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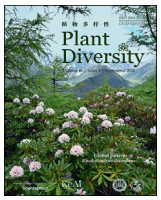

Research paper

Population genetic insights into the conservation of common walnut (*Juglans regia*) in Central Asia

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ABSTRACT

The common walnut (*Juglans regia*) is one of the most economically important nut trees cultivated worldwide. Despite its importance, no comprehensive evaluation of walnut tree population genetics has been undertaken across the range where it originated, Central Asia. In this study, we investigated the genetic diversity and population structure of 1082 individuals from 46 populations across Central Asia. We found moderate genetic diversity of *J. regia* across Central Asia, with 46 populations clustered into three groups with a weak relationship between genetic and geographic distance. Our findings reveal that the western Himalaya might be the core region of common walnut genetic diversity in Central Asia and that, except for two populations in Gongliu Wild Walnut Valley, humans might have introduced walnut populations to Xinjiang, China. The observed distribution of the genetic landscape has probably been affected by historical climate fluctuation, breeding system, and prolonged anthropogenic activity. We propose the conservation of the core genetic diversity resources in the western Himalaya and pay special attention to populations from Gongliu in Xinjiang. These findings enhance our understanding of the genetic variation throughout the distribution range of *J. regia* in Central Asia, which will provide a key prerequisite for evidence-based conservation and management.

1. Introduction

Genetic diversity, a key component of biodiversity, provides essential information on adaptation and the evolutionary capacity of plant species (Schaberg et al., 2008; Jump et al., 2009; Potter et al., 2017). The genetic diversity of forest trees is severely affected by increased harvesting (nuts, barks, timbers, etc.) and human interference (overexploitation, overgrazing, etc.), resulting in habitat fragmentation and considerable reduction of forestland area (MacDougall et al., 2013; Gaisberger et al., 2020). Consequently, genetic variation provides significant insights into conservation efforts and efficient utilization of plant resources (Loo et al., 2014). To date, the genetic diversity of endangered species has been widely studied, e.g., *Taxus wallichiana* (Liu et al., 2013), *Bertholletia excelsa* (Baldoni et al., 2020), and *Pinus koraiensis* (Wei

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et al., 2022). However, few studies have examined genetic diversity in tree species that face long-term and persistent anthropogenic pressures, particularly those planted in forest and agroforestry systems (Dawson et al., 2009; Gaisberger et al., 2020).

Juglans regia L. (Juglandaceae), popularly known as the common walnut, is a deciduous tree species typically distributed and/or cultivated in mountainous areas across subtropical and temperate regions (Lu et al., 1999; Yan et al., 2024). Its main distribution contains several countries from the Balkans eastward to Asia, including China, Iran, Kazakhstan, Afghanistan, and Pakistan (McGranahan and Leslie, 2012; Zohary et al., 2012). Presently, *J. regia* is cultivated in more than 60 countries, China being the leading world producer (with 31% of the total harvested) (FAOSTAT, 2023), and is one of the top three most-consumed nuts in the world (Avanzato et al., 2014; Ebrahimi et al., 2016). However, many ecosystems with walnut tree forests are seriously impacted by human activity (Beer et al., 2008; Lenda et al., 2017; Liu et al., 2023; Yan et al., 2024). In addition, post-destruction recovery of walnut forests can be challenging due to the trees' slow growth and reproduction, and late fruit ripening, which result in complex regeneration under natural conditions. Conservation efforts, including the effective use of plant germplasm, rely on a clear understanding of the population dynamics of a species, including its distribution, differentiation, as well as the factors that affect its genetic diversity (McNeely et al., 1990; Salgotra and Chauhan, 2023).

Due to the unique and fragile biodiversity in Central Asia, the region is considered as Mountains of Central Asia biodiversity hotspot (Mittermeier et al., 2004; Foggin et al., 2021; Liu et al., 2022). Because of the specific geomorphologic landscapes caused by the several Asian mountains (Tien-Shan, Western Himalaya, Hindu-Kush, and Karakoram Mountains) and Pamir Plateaus (Liu et al., 2022), Central Asia has an arid and semi-arid climate (Lioubimtseva and Henebry, 2009; Wang and Zhang, 2022). It is suggested that the diversity and spread of different species in this area were associated with the climatic transition characterized by enhanced aridification and global cooling since the late Eocene (Lioubimtseva and Henebry, 2009). In addition, Central Asia is also recognized as a center of origin of numerous fruit tree species, like the common walnut (*Juglans regia*) (Vavilov et al., 1992; Gaisberger et al., 2020), apricot (*Prunus armeniaca*) (Groppi et al., 2021), and wild apple (*Malus sieversii*) (Zhang et al., 2020). Regarding to walnut, previous studies have evaluated both genetic diversity and population structure of *J. regia* in some regions of Central Asia (Pollegioni et al., 2014; Aradhya et al., 2017; Roor et al., 2017; Mapelli, 2018; Torokeldiev et al., 2018; Gaisberger et al., 2020; Magige et al., 2022; Khan et al., 2023; Yan et al., 2024). However, despite the extensive distribution of *J. regia* in this area, there has been no comprehensive investigation into the genetic variation of this species across Central Asia. This is particularly true for walnuts in Afghanistan and the Xinjiang Autonomous Region of China.

In this study, we characterized the genetic diversity and population structure of 46 populations of *Juglans regia* across Central Asia. Specifically, we used 31 polymorphic microsatellite loci to genotype a total of 1082 individuals. Our findings will help develop genetically informed germplasm protection and management strategies as well as effective utilization of *J. regia*.

2. Materials and methods

2.1. Sample collection

From 2016 to 2018, we formed a walnut research network (see the author list) to collect walnut samples across Central Asia. This collection, which encompasses the whole range of walnuts, consists of 1082 *Juglans regia* individuals from 46 natural populations (Fig. 1 and Table S1) from six countries in the region: Afghanistan (4), China (21), India (3), Kyrgystan (4), Kazakhstan (2), and Pakistan (12). We collected a minimum of ten individuals per population, except in population 1 (from Ziarat in Pakistan). To avoid consanguinity, we only sampled neighboring trees that were at least 50 m apart. Within each population, fresh, healthy leaves were sampled and preserved in silica gel for DNA isolation. Voucher specimens have been deposited at the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Information on these populations can be found in Table S1.

2.2. DNA extraction and quality assessment

Genomic DNA was extracted from approximately 20 mg of silica gel dried leaves of *Juglans regia* using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987; Liu and Gao, 2011). Total DNA was dissolved in 50–100 mL 1 × TAE (pH = 8.0) and assessed by electrophoresis on 1.0% agarose gels. DNA concentration and quality were evaluated using a NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). All DNA samples were diluted to a final concentration of 30–50 ng/uL pending PCR procedures.

2.3. PCR amplification and SSR genotyping

A total of 1082 individuals from the 46 populations was genotyped using 31 nuclear microsatellite primer pairs following methods from our previous studies (Xu et al., 2020; Wambulwa et al., 2022; Xiahou et al., 2023). Genotype data of 12 populations from Pakistan and three populations from India were retrieved from our previous studies (Magige et al., 2022; Yan et al., 2024). The genotype data of both studies are publicly available from the publisher's website. For new samples, oligonucleotide tails were attached to the 5′ ends of all the forward primers and were labeled with FAM, HEX, or TAMRA fluorescent dyes (Optimus Bio., Kunming, China). PCR amplification was carried out in a final volume of 20 μL reaction containing 60–100 ng genomic DNA, 0.5 μM of each primer, 10 μL 2× Taq PCR MasterMix [Tiangen, 0.1 U Taq Polymerase/μL, 0.5 mM dNTP each, 20 mM Tris–HCl (pH = 8.3), 100 mM KCl, 3 mM $MgCl_2$]. PCR reaction conditions were as follows: an initial denaturation for 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, renaturation at the specified annealing temperature (T_m) of each primer for 30 s, elongation at 72 °C for 40 s, and a final extension step at 72 °C for 5 min. To genotype the samples, a multiplex PCR system was used on a Veriti® 96-Well Thermo-Cycler (Applied Biosystems, Foster City, California, USA), following Xiahou et al. (2023). Amplified fragments were initially visualized and assessed on 1.0% agarose gels with 1× TAE buffer, and the successful amplification products were separated on an ABI3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using GeneScan 500 LIZ (Applied Biosystems, Foster City, CA, USA) as an internal size standard.

2.4. Data analysis

Alleles of diploid genotypes were scored manually using GeneMarker v.4.0 (SoftGenetics, State College, Pennsylvania, USA). An allelic ladder of walnuts was used to calibrate the size of alleles among different batches of PCR products (Xiahou et al., 2023). Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004) was employed to examine the frequencies of null alleles at each locus in each population with 1000 randomizations. We detected Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between loci in Arlequin v.3.5 (Excoffier and Lischer, 2010)

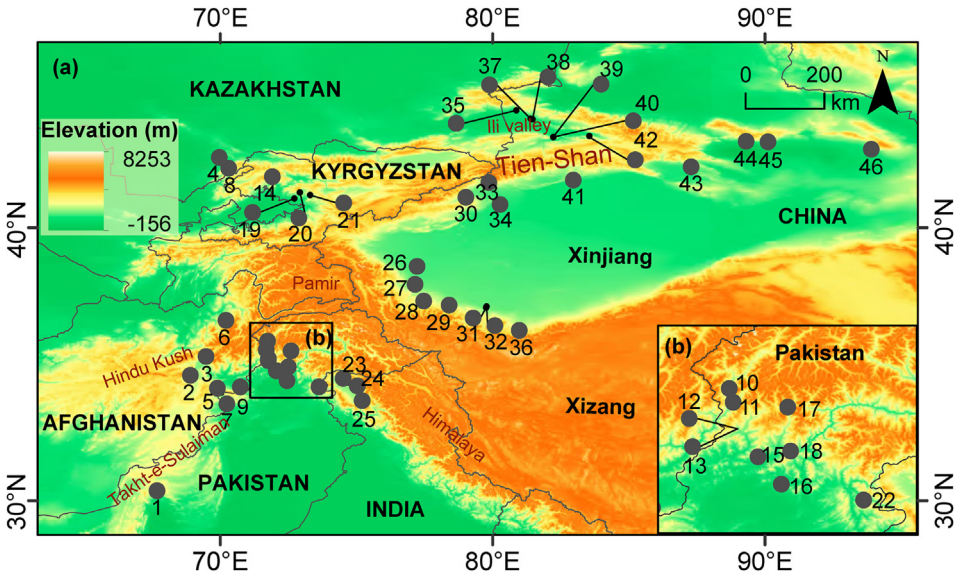

Fig. 1. Geographical location of the 46 *Juglans regia* populations (see Table S1 for detailed information). (a) Geographical location of all populations in China, Kazakhstan, Kyrgyzstan, Afghanistan, Pakistan, and India. (b) Right-lower inset shows the geographical distribution of nine populations in Pakistan.

using Bonferroni correction. We used GenAlEx v.6.5 (Peakall and Smouse, 2006) to estimate the following genetic diversity parameters: number of alleles (N_A), the effective number of alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), Shannon's information index (I), fixation index (F), gene flow (N_m), inbreeding coefficient (F_{IS}), unbiased expected heterozygosity (uH_E) across loci, Nei's genetic distances between populations, percentage of polymorphic loci (*PPL*), and private alleles (N_P). Allelic richness (A_R) and the polymorphic information content (*PIC*) were calculated by the *hierfstat* R package (Goudet, 2005) and PICcalc v.0.6 (Nagy et al., 2012), respectively. Principal co-ordinates analysis (PCoA) and analysis of molecular variance (AMOVA) (1000 permutations) were conducted in GenAlEx (Peakall and Smouse, 2006).

BayesAss v.3.0.4 (Wilson and Rannala, 2003) was used to compute recent migration rate between populations. In addition, the presence of population bottlenecks was computed using BOTTLENECK v.1.2.02 (Piry et al., 1999). A Two-Phase Mutation Model (TPM) was applied for the Wilcoxon (Cornuet and Luikart, 1996) and standardized differences tests. The parameter settings included 30% multi-step changes and a probability of 70%. The analysis was performed with 1000 permutations.

A Bayesian clustering algorithm in STRUCTURE v.2.3.4 (Pritchard et al., 2000) was utilized to assign each individual to a specific genetic cluster, determined by the pre-defined number of clusters (K). Each K value ranged from 1 to 10 and was repeated for 20 simulations. For each run, the burn-in period and Markov chain Monte Carlo (MCMC) generations were 100,000 and 1,200,000, respectively. All iterations were performed with the admixture model and correlated allele frequencies. The optimal K value was identified using the delta K method in STRUCTURE HARVESTER (Earl and vonHoldt, 2012). According to Wambulwa et al. (2016), an individual is assigned to a specific genetic cluster that is classified as a pure genetic group when the membership percentage is more than 80% ($Q \geq 0.80$), whereas those exhibiting a low membership coefficient ($0.20 < Q < 0.80$) are defined as 'admixture.' Genetic relationships among individuals were determined in Populations v.1.2.31 (Langella, 1999) using the unrooted neighbor-joining (NJ) tree method based on the D_A genetic distances calculated by Microsatellite Analyzer (MSA) v.4.05 (Dieringer and Schlötterer, 2003). The results were visualized using the *ggtree* R package (Yu et al., 2016). Pairwise Wright's F_{ST} values between populations were computed using Arlequin v.3.5 (Excoffier and Lischer, 2010), and a heatmap of D_A and F_{ST} estimates between various populations was visualized by the *ggplot2* R package (Wickham, 2016).

To analyze the impact of geographical isolation on patterns of genetic differentiation (F_{ST}), we conducted a Mantel test with the whole data and each group using the *vegan* R package (Oksanen et al., 2013). *P*-value was assessed through 1000 permutations. The geographic distance and F_{ST} matrix were generated using the *geosphere* R package (Hijmans et al., 2022) and Arlequin v.3.5 (Excoffier and Lischer, 2010), respectively. Moreover, to investigate correlations between environmental variables and genetic diversity (H_E) of *Juglans regia*, we evaluated the relationship between genetic diversity of walnut populations and annual mean temperature, annual mean precipitation, and elevation. Climatic data were downloaded from the WorldClim v.2.0 dataset (http://www.worldclim.org/bioclim), encompassing recent climate data (*ca.* 2000–2018), with a grid size of 30 arc-seconds (Fick and Hijmans, 2017).

Finally, the existence of genetic barriers among populations was evaluated using Monmonier's maximum difference algorithm implemented in Barrier v.2.2 (Manni et al., 2004). Microsatellite Analyzer (MSA) generated the pairwise genetic distance (D_A) with a bootstrap of 100 and geographical coordinates from Table S1. These matrices were then used to create a Delaunay triangulation net for linking neighboring populations and subsequently projecting the corresponding Voronoi tessellation. Every edge of the Voronoi polygon was connected based on Nei's genetic distance (D_A).

3. Results

3.1. Genetic diversity across SSR markers and populations

Our findings revealed that the 31 SSR markers were polymorphic. Micro-Checker analysis did not detect any evidence of scoring errors attributable to stuttering or significant allele dropout for any of the loci. A total of 39 out of 465 possible pairwise comparisons exhibited significant linkage disequilibrium ($P < 0.05$) (Table S2). A random association (linkage equilibrium) between all

the pairs of alleles was observed using Bonferroni correction. Only three loci (JR05, JM5446, and JS02) showed a departure from HW equilibrium, likely due to monomorphisms in some populations (Table S3). Hence, all data was used for downstream analyis. Genetic diversity parameters are presented for each locus in Table S4. All 31 nuclear SSR loci yielded 226 alleles overall. The average number of alleles throughout marker loci was 7.00, ranging from 5 for loci JR06, JR07, JR08, JR09, and CUJRD102 to 12 alleles for locus JS12 (Table S4). At the same time, the effective number of alleles (N_E) varied from 1.03 (JM5446) to 4.94 (BFU-Jr38), with an average of 2.62. The mean observed heterozygosity (H_O) and expected heterozygosity (H_E) were 0.44 and 0.56, respectively. The average Shannon's information index (I) and unbiased expected heterozygosity (uH_E) were 1.09 and 0.56, respectively. The inbreeding coefficient (F_{IS}) showed negative and significant values in loci JR02, JR03, JR04, JR06, JR07, JR08, JS02, JS04, JS14, and JS22 (Table S4), indicating heterozygosity excess.

At the population level, the genetic diversity parameters differed across populations of *Juglans regia* (Table 1). Total genetic diversity indices were typically moderate (N_E = 2.22, H_O = 0.46, H_E = 0.48). The average number of observed alleles (N_A), the effective number of alleles (N_E), and allelic richness (A_R) values for the whole dataset were 3.33, 2.22, and 3.04, respectively. The mean gene flow (N_m) between populations was 2.08. The maximum level of N_m was observed between populations 31 and 32 (244.85), while the minimum level was found between populations 39 and 45 (0.32) (Table S5). Populations 1 and 39 were varied considerably across all genetic diversity values. Particularly, populations 1, 15, 18, 22, and 25 exhibited high values of genetic diversity, whereas populations 39 and 40 showed relatively low values (Table 1). The fixation index (F) ranged from −0.09 (population 1) to 0.16 (population 36), with a mean of 0.03. The low value of the fixation index infers the deficiency of heterozygosity in the populations of *J. regia*.

Table 1
Genetic diversity and bottleneck effect analysis of the 46 populations of *Juglans regia* based on 31 microsatellite loci.

Code	PID	N	N_P	N_A	N_E	I	H_O	H_E	uH_E	F	*PPL (%)*	A_R	F_{IS}	TPM	Standardized difference test
ZTR	1	9	2	3.61	2.71	1.05	0.64	0.59	0.62	-0.09	1.00	3.65	-0.03	2.89	0.00
KPR	2	22	0	3.71	2.39	0.94	0.51	0.52	0.54	0.02	0.97	3.31	0.05	2.28	0.01
PBR	3	22	2	3.81	2.44	0.95	0.53	0.52	0.54	0.01	0.97	3.31	0.02	1.70	0.04
TUR	4	20	0	3.84	2.61	0.98	0.47	0.52	0.54	0.09	0.97	3.56	0.13	1.90	0.03
UKR	5	19	0	3.87	2.31	0.95	0.52	0.52	0.53	-0.02	0.97	3.62	0.02	0.54	0.30
BKR	6	22	0	3.68	2.28	0.90	0.48	0.49	0.50	0.00	0.97	3.32	0.03	0.62	0.27
KMR	7	19	1	3.74	2.48	1.00	0.56	0.56	0.58	-0.01	0.97	3.54	0.02	2.92	0.00
SUR	8	20	0	3.52	2.50	0.94	0.50	0.53	0.54	0.04	0.97	3.33	0.07	3.45	0.00
NSR	9	22	1	4.19	2.59	1.03	0.55	0.56	0.57	0.02	0.97	3.58	0.03	1.42	0.08
CLR	10	20	1	3.55	2.57	0.96	0.57	0.54	0.55	-0.06	0.94	3.31	-0.03	3.62	0.00
HCR	11	10	1	3.94	2.71	1.06	0.57	0.58	0.61	0.02	0.97	3.92	0.06	1.37	0.09
DUR	12	20	3	4.52	2.64	1.08	0.58	0.57	0.59	-0.01	0.97	3.80	0.01	0.45	0.33
DIR	13	20	0	3.19	2.33	0.90	0.51	0.52	0.53	0.03	0.97	3.00	0.05	3.72	0.00
TYR	14	10	0	3.16	2.24	0.86	0.50	0.49	0.52	-0.04	0.97	3.10	0.03	1.65	0.05
HDR	15	20	4	4.35	2.78	1.13	0.55	0.61	0.62	0.08	0.97	4.06	0.12	2.30	0.01
HSR	16	22	3	4.19	2.57	1.04	0.51	0.57	0.58	0.10	0.97	3.78	0.13	1.64	0.05
STR	17	11	0	3.65	2.64	1.02	0.56	0.57	0.61	0.00	0.97	3.62	0.10	-	-
SHR	18	14	1	3.94	2.78	1.07	0.61	0.58	0.60	-0.02	0.97	3.78	-0.01	2.59	0.00
KTR	19	10	0	2.61	1.94	0.69	0.39	0.42	0.44	0.05	0.87	2.70	0.11	2.11	0.02
ASR	20	20	0	2.81	2.04	0.71	0.41	0.42	0.43	0.01	0.90	2.60	0.03	2.26	0.01
KLR	21	20	0	2.58	1.96	0.70	0.45	0.43	0.44	-0.04	0.94	2.46	-0.04	3.22	0.00
KAR	22	20	0	4.13	2.79	1.09	0.63	0.59	0.61	-0.06	1.00	3.68	-0.04	2.79	0.00
AJR	23	19	1	3.68	2.51	0.96	0.53	0.54	0.55	0.01	1.00	3.28	0.04	2.49	0.01
BR	24	27	0	4.13	2.59	1.04	0.48	0.56	0.57	0.11	0.97	3.68	0.15	1.60	0.06
PR	25	26	2	4.32	2.82	1.12	0.59	0.59	0.60	0.03	1.00	4.04	0.02	2.55	0.01
SYR	26	30	0	3.13	2.11	0.79	0.43	0.46	0.47	0.08	0.87	2.69	0.10	3.08	0.00
SCR	27	30	1	3.10	1.95	0.72	0.41	0.42	0.43	0.03	0.90	2.64	0.05	1.21	0.11
YCR	28	30	0	3.03	2.05	0.75	0.40	0.44	0.45	0.09	0.87	2.62	0.11	2.83	0.00
PSR	29	30	0	3.16	2.07	0.76	0.39	0.44	0.45	0.09	0.87	2.72	0.12	2.39	0.01
AWSR	30	29	1	3.13	1.98	0.76	0.40	0.44	0.45	0.10	0.90	2.72	0.12	1.97	0.02
HTCR	31	49	0	3.10	2.04	0.77	0.45	0.45	0.46	0.02	0.90	2.69	0.02	3.26	0.00
HTR	32	30	1	3.23	2.02	0.77	0.44	0.44	0.44	0.00	0.90	2.91	0.01	1.64	0.05
TMR	33	30	0	2.94	1.96	0.71	0.38	0.43	0.43	0.12	0.87	2.54	0.13	2.53	0.01
WKMR	34	30	0	3.16	1.97	0.75	0.40	0.44	0.44	0.08	0.90	2.68	0.11	1.75	0.04
GZR	35	20	1	3.03	1.93	0.70	0.40	0.41	0.42	0.04	0.97	2.72	0.06	0.28	0.39
HCLR	36	30	0	3.13	1.94	0.73	0.35	0.42	0.43	0.16	0.94	2.84	0.19	1.12	0.13
PJR	37	30	0	2.81	1.92	0.71	0.39	0.42	0.42	0.06	0.97	2.72	0.08	2.24	0.01
TLR	38	14	0	2.71	1.85	0.66	0.39	0.39	0.40	0.00	0.81	2.54	0.04	1.16	0.12
DMR	39	30	0	1.74	1.47	0.37	0.21	0.25	0.25	0.13	0.55	1.74	0.15	4.15	0.00
DM2R	40	23	1	1.74	1.49	0.38	0.25	0.26	0.26	0.01	0.55	1.71	0.03	4.22	0.00
YXR	41	30	0	3.06	1.94	0.70	0.36	0.41	0.42	0.15	0.90	2.61	0.14	1.24	0.11
ALR	42	29	0	3.35	2.10	0.80	0.44	0.45	0.46	0.01	0.94	2.94	0.04	1.44	0.07
WSR	43	30	1	2.97	1.80	0.64	0.33	0.37	0.38	0.11	0.90	2.53	0.13	-0.23	0.41
QQR	44	30	0	2.74	1.72	0.60	0.35	0.36	0.37	0.05	0.94	2.33	0.04	0.57	0.28
PZR	45	30	0	2.39	1.71	0.57	0.37	0.35	0.36	-0.04	0.81	2.18	-0.05	2.63	0.00
YZR	46	30	0	2.97	2.03	0.76	0.41	0.45	0.46	0.08	0.97	2.78	0.11	2.62	0.00
Mean	-	23	-	3.33	2.22	0.84	0.46	0.48	0.49	0.03	0.92	3.04	0.06	-	-
Total	-	-	28	-	-	-	-	-	-	-	-	-	-	-	-

Notes: PID, Population ID; N, Sample size; N_P, Private alleles; N_A, Number of alleles; N_E, Effective number of alleles; I, Shannon's information index; H_O, Observed heterozygosity; H_E, Expected heterozygosity; uH_E, Unbiased expected heterozygosity; F, Fixation index; *PPL*, Percentage of polymorphic loci; A_R, Allelic richness; F_{IS}, Inbreeding coefficient; TPM, Two phase mutation model.

3.2. *Population structure of* Juglans regia

Our analyses grouped all walnut populations into three clusters (Figs. 2 and 3). Bayesian structure analysis revealed that the optimal number of K value was 3 (Fig. 2a). At $K = 3$, the 1082 individuals from the 46 populations of *Juglans regia* were classified into three principal genetic clusters (Fig. 2c). Specifically, Group 3 consists of 53 individuals from two populations in Gongliu Wild Walnut Valley in Xinjiang; Group 2 comprises 418 individuals from 22 populations from the western Himalaya, and Group 1 contains 611 individuals from the 22 remaining populations from the southern piedmont of Tien-Shan (Figs. 2c and S1). PCoA indicates that PCoA 1, PCoA 2, and PCoA 3 explained 8.52%, 5.54%, and 4.73% of the total genetic variation, respectively (Fig. 3a), categorizing individuals of *J. regia* into three major groups. NJ analysis also grouped all 1082 individuals into three principal clades (Fig. 3b). The three walnut population clusters identified by our analyses correspond to the geographical distribution of *J. regia* populations (For more details, see Fig. S1). The genetic divergence between the three groups is moderate to high (G1 vs G2, 0.137; G1 vs G3, 0.349; G2 vs G3, 0.304). Notably, our analyses indicated that Group 3 (populations 39 and 40) were genetically distinct from Group 1 and Group 2. However, some individuals from Group 1 and Group 2 exhibited admixed genetic composition from two groups, suggesting potential gene flow or limited genetic differentiation.

3.3. *Genetic differentiation and gene flow among populations of* Juglans regia

Genetic differentiation between the 46 walnut populations was moderate ($0.05 < F_{ST} < 0.15$). Nei's genetic distance values (D_A) were similar to genetic differentiation (F_{ST}) (Fig. S2 and Table S6). Measures of both genetic distance and genetic differentiation indicate that populations 39 and 40 diverged genetically from the remaining walnut populations (Fig. S2 and Table S6). Migration rates were low among walnut populations (Table S7). The Wilcoxon and/or standardized difference tests indicated that 28 out of 46 populations exhibit substantial bottlenecks ($P < 0.05$), suggesting excess heterozygosity and a recent decrease in effective population size at the population level (Table 1). AMOVA indicated that 74.23% of the overall variation was associated with differences within populations. The remaining 25.77% of variation resided among populations (Table 2), possibly due to the substantial gene flow ($N_m = 2.08$) between populations of *J. regia*. Furthermore, AMOVA indicated that most (82.03%) of the observed genetic diversity within groups was partitioned within populations, whereas 17.97% could be due to population differences (Table 2). A hierarchical AMOVA based on the findings of the genetic diversity analysis revealed that at the group level genetic diversity was higher within populations than among populations. Specifically, the genetic diversity among the populations from Gongliu Wild Walnut Valley (Group 3) was greater (93.18%) than that within the populations of the southern

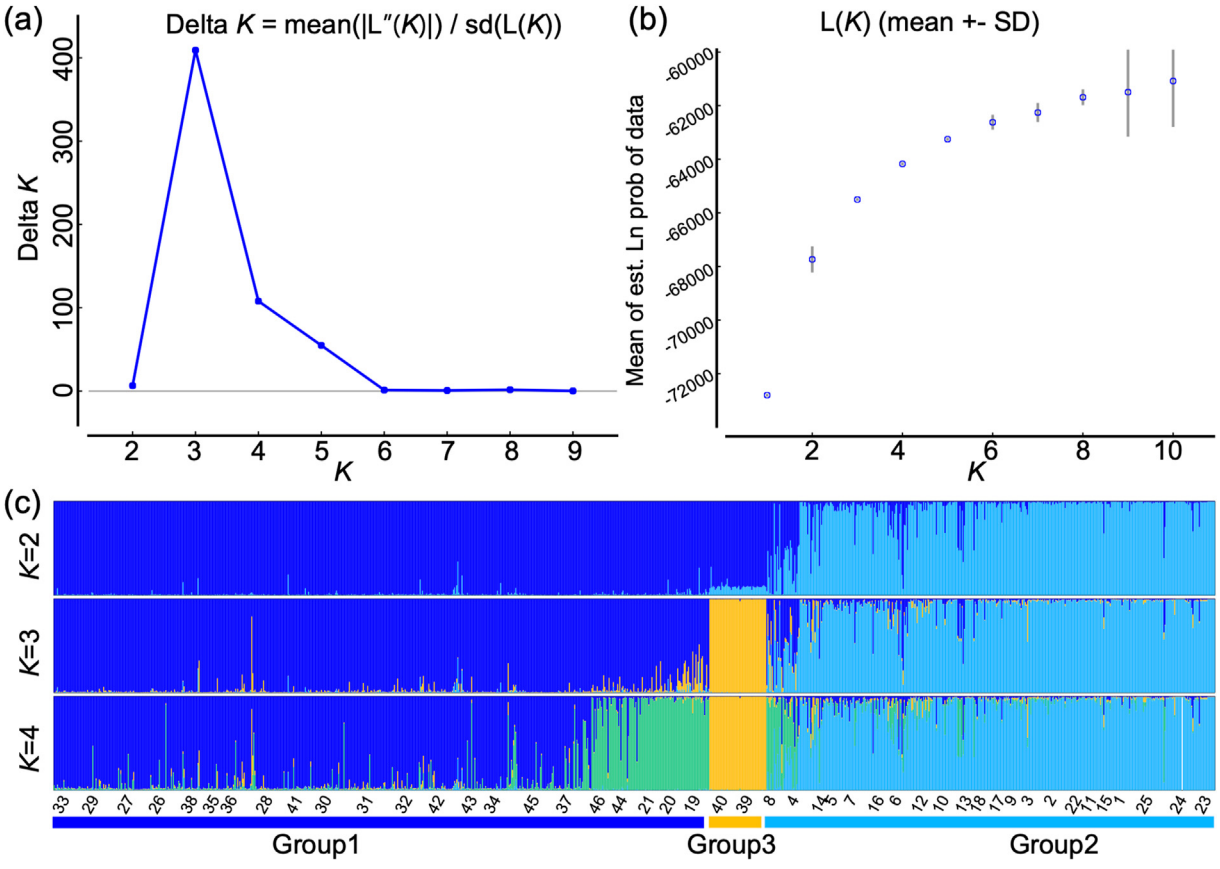

Fig. 2. Bayesian inference clustering STRUCTURE analysis of 1082 individuals from 46 populations of *Juglans regia*. (a) The optimal K value using the delta K (ΔK) method, maximum number of populations (K), was inferred at $K = 3$. (b) Mean log-likelihood [Ln(K) ± SD] of the data against the number of K. (c) A Bayesian clustering of 1082 individuals of *J. regia* from $K = 2$ to $K = 4$. Different colors indicate different genetic groups: blue for Group 1 (G1), light blue for Group 2 (G2), and yellow for Group 3 (G3).

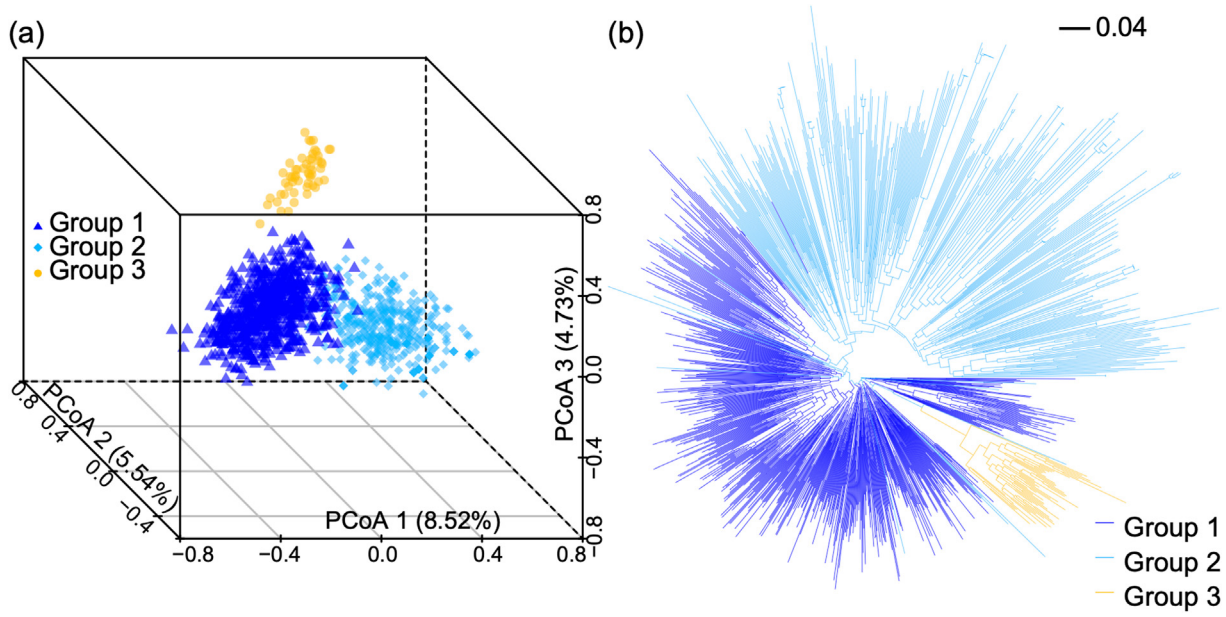

Fig. 3. Clustering relationship of 1082 *Juglans regia* individuals. (a) Principal coordinates analysis (PCoA). The first and second coordinates explain 8.52% and 5.54% of the total genetic variation, respectively. (b) A radial distance neighbor-joining tree of all *J. regia* individuals. The color scheme and group designation correspond to Fig. 2c.

Table 2
Analysis of molecular variance (AMOVA) determining the partition of genetic variation for all 46 populations of *Juglans regia* based on 31 microsatellite loci.

Scale	Source	d.f.	Sum of squares	Mean squares	Percentage of variation (%)
Total	Among Pops.	45	6621.79	147.15	25.77
	Within Pops.	1036	16666.55	16.09	74.23
	Total	1081	23288.34		100.00
Groups	Among Pops.	2	2431.95	1215.98	17.97
	Within Pops.	1079	20856.39	19.33	82.03
	Total	1081	23288.34		100.00
G1	Among Pops.	22	2408.26	109.47	18.88
	Within Pops.	608	9039.73	14.87	81.12
	Total	630	11447.99		100.00
G2	Among Pops.	20	1755.04	87.75	16.08
	Within Pops.	377	7161.20	19.00	83.92
	Total	397	8916.24		100.00
G3	Among Pops.	1	26.53	26.53	6.82
	Within Pops.	51	465.62	9.13	93.18
	Total	52	492.15		100.00

Note: d.f., degree of freedom.

piedmont of Tien-Shan (Group 2) (83.92%) and of the western Himalaya (Group 1) (81.12%) (Table 2).

3.4. Mantel test and geographic barriers

The Mantel test showed a weak positive correlation between genetic and geographic distance among populations in the whole investigated dataset of *Juglans regia* ($r^2 = 0.011$, $P = 1e\text{-}04$) as well as among populations in each specific group (Group 1, $r^2 = 0.133$, $P = 1e\text{-}04$; Group 2, $r^2 = 0.039$, $P = 1e\text{-}04$). The contribution of altitude to genetic differentiation was found to be statistically insignificant (Figs. 4 and S3). Correlation analysis between genetic diversity and environmental distances indicated a non-significant correlation between annual mean temperature, annual mean precipitation, and genetic diversity in the overall populations ($r^2 = 0.098$, $P = 2e\text{-}04$; $r^2 = 0.135$, $P = 1e\text{-}04$), respectively (Fig. S3). This relationship was identified within groups, except for the populations from the Gongliu Wild Walnut Valley (Group 3), where no correlation was detected due to small sample size (Fig. 4). Nevertheless, according to the Barrier analysis, two primary genetic barriers were determined, with a bootstrap support of > 90% (Figs. 5 and S4). The first geographical barrier (B1) separated population 1 from all other populations of *J. regia* in Central Asia. The second barrier (B2) isolated populations 10 and 11 from the remaining populations (Fig. 5 and S4). These two barriers align with the presence of the Takht-e-Sulaiman Mountains and Hindu Kush Range, respectively (Figs. 5 and S1).

4. Discussion

4.1. Genetic diversity of Juglans regia in Central Asia

We found that *Juglans regia* populations across Central Asia have moderate levels of genetic diversity. These levels of genetic diversity are comparable to that reported for *J. regia* from the Iranian Plateau (Shahi Shavvon et al., 2023), although our estimates are generally lower than those from previous studies (Table 3). Low levels of genetic diversity may be attributed to habitat

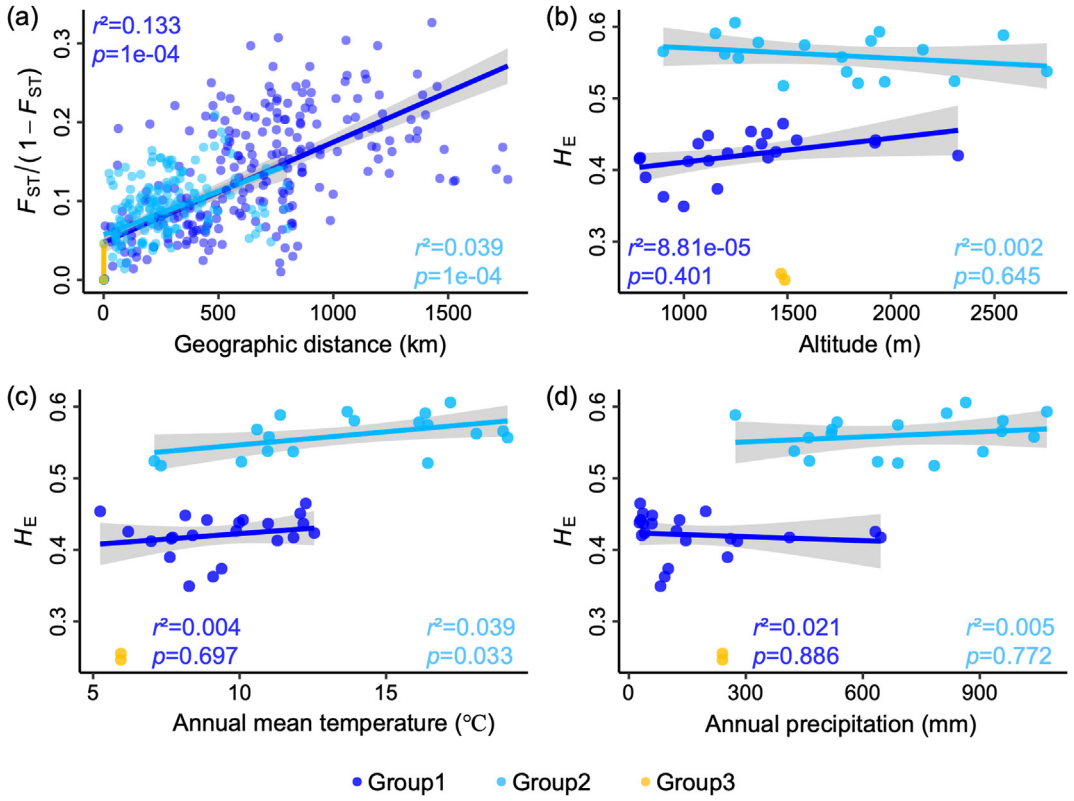

Fig. 4. The relationship between genetic component and environmental variables at the group level: (a) Mantel test of genetic distance (pairwise F_{ST}) and geographic distance (in kilometers), (b–d) correlations between genetic diversity (H_E) and altitude, annual mean temperature, and annual mean precipitation within three groups. The group designation corresponds to genetic structure analysis results (Fig. 2c).

Table 3
Genetic diversity of *Juglans regia* based on microsatellite markers in previous studies.

	Current study	Pollegioni et al. (2014)	Roor et al. (2017)	Gaisberger et al. (2020)	Magige et al. (2022)	Shahi Shavvon et al. (2023)	Yan et al. (2024)
H_O	**0.46**	0.56	0.62	0.57	0.56	0.44	0.42
H_E	**0.48**	0.59	0.64	0.59	0.56	0.44	0.47
N_A	**3.33**	4.78	7.54	5.10	3.79	3.14	4.52

fragmentation and/or human interference. Previous studies have shown that isolated habitat patches decrease population sizes, which in turn decreases genetic diversity owing to genetic drift, inbreeding, and bottleneck events (Bijma et al., 2000; González et al., 2020). In addition, human introduced populations may show low genetic diversity, as proved in *J. regia* populations from Xizang and Northern China (Qi et al., 2023; Yan et al., 2024). Alternatively, these moderate levels of genetic diversity might be ascribed to our use of SSR markers from *J. sigillata*. Primers transferred from closely related species, which are likely to produce more conservative results than those developed from the focal species. Although we believe the use of primers from *J. sigillata* has not greatly affected our estimations of *J. regia* genetic diversity in Central Asia, we recommend that future studies use population genomics with extensive geographical sampling.

Several genetic diversity parameters (i.e., H_E, H_O, N_E, and I) indicated that the highest values of genetic diversity were found in the *J. regia* population (population 25) located in mountainous areas of Jammu and Kashmir in the western Himalaya, followed by four populations from Pakistan. This is consistent with previous studies that found walnut populations from these areas have high levels of genetic diversity (Ahmed et al., 2012; Gaisberger et al., 2020; Magige et al., 2022; Yan et al., 2024). Hence, the western Himalaya (e.g., Jammu and Kashmir) can be considered core centers of genetic diversity. Pollen records at these sites date back to the Upper Pleistocene, nearly 30,000 years ago (Kotlia et al., 2000), suggesting that these sites might serve as refugia for walnuts during the LGM. This geographic area is situated at the intersection of Eurasia, with its boundaries extending towards Xizang and Xinjiang Autonomous Region of China in the northeast and Afghanistan and Pakistan in the northwest (Fig. 1). In addition, this area serves as the critical center for commercial walnut production (Shah et al., 2019). The high genetic diversity of four populations in Pakistan (i.e., 15, 22, 1, and 18) may be attributed to artificial seed selection and dispersal driven by the enduring economic and cultural significance of walnut cultivation in Asia, as suggested by Gunn et al. (2010) and Magige et al. (2022). Furthermore, we found multiple private alleles in three of these populations (i.e., populations 15, 18, and 12) (Table 1), which have important conservation implications (See below 4.3).

Our analysis indicates that the lowest genetic variation was typically found in populations from Xinjiang, China, indicating that the populations may have experienced a bottleneck event during the human-mediated introduction. This is consistent with previous work that suggested the low genetic diversity of walnut populations in northern China is attributable to a founder effect caused

by the introduction of individuals from West Asia (Ding et al., 2022; Qi et al., 2023). However, two walnut populations in Gongliu, Xinjiang (i.e., populations 39 and 40) are assumed to be a relic communities from the late Tertiary (Xi and Zhang, 1996), which do show distinct genetic structure and clear genetic differentiation (Figs. 2 and 3; Table S6). Furthermore, the low genetic diversity of these populations might have been caused by glaciation-driven bottleneck events. Our finding that a significant proportion of walnut populations in Central Asia have experienced genetic bottlenecks and a decrease in effective population size is likely due to farmer activities such as seed exchange (Alvarez et al., 2005; Shahi Shavvon et al., 2023).

Regarding populations in Ili valley, genetic diversity was higher in populations from the humid Ili valley than in populations of Gongliu Wild Walnut Valley (populations 39 and 40) (Fig. S1 and Table 1). Geographically, the distance is close between these populations. This genetic pattern may be the result of processes such as expanded quaternary glaciation and long-standing aridification in Central Asia. Researchers have also speculated that high genetic diversity is preserved, and isolated, in the moist valley areas of Tien-Shan, which are surrounded by areas subject to long-term aridification (Zhang et al., 2020). Hence the possible explanation for the genetic diversity of populations from Gongliu Wild Walnut Valley (populations 39 and 40) is long genetic isolation due to historical climatic oscillations. In contrary, the populations outside of Gongliu Wild Walnut Valley might be introduced from multiple resources by human being, which resulted in high genetic diversity.

4.2. Population structure and geographic barriers

The evolutionary potential and adaptability of a species to environmental changes are not only dependent on genetic diversity but also on population genetic structure (Stebbins, 1950). Population structure is determined primarily by genetic differentiation among populations (Frankham et al., 2002). As predicted for woody tree species, we observed a moderate level of genetic differentiation ($F_{ST} = 0.156$) across the 46 walnut populations (Table S6). This level of genetic differentiation in *Juglans regia* is consistent with results for most out-crossing and wind-pollinated species (e.g., *Quercus semecarpifolia*, *Taxus wallichiana*, and *Liquidambar formosana*) (Liu et al., 2013; Sun et al., 2016; Ginwal et al., 2023). Seeds of *J. regia* are spread mainly by rodents (Lenda et al., 2017; Zhang et al., 2017). Previous studies have shown that pollination and seed dispersal mechanisms of *J. regia* contribute to increased gene flow within and among populations, probably decreasing genetic differentiation among populations (Nybom, 2004).

Our analyses consistently categorized the 46 populations of *Juglans regia* in this study into three clusters: populations from the southern piedmont of Tien-Shan (Group 1), populations from the western Himalaya (Group 2), and populations from Gongliu Wild Walnut Valley (Group 3) (Figs. 2 and 3). Significant genetic differentiation was detected between the three groups. Meanwhile, consistent with previous studies (Torokeldiev et al., 2018; Shahi Shavvon et al., 2023; Yan et al., 2024), most genetic variation resides within populations of the three groups (Table 2). Moreover, we also found that geographical distance had a weak influence on genetic differentiation (Fig. 4). Although mountain ranges are known restrict gene flow between different populations in Central Asia (Zhang et al., 2020), our findings indicated that the distribution of genetic clusters is only weakly correlated with geographical barriers. In other words, although these populations are separated by the Pamir Plateau and Tien-Shan, these geographical barriers do not completely prevent gene flow between the populations. Consistent with this, non-significant correlation between genetic diversity and environmental variables might also indicate human activity reshape the genetic landscape (Fig. 4). One possible reason for this finding is that the extensive germplasm exchange among farmers and consumers from similar environmental regions may lead to the movement of plant material, thus aiding species dispersal. A similar case for human-mediated movement of germplasm of walnut was recently reported in the Himalaya (Yan et al., 2024).

Our finding that populations from Gongliu Wild Walnut Valley (Group 3) diverged from the other groups of walnut populations suggest restricted gene flow between Gongliu populations and those of southern Tien-Shan and of the western Himalaya (Table S5 and Table S7). Barrier analysis identified no distinct geographical barriers between Gongliu populations and those of other groups (Fig. 5). Thus, it can be inferred, as previously mentioned, that this separation might be due to the group originating from a distinct ancestry population with long isolation. Despite the geographical

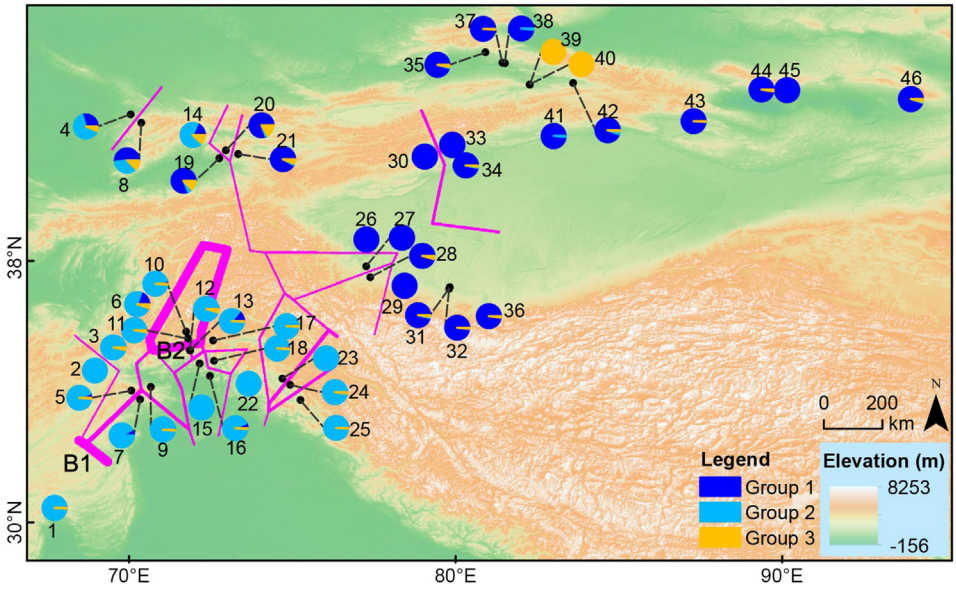

Fig. 5. Geographical distribution of genetic structure of walnuts in Central Asia. Colors and proportions in the pie charts correspond to the results of genetic structure analysis at $K = 3$ (Fig. 2c). Solid and thick lines represent the location of the two most probable barriers detected by Barrier analysis (B1 and B2). Population identifiers align with those found in Table S1.

distances between populations, mixtures were observed in most populations within the populations of southern Tien-Shan (Group 1) and those of the western Himalaya (Group 2), but not those of Gongliu (Group 3) (Fig. 2c). This phenomenon may be associated with factors such as a shared historical gene pool, dispersal of seeds facilitated by humans, and prior historical contact within the geographic range.

4.3. Management implications

Effective conservation strategies rely on assessing spatial and temporal changes in genetic diversity and population structure (Frankham, 2010; Hvilsom et al., 2022). For example, one key goal of conservation genetics is to identify and prioritize the protection of populations with high genetic variation and allele diversity values (Petit et al., 1998; Xiao et al., 2020). Our analyses indicate that populations of wild walnuts in the western Himalaya have high levels of genetic variation (Fig. 1 and Table 1). Thus, we recommend that these populations be prioritized for *in situ* conservation to prevent fragmentation of suitable walnut habitats and/or restore fragmented areas. Furthermore, because the Himalaya have been shown to undergo rapid environmental changes (Liu et al., 2018; Wambulwa et al., 2021), conservation efforts in this region should be adjusted in response to climate change. We also recommend that the western Himalayan accessions (particularly populations 1, 15, 16, 22, and 25) should be subject to *ex situ* conservation and breeding plans.

Walnut populations from Gongliu Wild Walnut Valley (Group 3), Xinjiang, China have low levels of genetic diversity (Table 1), although this region has populations with distinct genetic components, including Gongliu populations (39 and 40) (Fig. 5). We recommend that these populations be conserved using both *in situ* and *ex situ* approaches. Furthermore, the presence of two barriers limits natural genetic exchange and migration in several populations (1, 10, 11, and other populations). To limit the erosion of genetic diversity mediated by global climate change, we recommend that artificial assistant dispersal strategies be used within and between these populations.

5. Conclusions

This study provides comprehensive data on the genetic diversity and broad-scale population structure of *Juglans regia* in different eco-geographic regions across Central Asia. *J. regia* exhibits moderate genetic diversity and limited genetic differentiation, which can be attributed to both the breeding system and the human-mediated movement of plant material. The populations in Xinjiang, China may historically be introduced by human being except the two from Gongliu Wild Walnut Valley. Walnut populations from the western Himalaya showed high genetic diversity and therefore may be a rich genetic resource for future improvement programs and conservation actions. These populations should be protected by *in situ* and *ex situ* conservation measures. Special attention is needed for genetically distinct populations from Gongliu Wild Walnut Valley of Xinjiang, China. Our study paves the way for exploring the germplasm and conservation of common walnuts and serves as an exemplar for studies on other fruit trees in Central Asia.

Data availability

The datasets used in this study are available in Supplementary Material Table S8. Notably, the genotype data of 31 SSR loci of 12 populations from Pakistan and three populations in India were publicly available in previous studies (Magige et al., 2022; Yan et al., 2024), and were downloaded from the papers' data link.

CRediT authorship contribution statement

Linjiang Ye: Formal analysis, Funding acquisition, Writing – original draft – review & editing. **Robabeh Shahi Shavvon:** Formal analysis, Writing– original draft – review & editing. **Hailing Qi:** Data curation, Methodology, Formal analysis, Writing – review & editing. **Hongyu Wu:** Methodology, Formal analysis. **Pengzhen Fan:** Methodology, Formal analysis. **Mohammad Nasir Shalizi:** Investigation, Writing – review & editing. **Safiullah Khurram:** Investigation, Writing – review & editing. **Mamadzhanov Davletbek:** Investigation, Writing – review & editing. **Yerlan Turuspekov:** Investigation, Writing – review & editing. **Jie Liu:** Conceptualization, Investigation, Validation, Funding acquisition, Writing– original draft – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2024.06.001.

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