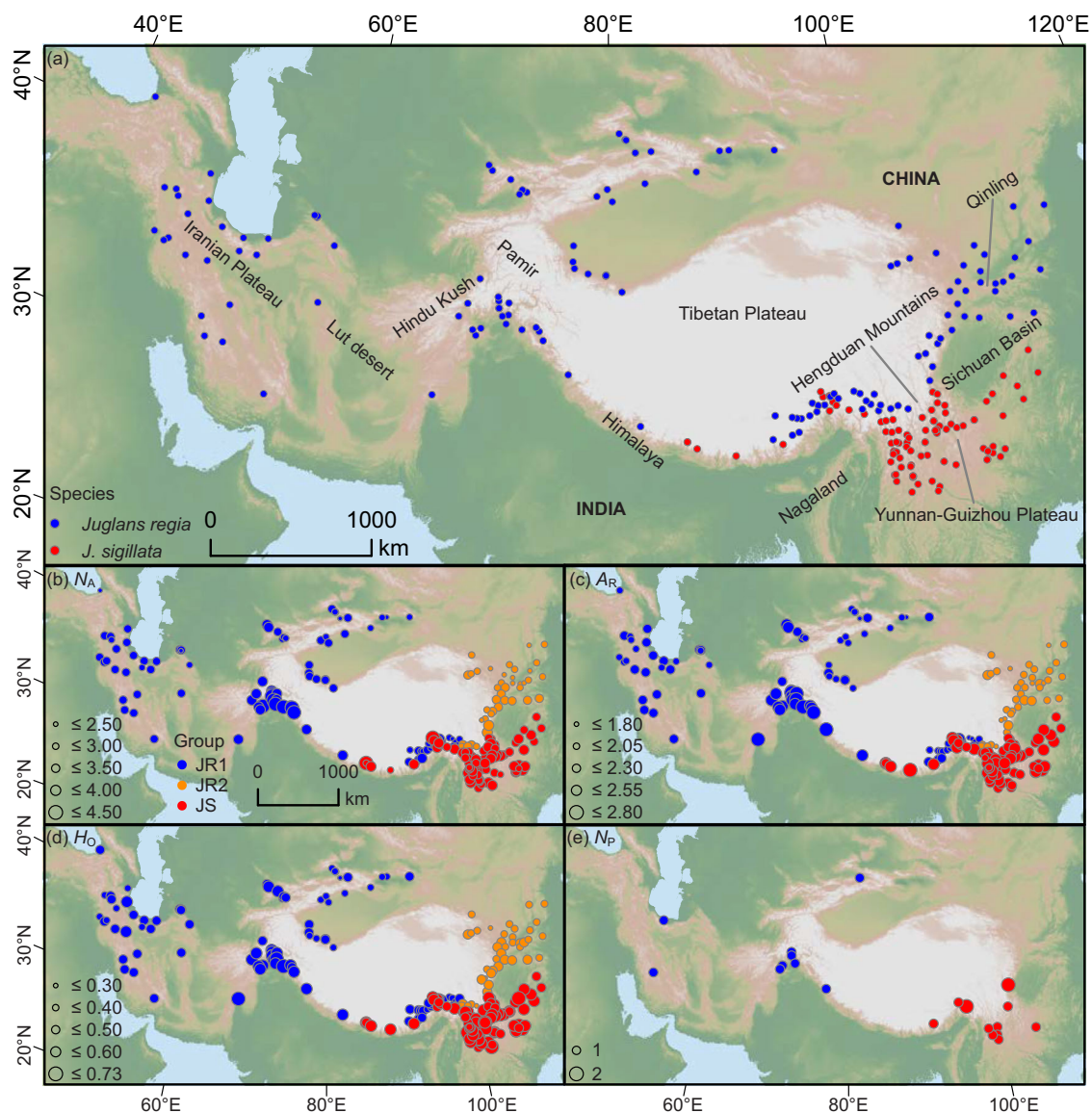


FIGURE 1 Sampling locations and genetic diversity of walnut populations in Asia: (a) distribution of 233 walnut populations sampled for genetic analyses (blue, *Juglans regia* [141 populations]; red, *J. sigillata* [92 populations]), (b) spatial patterns of mean number of alleles, (c) allelic richness for samples, (d) observed heterozygosity, and (e) number of private alleles (circle size, proportional to level of genetic diversity).

2003). Analyses were conducted for $K = 1$ to $K = 11$ (20 replicates for each K). The optimal K value (Evanno et al., 2005) was determined with STRUCTURE HARVESTER 0.6.94 (Earl & vonHoldt, 2012) and KFinder 1.0.0.0 (Wang, 2019). The greedy method was performed in CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007) to average admixture proportions over the 20 replicates. Bar charts illustrating the portion of cluster membership per individual were generated using DISTRUCT 1.1 (Rosenberg, 2004). We then mapped the proportion of cluster membership for each population with pie charts in ArcGIS Pro 3.0.2 (ESRI) (Figure 2b).

Population structure was further explored using discriminant analysis of principal components (DAPC), a hypothesis-free approach implemented in the R package ADEGENET 2.1.5 (Jombart, 2008). The DAPC was performed on the 3 groups

identified in the Bayesian clustering analysis. After assigning individuals to groups based on DAPC, we calculated the diversity parameters listed in the previous section for each group. A neighbor-joining (NJ) tree was constructed using PHYLIP 3.698 (Felsenstein, 1989) based on Nei's genetic distance matrix (D_A) (Nei et al., 1983) calculated with MSAnalyze 4.05 (Dieringer & Schlötterer, 2003). The tree was then visualized using iTOL 6.7.4 (Letunic & Bork, 2021).

We calculated the coefficient of pairwise genetic differentiation (F_{ST}) between populations and groups and estimated its significance levels with 10,000 permutations with Arlequin 3.5.2.2 (Excoffier et al., 2005). To evaluate genetic variance partition among populations and groups, an analysis of molecular variance (AMOVA) was performed in Arlequin with 1000 permutations.

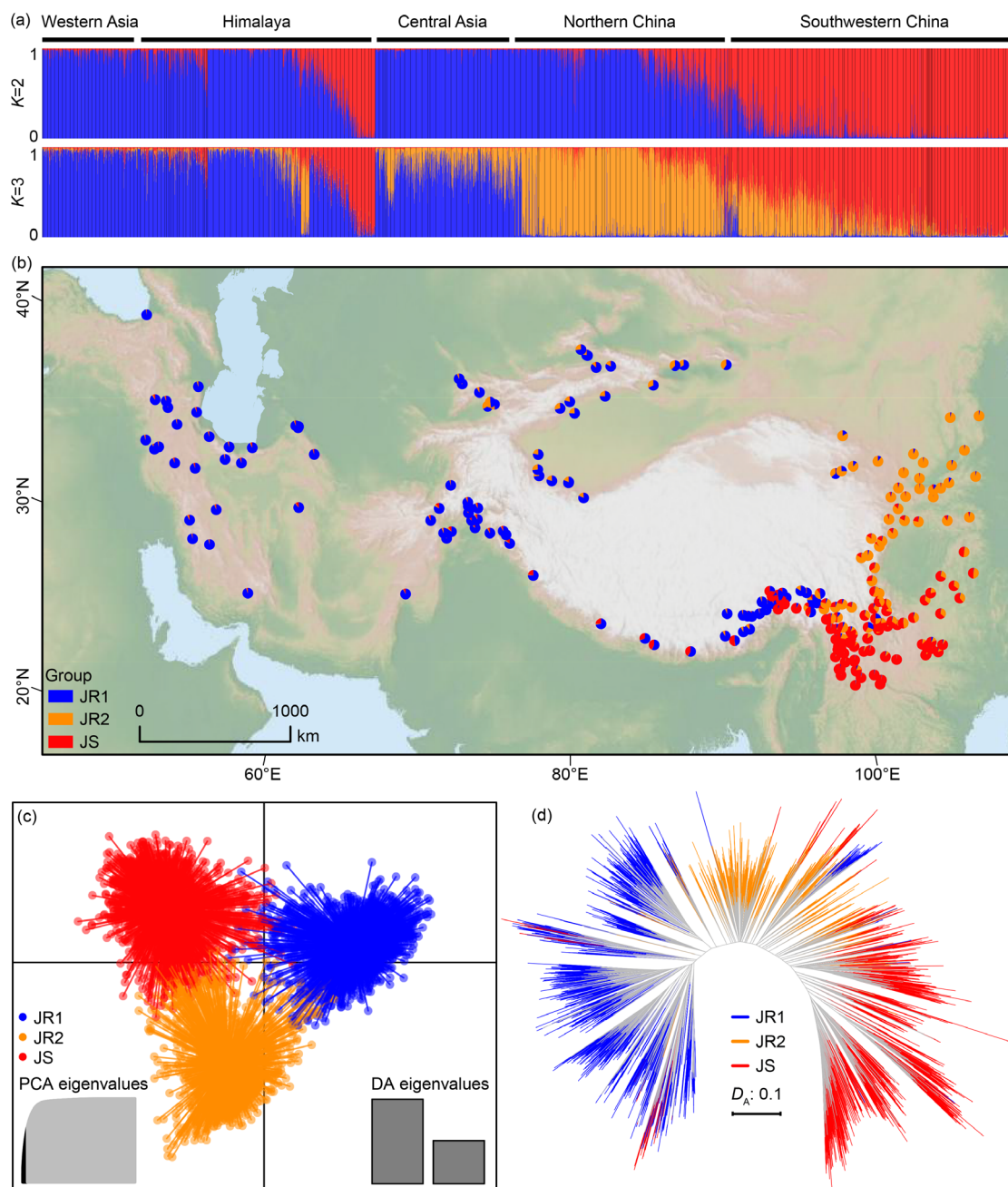


FIGURE 2 Genetic structure of 233 populations of *Juglans regia* (JR) and *J. sigillata* (JS) in Asia: (a) Bayesian clustering (STRUCTURE) at $K = 2$ and $K = 3$ (blue, ancestral gene pool JR1; orange, JR2; red, JS; vertical lines, individuals; colors, proportion of the genotype assigned to each group), (b) spatial genetic pattern inferred with STRUCTURE at $K = 3$ (proportions in the pie charts, frequency of each group in a population), (c) results of discriminant analysis of principal components (DAPC) among the gene pools (dots, individuals), and (d) unrooted neighbor-joining tree based on Nei's genetic distance (D_A).

Inferring demographic history

To explore the demographic history of the genetic groups, the approximate Bayesian computation (ABC) approach with supervised machine learning implemented in DIYABC Random Forest (DIYABC-RF) 1.2.1 (Collin et al., 2021) was applied. The analysis focused on 3 genetic groups identified through STRUCTURE and DAPC (Figure 2). Individuals with admixture coefficient $0.1 < (q) < 0.9$ were removed to limit potential

biases due to recent admixture events. We developed 9 plausible demographic scenarios (Figure 3a). All parameters, except t_1 , t_2 , and t_3 (t is time) and ra (genetic contribution of each source group), were set to default values (Appendix S3), and the generation time for walnut trees was assumed to be 15 years (Ji et al., 2021). We generated a reference table containing approximately 10,000 simulation data sets per scenario (90,000 in total) for scenario choice and parameter estimation. To evaluate compatibility, linear discriminant analysis (LDA) was performed on

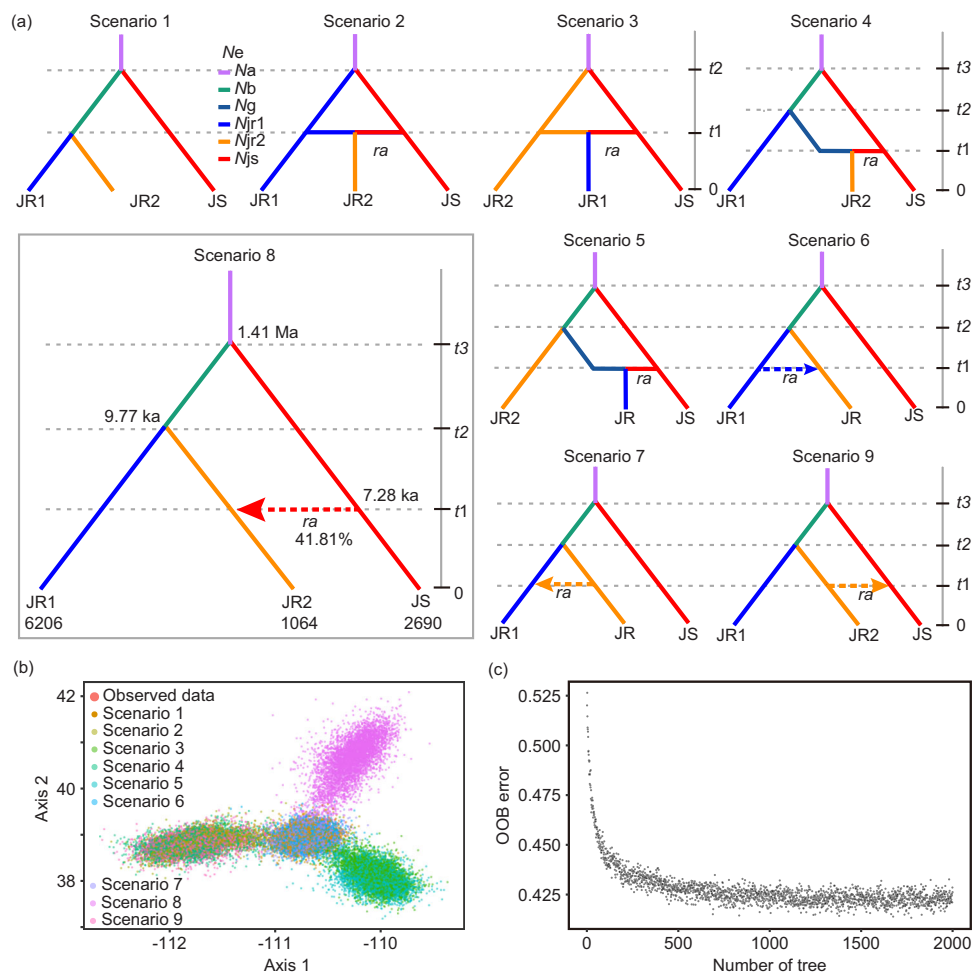


FIGURE 3 Demographic history of walnuts (*Juglans regia* [JR1 and JR2] and *J. sigillata* [JS]) in Asia inferred from DIYABC Random Forest (RF) analyses with (a) 9 demographic scenarios (6 effective population sizes: N_{JR1} , N_{JR2} , N_{JS} , N_b , N_g , and N_a ; 3 divergence or admixture time events: t_1 , t_2 , and t_3 , the time unit for t_1 is in million years (Ma), whereas t_2 and t_3 are measured in thousand years; genetic contribution of each source group, ra ; branch colors, discrete effective population size in the model with time measured in generations [not calibrated to scale]; details on prior distribution of parameters in Appendix S3); (b) first 2 linear discriminant analysis (LDA) axes used to project the 3 training data sets and the observed data set simultaneously (red point, location of observed data); and (c) effect of the number of RF trees on scenario selection (prior error rate stabilized at 2000 RF trees) (OOB, out-of-bootstrap).

the default summary statistics from the simulated data sets and the observed data set. The choice of scenario and the estimation of the posterior probability of the best-supported scenario were performed using the random forest algorithm module in DIYABC-RF, which produced 2000 random forest trees for each analysis (Collin et al., 2021). To assess the sufficiency of random forest trees, error and accuracy metrics were plotted as a function of the number of trees in the forest. Additionally, we performed Bayesian analysis of historical migration rates among the 3 genetic groups with Migrate-n 5.0.4 (Beerli et al., 2019).

Landscape connectivity analyses

To estimate spatial connectivity among populations, we employed estimated effective migration surfaces (EEMS) analysis to derive migration rates (Petkova et al., 2016). The EEMS

can also estimate the patterns of genetic diversity. We ran EEMS with 5000000 MCMC iterations and a thinning interval of 9999 after a burn-in of 1000000 for 2000 deme-sized grids. Three independent analyses were performed to ensure convergence. The migration and diversity surfaces were visualized using the R package rEEMSplots 0.1.0 (Petkova et al., 2016) and modified using ArcGIS Pro.

To further reveal the landscape connectivity among populations, we used the R package ResistanceGA 4.2-8 (Peterman & Jarman, 2018) to optimize landscape resistance values for continuous (elevation and human footprint) and categorical (land cover) surfaces at 15-km resolution. The F_{ST} value among populations was used as a response variable. The environmental variables were downloaded from 3 databases (Appendix S4) (Fick & Hijmans, 2017; Jung et al., 2020; Venter et al., 2016). We estimated the resistance distance matrices based on circuit theory (McRae & Beier, 2007). We used maximum-likelihood population-effects (MLPE) models (Clarke et al., 2002) to

model the effects of isolation by distance (IBD) and isolation by resistance (IBR) separately. Models were compared based on log likelihoods, Akaike's information criterion (AICc) for small sample sizes, and margin R^2 . We used the best model to create a cumulative current connectivity map. To predict the movement density according to the underlying resistance surface, we applied a SAMC model, which extends circuit-theoretic connectivity modeling by accounting for dispersal resistance and mortality in the R package *samc* 1.4.0 (Marx et al., 2020).

Ecological niche modeling

A total of 4819 georeferenced occurrence records were gathered from online databases (Appendix S5), literature sources, and our field investigation. To ensure the reliability of records, we removed records assigned to urban regions with the R package *CoordinateCleaner* 2.0-20 (Zizka et al., 2019). To reduce the effects of spatial sampling biases, we applied the R package *spThin* 0.2.0 (Aiello-Lammens et al., 2015) to keep a distance of at least 5 km between any 2 points. For ambiguous occurrence records, we implemented a 2-step classification approach. First, we assigned uncertain occurrence records to their most plausible genetic and geographical group using the function *biodecrypt* in the R package *recluster* 2.9 (Platanía et al., 2020) based on our genetic structure map. For occurrence records in species-overlapping regions, assignments were made based on morphological characteristics and verified through field investigations, virtual specimens, and photographs. Records lacking sufficient information for reliable identification were excluded. Ultimately, we retained 890 records for the *J. regia* JR1 group, 981 for the *J. regia* JR2 group, and 567 for *J. sigillata* (JS) (Appendix S6). We delineated the alpha-hull polygon based on occurrence points and then delineated a 200-km buffer for the ENM with the R package *rangeBuilder* 2.1 (Davis Rabosky et al., 2016).

We downloaded 26 environmental variables from 4 databases and classified them into 4 categories (Appendix S4): bioclimatic, topographic, pedologic, and anthropogenic variables (Batjes et al., 2020; Chen et al., 2020; Fick & Hijmans, 2017; Venter et al., 2016). Future bioclimatic data sets were acquired from the Beijing Climate Center climate system model 2 (BCC-CSM2-MR) (Wu et al., 2019). To minimize multicollinearity among variables, we selected variables based on the variance inflation factor ($VIF < 5$) with the R package *usdm* 1.1-15 (Naimi et al., 2014). We retained 14 variables for analyses (Appendix S4). For future climate change projections, we incorporated bioclimatic variables from 2 Shared Socioeconomic Pathways: SSP126 (a scenario with low forcing) and SSP585 (a scenario with high forcing) for 2090 (2081–2100). We assumed that the 2 topographic variables and 2 pedologic variables would remain static over the next 70 years (Stanton et al., 2012).

The ENMs were constructed with the R package *biomod2* 4.2-3 (Thuiller et al., 2009) for the 3 geographical groups with the ensemble modeling approach that combines outputs of 7 different algorithms: generalized linear models, generalized boosted models, classification tree analysis, flexible discriminant analysis, multivariate adaptive regression splines, random for-

est, and maximum entropy. We calibrated ENMs with a 5-fold cross-validation with 2 iterations and evaluated model performance with the true skill statistic (TSS) and the area under the receiver operator characteristic curve (AUC). Models with TSS > 0.6 and AUC > 0.8 were retained, and ensemble models were constructed by weighted averaging of AUC statistics (Araujo & New, 2007). Variable importance was evaluated by the internal jackknife test. Suitability maps were transformed into binary maps (suitable or unsuitable) with the maximum sum of sensitivity and specificity (maxSSS) threshold method (Liu, White, et al., 2013). Range shifts from present to 2090 were calculated and visualized with ArcGIS Pro and Sankey diagrams in the R package *ggalluvial* 0.12.5 (Brunson, 2020).

Assessment of genetic erosion

To estimate the potential change in genetic diversity and population structure under future climate scenarios considering the sessile nature of plant species, we assumed that only populations in suitable areas under future climate scenarios would persist without migration and local adaptation (Abreu-Jardim et al., 2021; Lima et al., 2017; Wambulwa et al., 2025). To estimate the change in genetic diversity, we recalculated the genetic diversity indices mentioned above after excluding populations outside the projected suitable area in 2090, following Wambulwa et al. (2025).

Population genetic structure dynamics were predicted using spatially explicit simulations implemented in POPS 1.2 (Jay et al., 2012, 2015). Principal component analysis (PCA) was performed on the 14 environmental variables retained for use in ENM (Appendix S4). Eigenvectors with a cumulative contribution of $> 80\%$ were retained as representative components of the environmental variables. Four independent replicates were run for $K = 3$ under the present climate conditions. The MCMC runtime was set at 100,000 sweeps and the burn-in period at 10,000 sweeps with admixture models. The resulting output files were loaded into CLUMPP to average the 4 replicates. We forecasted the genetic structure under the SSP126 and SSP585 scenarios. A surface map depicting genetic structure was plotted using the R script POPSutilities (Jay et al., 2012) and further optimized in ArcGIS Pro. Predictions were projected in the ENM-calibrated region. Finally, we measured and visualized the genetic turnover between present and future for the SSP126 and SSP585 scenarios with ArcGIS Pro.

RESULTS

Genetic diversity and population structure

We genotyped 5282 samples from 233 populations (Figure 1; Appendix S1) based on 31 microsatellite markers. Missing data comprised 0.49% of the data set (Appendix S7). The loci showed relatively high polymorphism with minimal stuttering. The data set contained 5047 MLGs. Kinship filtering further excluded 129 individuals; thus, we had 4918 individuals for

TABLE 1 Sample sizes and genetic diversity indices for *Juglans regia* (JR) and *Juglans sigillata* (JS) under present and future (2090) climate scenarios based on 31 microsatellites.

Scenario	Group	<i>n</i>	MLG	<i>N_A</i>	<i>N_P</i>	<i>N_E</i>	<i>A_R</i>	<i>H_O</i>	<i>H_E</i>	<i>I</i>	<i>F_{IS}</i>
Current	JR1+JR2	3604	3517	8.26	21	2.73	4.72	0.42	0.57	1.12	0.25
	JR1	2321	2236	8.06	17	2.69	4.77	0.44	0.56	1.12	0.22
	JR2	1283	1281	6.42	0	2.15	3.81	0.40	0.47	0.88	0.15
	JS	1678	1530	8.45	25	2.83	4.61	0.52	0.61	1.17	0.15
2090 SSP126	JR1+JR2	2525	2475	7.94	18	2.68	4.63	0.42	0.57	1.10	0.25
	JR1	1537	1488	7.52	14	2.69	4.70	0.45	0.56	1.11	0.20
	JR2	988	987	5.84	0	2.06	3.58	0.39	0.45	0.83	0.12
	JS	1646	1500	8.35	25	2.82	4.58	0.52	0.61	1.16	0.15
2090 SSP585	JR1+JR2	1956	1906	7.68	17	2.65	4.59	0.42	0.56	1.09	0.25
	JR1	1263	1214	7.45	13	2.67	4.69	0.45	0.56	1.11	0.20
	JR2	693	692	5.35	0	1.96	3.41	0.37	0.42	0.78	0.12
	JS	1456	1325	8.23	24	2.83	4.59	0.52	0.61	1.17	0.15

Note: *n*, total number of samples per population; MLG, number of multilocus genotypes; *N_A*, mean number of alleles; *N_P*, number of private alleles in each population; *N_E*, mean number of effective alleles; *A_R*, allelic richness of 50 samples; *H_O*, average observed heterozygosity per locus; *H_E*, average expected heterozygosity per locus; *I*, Shannon's information index; *F_{IS}*, inbreeding coefficients.

downstream analyses. Moreover, the quality of the data set was not affected by null alleles (Appendix S8), scoring errors, or large allele dropout. No pairwise locus comparisons showed significant gametic disequilibrium (Appendix S9). Based on these results, all loci were retained for subsequent analyses. A total of 284 alleles were identified, ranging from 5 alleles (primer JR07) to 13 alleles (primers JR11 and JS22) (Appendix S10).

Bayesian analysis of population structure revealed that $K = 2$ was the optimal value of K based on the delta K method (Appendix S11), whereas the parsimony method suggested $K = 7$ was the optimal value. At $K = 2$, one genetic group comprised 2612 individuals ($q > 0.9$) of *J. regia* collected from western Asia, southern Asia, central Asia, and northern China (Figure 2a,b). The second genetic group comprised 1416 individuals of *J. sigillata* from southwestern China ($q > 0.9$). However, 1254 individuals from the mountains around the Sichuan basin and the Himalaya exhibited varying degrees of admixture and hence did not fall fully in either group (Figure 2a,b). At $K = 3$, the *J. regia* genetic group was further split into 2 subgroups: JR1, comprising 1188 individuals from western Asia, southern Asia, central Asia, and Tibet of China, and JR2, comprising 610 individuals from northern China. A total of 431 individuals from the Himalaya showed admixture between group JR1 and *J. sigillata*, whereas 1054 individuals from the mountains surrounding the Sichuan basin showed admixture between groups JR2 and *J. sigillata* (Figure 2a,b). After comparing the results across different K values (Appendices S12 & S13), we selected $K = 3$ for subsequent analyses. The population structure of subdivision at $K = 3$ was also supported by the DAPC and NJ tree results (Figure 2c,d).

We found that *J. sigillata* ($N_A = 8.45$, $A_R = 4.61$, $H_O = 0.52$) had higher genetic diversity than *J. regia* (JR1+JR2, $N_A = 8.26$, $A_R = 4.72$, $H_O = 0.42$). At the group level, JS had the highest genetic diversity, followed by JR1 ($N_A = 8.06$, $A_R = 4.77$, $H_O = 0.44$) and JR2 ($N_A = 6.42$, $A_R = 3.81$, $H_O = 0.40$)

(Table 1). A total of 189 (67% of total alleles) alleles were shared among the 3 groups (Appendix S14). Moreover, JS had more private alleles ($N_P = 25$) than JR1 ($N_P = 17$); JR2 had no private alleles (Table 1; Figure 1c; Appendix S14).

At the population level, the average number of observed alleles (N_A) varied from 1.74 in population PJR-56 to 4.48 in PJR-38 (mean = 3.22). Rarefied allelic richness (A_R , for 5 samples) ranged from 1.51 (PJR-56) to 2.80 (PJR-47) (mean = 2.16) (Figure 1; Appendix S15). Observed heterozygosity varied from 0.21 (PJR-56) to 0.73 (PJS-15) (mean = 0.47). Private alleles (N_P) varied from 0 to 2. For *J. regia*, populations from the Western Himalaya showed the highest genetic diversity, whereas *J. sigillata* populations from the Yunnan–Guizhou Plateau showed the highest genetic diversity (Figure 1; Appendix S16).

Genetic differentiation analyses revealed significant divergence between the 2 species: $F_{ST} = 0.15$ ($p < 0.001$). At the group level, we observed substantial genetic differentiation: $F_{ST} = 0.17$ for JS versus JR1, 0.20 for JS versus JR2, and 0.15 for JR1 versus JR2. All were statistically significant ($p < 0.001$) (Appendix S17). The F_{ST} among the 233 populations varied from -0.11 (between PJS-63 and PJS-64) to 0.63 (between PJR-127 and PJS-91) (mean = 0.25) (Appendix S18). According to the AMOVA, 15.26% of the genetic variation was partitioned between the 2 species (Appendix S17), 17.41% among the 3 groups, 25.68% among populations, and 74.32% within populations.

Demographic history

The first 2 LDA axes showed that priors and scenarios were compatible with the observed data set and had substantial power to discriminate among the tested scenarios (Figure 3b). A total of 2000 trees in the random forest were sufficient to estimate the global accuracy metrics (Figure 3c). The ABC-RF

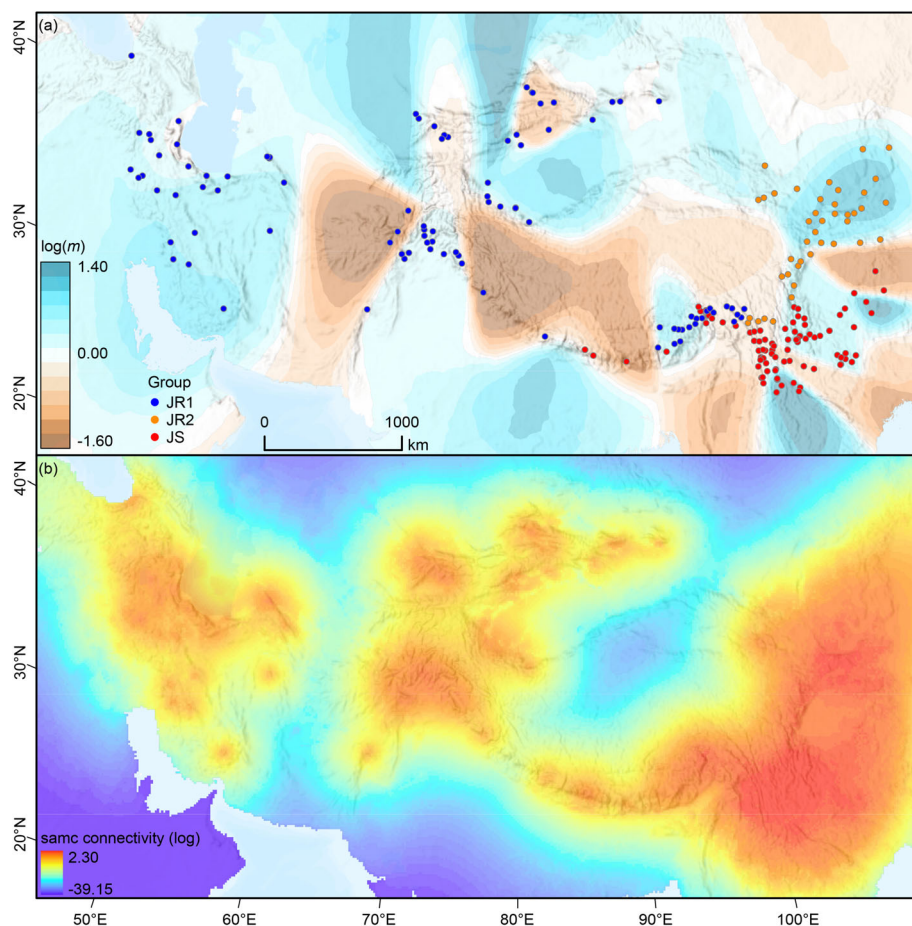


FIGURE 4 Landscape connectivity of walnut populations in Asia: (a) estimated posterior mean of the effective migration surface (blue, dispersal corridors; white, isolation by distance; orange, barriers; dots, population localities; colors, genetic groups for *Juglans regia* [JR1, JR2] and *Juglans sigillata* [JS]) and (b) landscape connectivity estimates based on a pairwise dispersal model with spatially absorbing Markov chains (SAMC) (red, regions of high connectivity; blue, regions of restricted connectivity).

analyses showed that the best fit model was scenario 8 (822 out of 2000 votes) (posterior probability of 0.61). This model indicated that *J. sigillata* split from *J. regia* around 1.41 Ma BP (95% confidence interval [CI] 0.78–3.12 Ma BP), followed by the split of JR1 and JR2 around 9.77 ka BP (95% CI 2.72–14.55 ka BP). However, 41.81% (95% CI 23.19–48.14%) of the genetic composition of JR2 originated from JS around 7.28 ka BP (95% CI 1.22–12.00 ka BP). The effective population sizes of JR1, JR2, and JS were 6206 (95% CI 2335–9628), 939 (95% CI 263–3147), and 2690 (95% CI 325–6443), respectively (Appendix S3). Gene flow estimates revealed relatively symmetrical historical migration rates between groups (Appendix S19). The highest migration rate, 154.41, was observed from JR2 to JS (95% CI 132.00–176.67), whereas the lowest rate, 119.04, was detected from JS to JR1 (95% CI 96.67–140.67).

Landscape connectivity

The EEMS highlighted several dispersal barriers associated with the Tibetan Plateau, the Pamir, the Hindu Kush Mountains, and the Lut desert (Figure 4a). Moreover, migration corri-

dors appeared to align with historical trade routes, such as the Silk and Tea Horse Roads. The MLPE describing IBR clearly performed better than the IBD model (Appendix S20). The composite resistance surface with the minimum AICc value consisted of elevation, human footprint, and land cover (mean AICc = −40,159) (Appendix S20). No competing models were within 10 AICc units. The connectivity map (Figure 4b) based on the best-supported model (Appendix S20) revealed low connectivity in the Tibetan Plateau and the deserts in northwestern China, Iran, and Afghanistan. In contrast, high connectivity was observed in southwestern and northern China.

Range dynamics under future climate change

Our ENM demonstrated high predictive performance (JR1: AUC = 0.94, TSS = 0.72; JR2: AUC = 0.97, TSS = 0.80; JR1+JR2: AUC = 0.91, TSS = 0.67; JS: AUC = 0.99, TSS = 0.89) (Appendix S21). Notably, the group-level ENM captured more potential suitable areas for *J. regia* than the species-level ENM (Appendices S22 & S23); thus, the following results are based on group-level analysis. The present poten-

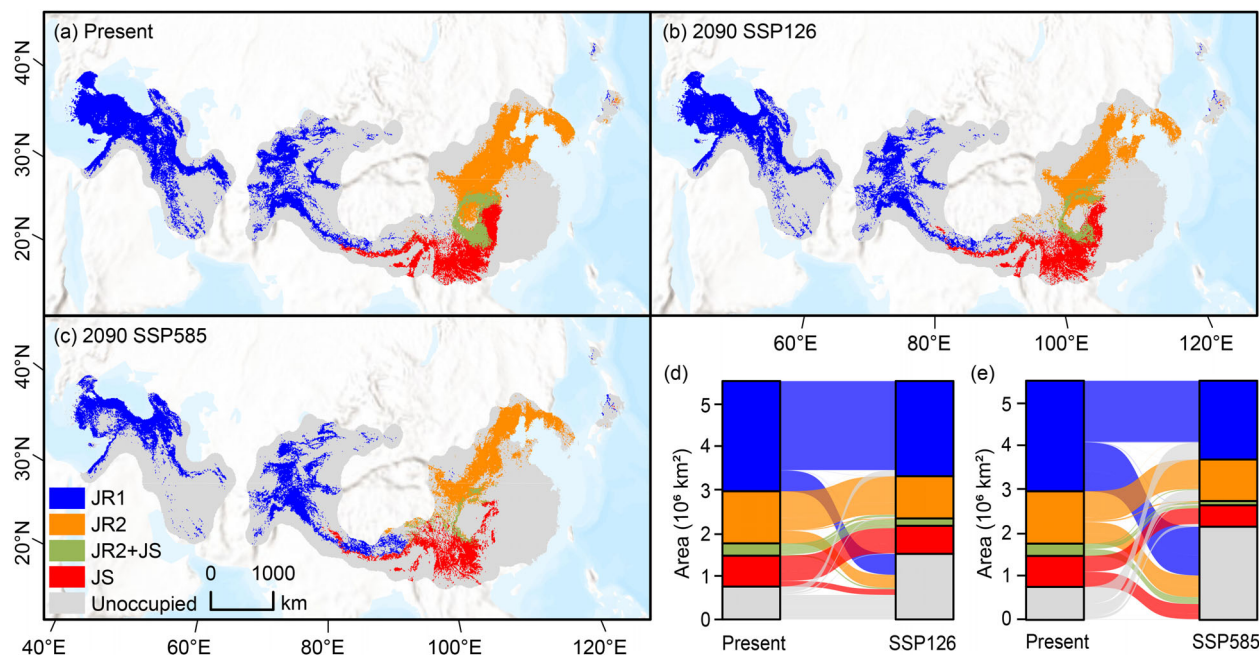


FIGURE 5 Predicted changes in suitable areas for 3 walnut genetic groups (*Juglans regia* [JR1 and JR2] and *Juglans sigillata* [JS]) in Asia under present and future climate scenarios: (a–c) potentially suitable areas for JR1 (blue), JR2 (orange), and JS (red) under different periods and scenarios (green, areas of overlap between JR2 and JS; overlaps between JR1 and JS and between JR1 and JR2 are too small to be visually displayed) and (d, e) predicted changes in habitat area occupied by the 3 groups under SSP126 and SSP585 emission scenarios.

tial suitable areas identified were as follows: *J. regia* (JR1+JR2) in temperate regions of Asia (Appendices S22 & S23); JR1 in parts of western Asia, central Asia, and the Western Himalaya (Figure 5a); JR2 in parts of northeastern China and the Korean Peninsula (Figure 5a); and JS in southwestern China, the Eastern Himalaya, and the Naga Hills (Figure 5a). The potential suitable areas overlapped around the Sichuan basin for JS and JR2, and for JS and JR2 in a small portion of the Central Himalaya. The JR1 had the largest potential suitable area (2.53×10^6 km²), followed by JR2 (1.51×10^6 km²) and JS (1.16×10^6 km²). Mean temperature (bio9), precipitation of wettest month (bio13), and precipitation of coldest quarter (bio19) were the most critical factors in ENM. Aspect (aspt) and vegetation (frt) exhibited generally low relative significance (Appendix S24).

The potential distribution range remained mostly stable under the 2 future emission scenarios, except for a dramatic contraction in the areas of overlap around the Sichuan basin (JR2+JS) and in the Iranian Plateau (JR1) (Figures 5b,c). The range contraction ratio of the 3 groups depends on the climatic scenario. The future total range for the JR1 was estimated to decline by 13.79% (SSP126) or 27.02% (SSP585), JR2 by 21.13% (SSP126) or 27.45% (SSP585), and that of JS by 14.92% (SSP126) or 30.71% (SSP585; Figure 5d,e).

Climate-driven changes in genetic diversity and population structure

The reestimation of genetic diversity under future climate scenarios (Figure 5) suggested genetic diversity loss in all 3 groups. This decrease was greater under the SSP585 emission scenario

compared to SSP126. The projected number of alleles lost was highest in JR2 (N_A : 9.03%, SSP126; 16.67%, SSP585), followed by JR1 (N_A : 6.70%, SSP126; 7.57%, SSP585) and JS (N_A : 1.18%, SSP126; 2.60%, SSP585) (Table 1).

Regarding the population genetic structure shift caused by climate change, the correlation between the estimated admixture coefficients and those predicted from climatic variables was 0.92, confirming the predictive accuracy of our climatic variables. Overall, spatially explicit simulations showed more dramatic genetic structure turnover under the SSP585 scenario than under SSP126. The Himalaya and Hengduan Mountains were projected to experience high genetic turnover (Figure 6). We found that JR1 was predicted to be substantially replaced by JS in the Eastern Himalaya and that JS was predicted to expand northward to the JR2 region in the Hengduan Mountains (Figure 6; Appendix S25).

DISCUSSION

To the best of our knowledge, our study is the first comprehensive analysis of walnuts in Asia. Our results provide novel insights into the origin of the genetic diversity of walnuts in Asia and how they might be affected by future climate change.

Genetic diversity origin of walnuts in Asia

Our results indicated distinct genetic and geographical separation between *J. regia* and *J. sigillata* (Figures 2 & 5; Appendix S17) in the middle Pleistocene (Figure 3). These findings, combined

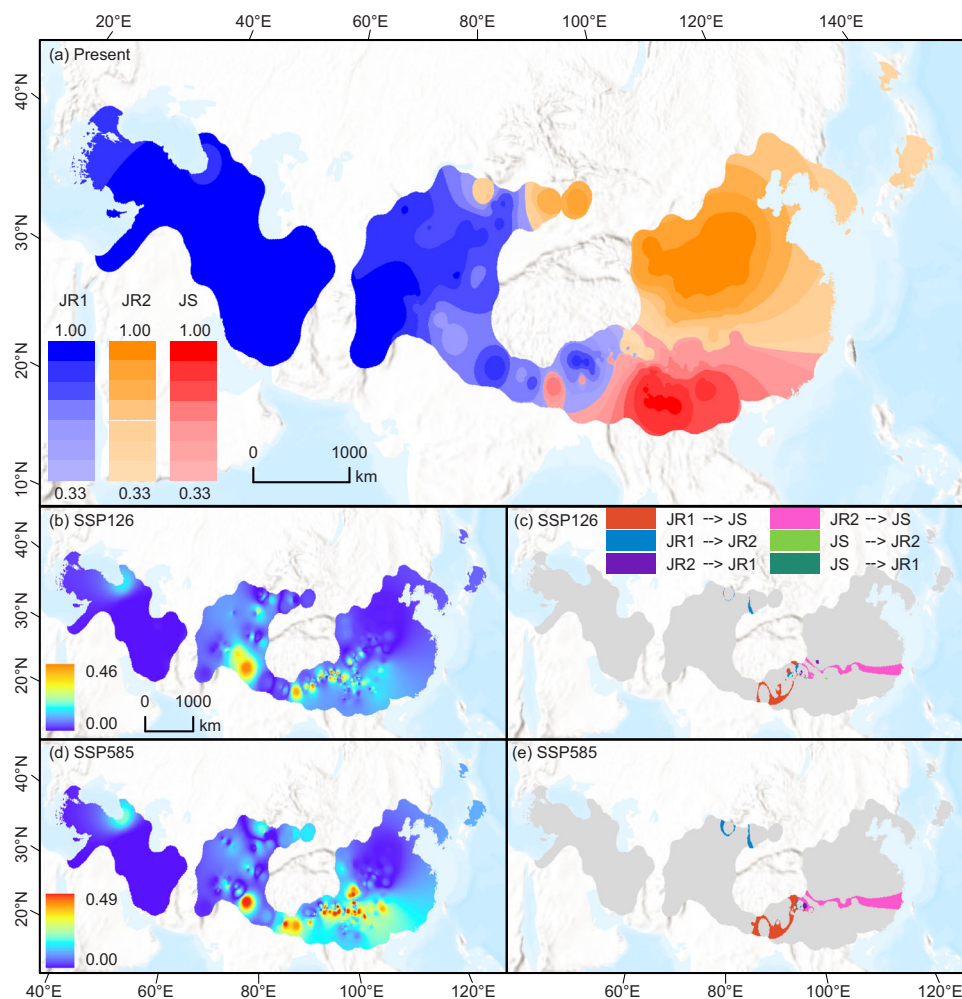


FIGURE 6 Present genetic structure and projected turnover under future climate scenarios: (a) present population genetic structure inferred with POPS software (JR, *Juglans regia*; blue, JR1; orange, JR2; red, JS, *Juglans sigillata*; admixture coefficients spatially displayed at $a > 0.33$ threshold for each group) and projected genetic turnover intensity and trends under (b, c) SSP126 (2090) and (d, e) SSP585 (2090).

with well-documented morphological distinctions (Lu et al., 1999), strongly support the separation of *J. regia* and *J. sigillata* into 2 species (Dode, 1906; Lu et al., 1999) and contradict the view that *J. sigillata* is an ecotype, landrace, or subspecies of *J. regia* (Yuan et al., 2018; Zhang et al., 2019; Zhao et al., 2018).

At the infraspecific level, the substructuring in *J. regia* aligned with previous findings (Ding et al., 2022). The JR2 genetic group had a low effective population size (Figure 3; Appendix S3), lower heterozygosity, and an absence of private alleles (Table 1; Figure 1; Appendix S14), suggesting that JR2 likely experienced a historical bottleneck, potentially caused by a founder event. In addition, the spatial correlation between ancient trade routes and JR2 migration corridors was coupled with the significant influence of human footprint on walnut gene flow in the region (Figure 4). These evidences, plus the apparent bottleneck and evidence from demographic history analysis, indicated that JR2 originated from anthropogenic introduction via ancient trade routes. Meanwhile, introgression from *J. sigillata* into JR2 could be involved in the local adaptation of JR2 in northern China, as with other tree crops

(Julca et al., 2020; Martin, Cottin, et al., 2023; Xiao et al., 2023).

The high genetic diversity and abundance of private alleles of *J. regia* detected in the Western Himalaya (Figure 1) indicate that this region is a center of genetic diversity for the species. This finding is supported by other recent population genetic studies in which microsatellite markers in walnut were used (synthesized in Appendix S26) (see also Appendix S27). For instance, northern Pakistan and surrounding regions (notably northeastern Afghanistan, southern Tajikistan, and northwestern India) have been identified as genetic diversity hotspots of *J. regia* (Aradhya et al., 2017; Roor et al., 2017). More recent studies highlight the Western Himalaya as a center of genetic diversity for *J. regia* (Yan et al., 2024; Ye et al., 2024). However, interstudy comparisons should be made with caution because of differences in sample sizes and microsatellite markers used (Wambulwa et al., 2022). Notably, this pattern is further reinforced by a recent population genomic study (Luo et al., 2022). In addition, palynological records indicated persistent *Juglans* pollen deposition in this region spanning the Last Glacial Maximum (LGM) and

Holocene (Bhattacharyya et al., 2011; Chauhan et al., 2022; Khan et al., 2022; Quamar et al., 2023). The ENM also suggested the presence of walnut habitats in this region during the LGM (Aradhya et al., 2017). Morphologically, populations from Pakistan and India retain ancestral nut traits, such as the highest nut density (Roor et al., 2017). Collectively, these findings suggest that the Western Himalaya likely served as a Pleistocene glacial refugium for *J. regia*. This challenges the hypothesis of multiple glacial refugia during the LGM across Eurasia, including the Transcaucasus, northern Iran, and northern China (Aradhya et al., 2017; Bottema & Woldring, 1984; Feng et al., 2018; Graham, 1972; Tiffney, 1985), and particularly undermines the idea of multiple independent refugia in Asia (Feng et al., 2018; Pollegioni et al., 2017). These findings further suggest that the Western Himalaya may represent the center of origin for *J. regia*. The cultivated walnut likely originated from this region prior to approximately 10,000 years ago (Figure 3) and subsequently spread to Western Asia and northern China, as evidenced by the spatial pattern of genetic diversity (Figures 1 & 4).

Our results uncovered higher genetic diversity in *J. sigillata* than in *J. regia* (Table 1), consistent with findings from Tibetan (Wang et al., 2015) and Yunnan populations (Liu et al., 2023). However, among the limited number of genetic diversity studies available in *J. sigillata* (Appendices S26 & S27), lower genetic diversity has also been reported (Dang et al., 2025; Feng et al., 2018). This discrepancy may be attributed to differences in the spatial coverage and loci of microsatellites across studies. Spatially, the Eastern Himalaya and western Yunnan emerged as genetic diversity hotspots for *J. sigillata*, potentially serving as glacial refugia for the species. This region has also been identified as a glacial refugium for other plants (Ahmad et al., 2025; Wambulwa et al., 2025), such as yew trees (*Taxus wallichiana*) (Liu, Möller, et al., 2013) and cypress species (Xu et al., 2010). Regrettably, our data here did not allow us to clearly determine the specific center of origin of cultivated *J. sigillata*. Additionally, the Sichuan basin and surroundings, as well as the Central Himalaya, exhibited elevated genetic diversity but lacked genetic endemism. Likely, this diversity is due to human-mediated hybridization between the 2 species (Yan et al., 2024) that facilitated the introgression of genetic components from both parental lineages (Figures 1 & 2) and ultimately led to the formation of 2 distinct hybrid zones. The genetic diversity in the hybrid regions may confer an evolutionary advantage by enhancing adaptive potential in response to climate change (Brauer et al., 2023; Hansen, 2023; Turbek & Taylor, 2023).

Future genetic erosion in Asian walnuts

The ENM showed that bioclimatic variables explained the environmental suitability of the walnuts in Asia better than topographical, pedologic, and anthropogenic variables, as has also been found in other tree crops (Bothwell et al., 2021; Zhang et al., 2016). This finding underscores the need to incorporate climatic considerations into conservation plans for walnuts and other tree crops. Our predictions clearly showed that potential

suitable areas of the 3 walnut genetic groups would decrease overall, with losses exceeding gains, under future climate change (Figure 5; Appendix S22). This finding, combined with results from ancestry distribution models (Figure 6), can guide the identification of environmentally appropriate locations for the introduction, cultivation, and management of walnuts in Asia.

All 3 groups were predicted to experience a loss in genetic diversity due to the loss of potential suitable areas under future climate change. Such a loss would lead to a notable change in population genetic structure (Figure 6). Specifically, JR2 had the lowest genetic diversity, suggesting that its capacity to adapt to changing climatic conditions is the lowest. Future genetic erosion may exacerbate inbreeding depression, potentially posing the threat of local extinction. These results support the claim that genetic diversity determines the ability of plant populations to survive in nature through adaptability to climate change (Müller et al., 2021).

Stable climatic conditions do not necessarily correlate with high genetic diversity in populations due to long-term population persistence (De Kort et al., 2021). Although environmental instability poses significant challenges, it may also create evolutionary opportunities. Mountainous regions, such as the Himalaya and Hengduan Mountains, are more vulnerable to climate change than other areas (Alba et al., 2022; Chen et al., 2022; Knight, 2022; Mendes et al., 2024; Schmeller et al., 2022). As one of the world's most notable biodiversity hotspots (Liu et al., 2022; Myers et al., 2000), the Himalaya has experienced accelerated warming rates surpassing global averages over recent decades (IPCC, 2021). However, the Himalaya and Hengduan Mountains not only harbor high levels of genetic diversity but are also predicted to undergo substantial genetic turnover under future climate change (Figures 1 & 6; Appendix S25). This region's high environmental complexity may facilitate species in tracking suitable climatic conditions within relatively small ranges, a pattern partially captured in plant species distribution modeling (Wambulwa et al., 2025). Such areas are fundamental to species evolution and are frequently associated with high genetic diversity (Ohlemüller et al., 2008; Perrigo et al., 2019), indicating the need for increased conservation attention and long-term monitoring (Ahmad et al., 2025).

Conservation implications for Asian walnuts

Our projections indicated that areas of high habitat suitability for the 3 genetic groups would remain relatively stable under future climate scenarios. Therefore, in situ conservation is the optimal strategy for protecting walnuts in Asia because it allows for the evolution of genotypes in their natural environment and preserves ancient trees (Fanelli et al., 2022). Conservation priority should be given to regions that show both high genetic diversity and the presence of future habitat, such as the Western Himalaya for *J. regia* and the Eastern Himalaya and western Yunnan for *J. sigillata*.

Regarding JR2, which exhibited the lowest genetic diversity and might experience serious genetic erosion in the future, we recommend implementing assisted gene flow strategies. This

would involve introducing genotypes from JR1 to enhance population size and genetic diversity, thereby mitigating the risks of genetic drift, inbreeding, and extinction. Although this approach poses potential risks of outbreeding depression and disruption of local adaptation, these risks can be effectively mitigated through rigorous selection of viable hybrid saplings and careful long-term monitoring (Aitken & Whitlock, 2013).

The natural hybrid populations in the Central Himalaya and around the Sichuan basin may enhance overall genetic diversity compared to parental species, thereby reducing genomic vulnerability to climate change (Brauer et al., 2023; Karunarathne et al., 2024). Additionally, these populations may act as a reservoir of adaptive alleles, facilitating the introgression of beneficial traits from crop wild relatives and landraces into elite breeding lines. However, introgression from the domesticated *J. regia* could potentially lead to homogenization and the loss of local alleles and ecotypes of *J. sigillata*, hence threatening its evolutionary potential. Therefore, management and conservation efforts should be upscaled for the pure natural populations of *J. sigillata* (southern Tibet and western Yunnan) to maintain their genetic integrity and purity (Yan et al., 2024). Furthermore, urgent measures should be taken to mitigate the effects of deforestation and to prevent the widespread replacement of natural *J. sigillata* populations with modern cultivars (Liu et al., 2023).

Despite the rich diversity in the walnut germplasm worldwide, current collections predominantly consist of improved cultivars and landraces, and there is limited representation of natural populations (Guney et al., 2021; Wambulwa et al., 2022). We strongly recommend preserving more wild walnut germplasm, particularly *J. regia* from the Western Himalaya and *J. sigillata* from the Eastern Himalaya and western Yunnan, in germplasm banks and specialized walnut nurseries. In addition, the populations located in areas projected to experience range loss or high genetic turnover (Figure 6) should be prioritized for ex situ conservation.

Addressing climate change solely through management strategies is unsustainable (Zhang & Batley, 2020). Instead, natural populations serve as critical germplasm resources for breeding climate-resilient cultivars (Wang et al., 2025) and play a key role in targeted introgression for agronomic improvement (Hajjar & Hodgkin, 2007; He et al., 2019). This approach also helps preserve genetic heritage shaped by a long evolutionary history. For the walnut species we studied, future breeding should integrate climate adaptation alongside yield and quality because modern cultivars suffer from narrow genetic backgrounds (Wambulwa et al., 2022). Our study provides strong evidence that Himalayan populations of both species can be effectively utilized in future breeding programs. Moving forward, precise genomic evaluation of genetic load and genomic vulnerability is urgently needed to identify specific superior parental candidates for this purpose.

AUTHOR CONTRIBUTIONS

Jie Liu and De-Zhu Li conceived and designed the study. Jie Liu, Peng-Zhen Fan, Guang-Fu Zhu, Robabeh Shahi Shavvon, Debabrata Maity, Raees Khan, Ya-Huang Luo, Zeng-Yuan Wu, Yerlan Turuspekov, and Lian-Ming Gao carried out field work.

Hai-Ling Qi, Hong-Yu Wu, and Xing Kong performed lab work. Peng-Zhen Fan, Guang-Fu Zhu, and Jie Liu analyzed the data. Peng-Zhen Fan wrote the first draft, with input from Jie Liu, Guang-Fu Zhu, Moses C. Wambulwa, and Richard I. Milne. All authors contributed to the revision and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data that support our findings are in the Supporting Information of this article (genotyping data in Appendix S28). Genotype data on 31 SSR loci of some regional data sets are publicly available from Magige et al. (2022), Qi et al. (2023), Shahi Shavvon et al. (2023), Yan et al. (2024), and Ye et al. (2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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