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Gene Introgression among Closely Related Species in Sympatric Populations: A Case Study of Three Walnut (*Juglans*) Species

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Abstract: Gene introgression usually results from natural hybridization occurring among closely related species in sympatric populations. In this study, we discussed two rare and frequent gene flow phenomena between three species of *Juglans* plants and analyzed the possible causes for the difference. We collected 656 individuals from 40 populations of Persian walnut (*Juglans regia* L.), Chinese walnut (*J. cathayensis* Dode), and Iron walnut (*J. sigillata* Dode) that were genotyped at 17 expressed sequence tag simple sequence repeat (EST-SSR) loci to analyze the introgressions between *J. regia* and *J. cathayensis*, and *J. regia* and *J. sigillata*. Our study compared the spatial patterns of expected heterozygosity (H_E), allelic richness (R_s), and private allele richness (P_{AR}) so as to vividly infer the biogeographic history of related species of *Juglans* in the two regions. The results of the PCoA, UPGMA, and STRUCTURE analyses showed that all *J. regia* and *J. sigillata* populations clustered into one group, and the *J. cathayensis* populations clustered into the other group. The results of the historical gene flow analysis indicated that *J. regia* and *J. sigillata* have no genetic barriers, and the directional gene flow is mainly from *J. regia* to *J. sigillata*. For the three species of *Juglans*, all the above results indicated that gene flow was common among the same group of *Juglans*, and only rare and low-level gene flow appeared in distinct groups. Therefore, our study revealed multiple phenomena of gene flow and introgression among closely related species in sympatric populations, thereby providing a theoretical basis for the genetic evolution of the genus *Juglans*.

Keywords: gene introgression; microsatellites; genetic diversity; sympatric populations; *Juglans*

1. Introduction

Interspecies introgression can cause base substitution and deletion of DNA fragments, leading to a decrease in species specificity, which is likely to promote adaptive evolution of the infiltrated species [1–3]. Interspecific hybridization occurs frequently in sympatric forest trees. Morphology may provide some useful information to identify hybrids, but molecular markers are more reliable at identifying hybrids. Nuclear DNA sequences can be used to differentiate among closely related lineages, to clarify cases of sympatric speciation, and to identify hybrid individuals [4–7]. Studies have shown that hybridization and gene flow between species occur extensively in the genus *Populus* [8–12]. One of these studies showed that *Populus × jrtyschensis* is typical of F1 dominated hybrid zones between the distantly related species, *P. nigra* and *P. laurifolia* [12]. In the genus *Eriobotrya* Lindl., compelling evidence indicated that most haplotypes of *E. prinoides* var. *Daduheensis* were also shared with those of *E. japonica* and *E. prinoides* to produce a hybrid status for *E. prinoides* var. *Daduheensis* [13]. However,

most of these studies only focused on investigating the frequent gene flow between two closely or distantly related species and rarely reported on a variety of gene flow among a single genus.

Walnuts (*Juglans*), one of the eight living genera in the family Juglandaceae, have about 21 species in the world, with a distribution from tropical to temperate zones, and from Asia to Europe and the Americas [14–19]. The walnut (*Juglans*) has always been the focus of biological research because of its economic, culinary, and medicinal value. Although more and more scholars have revealed the genetic diversity and genetic structure of walnut populations, few studies have been done on the interspecific hybridization and gene flow between walnuts. Hybridization between species of the genus *Juglans* is common because of the weak reproductive isolation mechanisms between phylogenetically close species [20–24]. This study is the first to explore the interspecific hybridization of Chinese *Juglans* plants and provides a theoretical basis for the genetic evolution of the genus *Juglans*.

Previous studies on the morphology, molecular biology, phenological, and fossils indicated that *Juglans* plants are classified into two sections that are distributed in China, section *Juglans* and section *Cardiocaryon* [15,25–27]. Section *Juglans* includes the two cultivated walnuts, *J. regia* and *J. sigillata*. Section *Cardiocaryon* contains three taxa, *J. manshurica* M., *J. cathayensis*, and *J. hopeiensis* Hu. These five species are different from each other in numerous ways, from leaves to branches and flowers [17,25–30]. In order to further investigate the gene introgression between *Juglans* species, we selected the aforementioned two groups (section *Juglans* and section *Cardiocaryon*) of walnut species to study (*J. regia*, *J. sigillata*, and *J. cathayensis*); the two cultivated walnuts of section *Juglans* (*J. regia* and *J. sigillata*) were from Yunnan province, while *J. regia* of section *Juglans* and *J. cathayensis* of section *Cardiocaryon* were from the Qingling Mountains. Yunnan province is the most abundant area of biodiversity in China. This province harbors many rare, endemic, and ancient groups and is one of the most important focuses of global research and one of the key areas of biological diversity taxa distribution. The Qingling Mountains are rich in natural resources and are located in the transition zone between the temperate zone and subtropical zone in China, and therefore, have become an important biogeographical boundary in China. Studies have shown that the Qingling Mountains were one of the shelters for walnut plants during the glacial period [31–33].

In recent years, researchers have used different molecular genetic markers (RAPD, RFLP, ITS, cpDNA, SSR, and ISSR) to study the genetic relationship, phylogeography, and genetic structure of *Juglans* [27,28,30,34–43]. Simple sequence repeats (SSR) [34], with the advantage of high polymorphism and co-dominant inheritance, are increasingly used in walnut germplasm resources' research and the genetic study of many crops. Currently, hundreds of SSR primer pairs with high polymorphism are available to *Juglans* and have been demonstrated to be useful in many kinds of genetic analyses on walnut [23,31,37–41]. A previous molecular study used 12 microsatellite markers from samples of several individuals from three regions found in Hubei Province to identify the hybrids between *J. regia* and *J. cathayensis*; that study also suggested that hybridization between *J. regia* and *J. cathayensis* was rare [42]. The SSR data analysis method was also applied to *J. regia* and *J. sigillata* populations. The results revealed that introgression has occurred between the two species (*J. regia* and *J. sigillata*) in southwest China and the direction of gene flow is mainly from *J. regia* to *J. sigillata* [27,43].

In this study, we extended the sample group to cover more natural populations of three species and first explored the interspecific hybridization of Chinese *Juglans* plants, which provides a theoretical basis for the genetic evolution of the genus *Juglans*. We collected 656 individuals from 40 populations of sympatric *J. regia* and *J. cathayensis* in the Qinling Mountains and sympatric *J. regia* and *J. sigillata* in Yunnan Province that were genotyped at 17 expressed sequence tag simple sequence repeat (EST-SSR) loci to address the following two questions: (1) What is the genetic diversity and genetic structure of walnut plants, taking three species of *Juglans* as an example to explore the phenomenon of gene flow among closely related species in sympatric populations? (2) Are *J. sigillata* and *J. regia* completely different species or are they simply different ecological types?

2. Materials and Methods

2.1. Sampling, DNA Extraction, and PCR Amplification

Leaves from 656 samples were collected from 40 typical populations of *J. regia*, *J. cathayensis*, and *J. sigillata* (details in Table 1; Figure 1). In this group, 4 pairs of the sympatric *J. regia* ($n = 58$, labeled with a Y-r postfix) and *J. sigillata* ($n = 120$, labeled with a Y-s postfix) populations were from Yunnan province; 12 pairs of the sympatric *J. regia* ($n = 183$, labeled with an r postfix) and *J. cathayensis* ($n = 186$, labeled with a c postfix) populations were from the Qingling Mountains; 4 pairs of the sympatric *J. regia* ($n = 49$, labeled with an r postfix) and *J. cathayensis* ($n = 60$, labeled with a c postfix) populations were from the four other provinces, Sichuan, Yunnan, Gansu, and Henan. Samples were identified and collected according to the morphological characteristics (leaf, flower, fruit, nut, etc.) of the three *Juglans* species [17,25–30]. All fresh leaves were completely dried with silica gel, which followed the methods of Doyle and Doyle (1987) [44] and Zhao and Woeste (2011) [24], and were stored at $-20\text{ }^{\circ}\text{C}$. Following DNA extraction, we further examined the gene flow of the three species via 17 pairs of polymorphic EST-SSR primers, which have been reported before (Table S1). All primers were synthesized and labeled with fluorescent dye, 6-FAM, TAMRA, or HEX by Sangon Biotech in Shanghai, China. PCR amplification was carried out on a SimpliAmp Thermal Cycler (Applied Biosystem, Hercules, CA, USA) in a 20 μL reaction volumes (10 μL 2 $\times$ PCR master mix, 0.4 μL each primer, 2 μL BSA, 2 μL of 10 ng/ μL DNA, and 5.2 μL ddH₂O). PCR amplifications were performed as follows: an initial denaturation step at $94\text{ }^{\circ}\text{C}$ for 5 min, followed by 30 cycles of 45 s at $94\text{ }^{\circ}\text{C}$, 30 s annealing temperature (T_m ; Table S1), and 90 s at $72\text{ }^{\circ}\text{C}$, and a final extension step at $72\text{ }^{\circ}\text{C}$ for 5 min. Fragment sizes were evaluated using GeneMapper v3.7 (Applied Biosystems) and were checked manually, twice, to reduce the scoring error.

2.2. Microsatellite Data Analysis

The evaluation of the genetic diversity for each locus and population was based on the following descriptive summary statistics: the number of alleles (A), the number of effective alleles (N_e), the observed (H_O) and expected (H_E) heterozygosity, the unbiased expected heterozygosity (U_{H_E}), the polymorphic information content (PIC), the within-population inbreeding coefficient (F_{IS}), the total population inbreeding coefficient (F_{IT}), and the among-population genetic differentiation coefficient (F_{ST}) using GenAlEx6.5 [45]. Allelic richness (R_s) and private allele richness (P_{AR}) were calculated by rarefying to the minimum sample size across populations [46] (Tables S1 and S2). Subsequently, the expected heterozygosity (H_E), the allelic richness (R_s), and the private allele richness (P_{AR}) of the 40 populations sampled were used to show the geographic pattern by using the inverse distance weighted (IDW) interpolation function implemented in the Geographic Information System software ArcGIS 10.0 (ESRI, Redlands, CA, USA). The program, Arlequin version 3.5 [47], was used to analyze the molecular variance (AMOVA) of each species, among populations within species, and within populations among individuals. We calculated F_{ST} to identify the outlier loci potentially under selection [48]. Loci under selection pressure can bias the population's genetic analysis because most methods for evaluating genetic structure assume neutrality [49,50] ($p \leq 0.05$; Figure S2). GENEPOP Version 4.2 [51] was used to test all loci of the Hardy–Weinberg equilibrium (HWE). Markers were also tested for the linkage disequilibrium (LD) at each population, and the locus was tested using FSTAT [52]. Significance was tested using 1000 permutations. CERVUS version 3.0 [53] was used to calculate the polymorphic information content (PIC). Each locus was evaluated for the possible presence of a null allele using MICRO-CHECKER 2.2.3 [54].

Table 1. Details of sample location, sample size, and descriptive statistics of genetic variability for 18 *Juglans regia* L. populations, four *Juglans sigillata* Dode populations, and 14 *Juglans cathayensis* Dode populations sampled in China.

Order	Code	Species	Sample Site	Samples	Latitude	Longitude	A	Ne	Ho	H _E	U _{H_E}	F _{IS}	Rs	P _{AR}
1	QS-r	<i>J. regia</i>	Qishan, Shaanxi	28	34°26'36" N	107°37'16" E	3.706	2.069	0.456	0.422	0.430	−0.092	2.780	0.010
2	HHG-r	<i>J. regia</i>	Honghe, Shaanxi	12	34°16'27" N	107°44'59" E	3.059	1.923	0.450	0.415	0.433	−0.088	2.870	0.020
3	HZ-r	<i>J. regia</i>	Hanzhong, Shaanxi	4	32°37'01" N	107°24'29" E	1.941	1.596	0.324	0.250	0.286	−0.284	1.940	0.000
4	BJ-r	<i>J. regia</i>	Baoji, Shaanxi	15	34°21'36" N	107°52'12" E	2.294	1.631	0.203	0.304	0.315	0.266	2.110	0.000
5	NS-r	<i>J. regia</i>	Ankang, Shaanxi	19	33°45'17" N	108°45'52" E	3.765	2.253	0.501	0.515	0.529	0.039	3.230	0.050
6	HH-r	<i>J. regia</i>	Honghegu, Shaanxi	12	34°16'12" N	108°57'00" E	2.118	1.555	0.208	0.288	0.301	0.293	2.030	0.050
7	LN-r	<i>J. regia</i>	Shangluo, Shaanxi	20	34°05'27" N	110°08'55" E	2.882	1.809	0.350	0.374	0.384	0.039	2.440	0.000
8	LT-r	<i>J. regia</i>	Lantian, Shaanxi	10	34°09'06" N	109°19'25" E	3.000	1.949	0.501	0.451	0.475	−0.059	2.910	0.110
9	PL-r	<i>J. regia</i>	Pingli, Shaanxi	14	32°23'24" N	109°21'36" E	2.471	1.723	0.290	0.322	0.335	0.125	2.290	0.000
10	SZ-r	<i>J. regia</i>	Shangluo, Shaanxi	23	33°51'46" N	109°56'28" E	3.118	1.813	0.341	0.385	0.394	0.138	2.540	0.020
11	ZS-r	<i>J. regia</i>	Shangluo, Shaanxi	15	33°31'48" N	109°52'48" E	2.059	1.619	0.263	0.300	0.310	0.169	2.010	0.010
12	SL-r	<i>J. regia</i>	Shangluo, Shaanxi	11	33°41'45" N	110°19'38" E	3.000	2.020	0.566	0.436	0.457	−0.282	2.840	0.000
13	EM-r	<i>J. regia</i>	Emei, Sichuan	14	30°39'00" N	102°04'48" E	2.588	1.814	0.285	0.367	0.382	0.193	2.420	0.010
14	YN-r	<i>J. regia</i>	Wenshan, Yunnan	12	23°24'00" N	104°13'12" E	2.529	1.791	0.226	0.366	0.383	0.398	2.430	0.000
15	GS-r	<i>J. regia</i>	Tianshui, Gansu	9	34°18'00" N	106°03'00" E	2.059	1.596	0.215	0.291	0.310	0.231	2.060	0.060
16	HN-r	<i>J. regia</i>	Zhenzhou, Henan	14	34°46'12" N	113°45'00" E	2.353	1.657	0.288	0.315	0.327	0.088	2.200	0.000
17	QZ-Y-r	<i>J. regia</i>	Qizong, Yunnan	22	27°34'00" N	99°31'00" E	3.294	2.317	0.369	0.443	0.454	0.177	2.840	0.000
18	LM-Y-r	<i>J. regia</i>	Liming, Yunnan	10	27°9'00" N	99°48'00" E	1.176	1.118	0.165	0.092	0.097	−0.634	2.110	0.040
19	SG-Y-r	<i>J. regia</i>	Shigu, Yunnan	11	26°52'00" N	99°57'00" E	2.529	1.747	0.278	0.335	0.352	0.201	2.440	0.060
20	BS-Y-r	<i>J. regia</i>	Baoshan, Yunnan	15	25°17'00" N	98°47'00" E	1.765	1.461	0.249	0.251	0.261	0.033	2.660	0.000
Average							2.585	1.773	0.326	0.346	0.361	0.048	2.458	0.022
21	QZ-Y-s	<i>J. sigillata</i>	Qizong, Yunnan	30	27°34'00" N	99°31'00" E	3.176	2.183	0.346	0.387	0.394	0.150	3.510	0.010
22	LM-Y-s	<i>J. sigillata</i>	Liming, Yunnan	30	27°9'00" N	99°48'00" E	2.882	2.077	0.387	0.367	0.374	−0.030	3.230	0.050
23	SG-Y-s	<i>J. sigillata</i>	Shigu, Yunnan	30	26°52'00" N	99°57'00" E	2.353	1.604	0.258	0.312	0.317	0.193	2.900	0.000
24	BS-Y-s	<i>J. sigillata</i>	Yongping, Yunnan	30	25°27'00" N	99°32'00" E	2.294	1.418	0.336	0.254	0.259	−0.215	2.730	0.000
Average							2.676	1.821	0.332	0.33	0.336	0.025	3.093	0.015
25	BY-c	<i>J. cathayensis</i>	Baoji, Shaanxi	21	34°26'36" N	107°37'16" E	4.765	2.780	0.476	0.515	0.527	0.211	3.820	0.040
26	GX-c	<i>J. cathayensis</i>	Baoji, Shaanxi	16	34°21'47" N	107°14'16" E	4.471	2.978	0.475	0.458	0.473	0.057	3.860	0.090
27	HZ-c	<i>J. cathayensis</i>	Hanzhong, Shaanxi	20	32°37'01" N	107°24'29" E	4.824	2.923	0.487	0.486	0.499	0.037	3.830	0.050
28	BJ-c	<i>J. cathayensis</i>	Baoji, Shaanxi	9	34°21'33" N	107°52'30" E	3.412	2.226	0.434	0.441	0.468	0.041	3.400	0.000
29	NS-c	<i>J. cathayensis</i>	Ankang, Shaanxi	18	33°32'60" N	108°32'58" E	4.353	2.658	0.481	0.484	0.498	0.016	3.570	0.030
30	HH-c	<i>J. cathayensis</i>	Honghe, Shaanxi	24	34°15'56" N	108°57'16" E	5.706	3.134	0.527	0.497	0.508	−0.033	4.060	0.020
31	LN-c	<i>J. cathayensis</i>	Shangluo, Shaanxi	19	34°15'56" N	108°57'16" E	3.412	2.024	0.452	0.411	0.423	−0.093	2.930	0.010
32	LT-c	<i>J. cathayensis</i>	Lantian, Shaanxi	5	34°09'06" N	109°19'25" E	3.882	3.153	0.552	0.611	0.698	0.095	3.880	0.160

Table 1. Cont.

Order	Code	Species	Sample Site	Samples	Latitude	Longitude	A	Ne	Ho	H _E	U _{H_E}	F _{IS}	Rs	P _{AR}
33	PL-c	<i>J. cathayensis</i>	Ankang, Shaanxi	18	32°23'20" N	109°21'43" E	5.353	3.210	0.506	0.499	0.513	0.030	4.260	0.080
34	SZ-c	<i>J. cathayensis</i>	Shangluo, Shaanxi	10	33°51'38" N	109°52'43" E	3.588	2.400	0.481	0.467	0.493	0.070	3.480	0.000
35	ZS-c	<i>J. cathayensis</i>	Shangluo, Shaanxi	15	33°31'56" N	109°52'56" E	3.706	2.268	0.506	0.467	0.484	−0.063	3.290	0.030
36	SL-c	<i>J. cathayensis</i>	Shangluo, Shaanxi	11	33°27'49" N	110°26'30" E	3.647	2.483	0.473	0.462	0.484	0.014	3.400	0.000
37	EM-c	<i>J. cathayensis</i>	Baoxing, Sichuan	9	30°22'05" N	102°48'52" E	3.294	2.158	0.399	0.404	0.428	−0.007	3.240	0.060
38	YN-c	<i>J. cathayensis</i>	Wenshan, Yunnan	11	23°24'02" N	104°12'58" E	3.471	2.463	0.494	0.507	0.532	−0.014	3.340	0.120
39	GS-c	<i>J. cathayensis</i>	Tianshui, Gansu	20	34°34'13" N	105°53'22" E	4.588	2.664	0.426	0.431	0.442	0.056	3.570	0.080
40	HN-c	<i>J. cathayensis</i>	Linzhou, Henan	20	36°04'59" N	113°49'12" E	3.765	2.430	0.436	0.446	0.457	0.023	3.290	0.080
Average							4.140	2.622	0.475	0.474	0.495	0.028	3.576	0.053

Note: Each population's total number of alleles (A), number of effective alleles (Ne), observed (Ho) and expected (H_E) heterozygosity, unbiased expected heterozygosity (U_{H_E}), inbreeding coefficient (F_{IS}), allelic richness (Rs), and private allele richness (P_{AR}).

Figure 1. The sample collection information for the three walnuts. (a) A total of 656 trees from 40 sites of sympatric *Juglans regia* and *J. cathayensis* in the Qinling Mountains and sympatric *J. regia* and *J. sigillata* in Yunnan Province. The black dots indicate the population locations. (b) The map information for a total of 369 samples from 24 populations from the Qinling Mountains. The red dots and blue dots indicate the locations of the sympatric *J. regia* (183 individuals) and *J. cathayensis* (186 individuals), respectively. (c) The map information for a total of 178 samples of 8 populations from the Qinling Mountains. The red dots and green dots indicate the locations of the sympatric *J. regia* (58 individuals) and *J. sigillata* (120 individuals) populations, respectively.

Genetic structure analysis was implemented in STRUCTURE version 2.3.4 using a Bayesian clustering approach [55]. A total of nine independent simulations were run for each K (1–10), with 50,000 burn-in steps followed by 100,000 MCMC steps, using the LOCPRIOR model (admixture model with sample locations as prior information) with dependent allele frequencies. The program STRUCTURE HARVESTER was used to calculate the optimal value of K using the delta K criterion [56], while the inferred clusters were drawn as colored box plots using the program DISTRUCT [57]. GenAlEx 6.5 was used for principal coordinate analysis (PCoA) to explore the overall genetic variation of the EST-SSR data. The unweighted pair-group method with an arithmetic means (UPGMA) tree based on Nei's genetic distance [58] was utilized using POPTREE2 [59] and visualized with the software Fig Tree 1.4.2 [60]. MIGRATE 3.6.4 [61] was used to estimate the historical gene flow parameter (M) and the frequency of migration events through the coalescent history between sympatric *J. regia* and *J. sigillata* populations in Yunnan Province and *J. regia* and *J. cathayensis* populations in the Qinling Mountains for the EST-SSRs. The mode and 95% highest posterior density were then estimated after checking for data convergence.

3. Results

3.1. Nuclear Microsatellite Diversity

Genetic diversity estimates varied among the microsatellite loci (Table S1) and among the population (Table 1). The allelic diversity across the loci was highly polymorphic and is summarized in Table S1. Most loci showed a significant departure from the Hardy–Weinberg equilibrium (HWE) test, which was performed for all individuals using Genepop with 1000 permutations. A total of 55 alleles were detected over the 17 loci among the 656 sampled individuals, with the total number of alleles (A) per locus ranging from 2 to 7 and having a mean of 3.22 (Table S1). Locus JR1817 had the largest number of alleles ($A = 7$). A private allele was identified at a high frequency (0.19) at the GS-r sample site. The observed (H_o) and expected (H_E) heterozygosity varied from 0.117 to 0.668 and from 0.135 to 0.671, with averages of 0.386 and 0.396, respectively (Table S1). The within-population inbreeding coefficient (F_{IS}) was significantly negative for JC8125, JH89978, JM5969, JR4616, JH42753, JH86514, JH6044, and JH2096, and was significantly positive for the remaining nine loci. An F_{IS} value significantly greater than zero indicated heterozygote deficiency, which may be the result of the inbreeding extent of the larger population. The inbreeding coefficient was determined (F_{IT}) at each locus, ranging from 0.107 to 0.784, with an average of 0.441. The genetic differentiation (F_{ST}) ranged from 0.188 to 0.77, with a mean of 0.429, indicating strong genetic differentiation among the populations (Table S1). Eight of the seventeen loci were detected to be F_{ST} outliers: JC3412, JM5969, JC7329, JR4964, JR4616, JM78331, JR6160, and JR1817 (Figure S2).

3.2. Genetic Diversity within and among *Juglans* Population

Genetic diversity estimates for each population based on the 17 EST-SSRs are reported in Table 1. The values of the A , N_e , H_o , H_E , U_{H_E} , R_s , and P_{AR} of each population ranged from 1.176 to 5.706, 1.118 to 3.21, 0.165 to 0.566, 0.092 to 0.611, 0.097 to 0.698, 1.960 to 4.260, and 0.000 to 0.160, respectively (Table 1). Overall, F_{IS} ranged from -0.634 (18-LM-Y-r) to 0.398 (14-YN-r), which was significantly greater than zero in 27 of 40 sampled sites, indicating a surplus of homozygotes in 14, 2, and 11 of *J. regia*, *J. cathayensis*, and *J. sigillata*, respectively. Figures 2 and 3 display, geographically, the values of the expected heterozygosity (H_E), the allelic richness (R_s), and the private allele richness (P_{AR}) reported in Table 1. The geospatial interpolation of these indices enables us to better compare the new spatial data representing the genetic diversity of sympatric *J. regia* versus *J. cathayensis* populations in Qinling Mountains and sympatric *J. regia* versus *J. sigillata* populations in Yunnan Province (Figure 2 versus Figure 3). In the Qinling Mountains, the expected heterozygosity (H_E) values of *J. regia* and *J. cathayensis* ranged from 0.25 (3-HZ-r) to 0.52 (5-NS-r) and 0.41 (31-LN-c) to 0.61 (32-LT-c), respectively (Table 1). A high value of H_E was observed in the *J. regia* populations located in the central, western, and eastern parts of the Qinling Mountains (Figure 2a), while high values of H_E were taken from the central and western parts of the Qinling Mountains of *J. cathayensis* populations (Figure 2b). The difference was that lower values of H_E appeared in the *J. cathayensis* populations from the eastern Qinling Mountains. High values of allelic richness (R_s) and H_E showed the same distribution areas in *J. regia* populations and *J. cathayensis* populations (Figure 2c,d). However, the western and eastern parts of the Qinling Mountains in *J. regia* populations revealed lower values for the private allele richness (P_{AR}), which was inconsistent with the results of H_E and R_s (Figure 2e). The difference was that the P_{AR} results of the *J. cathayensis* populations showed the same trends as those of H_E and R_s (Figure 2f). In Yunnan Province, the expected heterozygosity (H_E) values of *J. regia* and *J. sigillata* ranged from 0.10 (18-LM-Y-r) to 0.43 (17-QZ-Y-r) and from 0.25 (24-BS-Y-s) to 0.39 (21-QZ-Y-s), respectively (Table 1). For *J. sigillata*, higher values of H_E , R_s , and P_{AR} were observed in northern Yunnan, while some of the populations from south Yunnan province had the lowest numbers (Figure 3b,d,f). For *J. regia*, a high value of H_E was found in northern Yunnan (Figure 3a). Interestingly, the result of R_s in *J. regia* populations from Yunnan Province was the opposite of the P_{AR} result (Figure 3c,e). AMOVA showed that the percentage of variation and the coefficient of genetic differentiation at these loci (F_{ST}), between *J. regia* and *J. sigillata*, *J. regia*, and

J. cathayensis, was 26.53% ($p < 0.001$), 33.77% ($p < 0.001$), and 0.406 ($p < 0.001$) and 0.412 ($p < 0.001$), demonstrating that the difference between *J. regia* and *J. cathayensis* was significantly higher than that of *J. regia* and *J. sigillata* (Table 2). The AMOVA results of *J. regia*, *J. sigillata*, and *J. cathayensis* species showed that the proportion of variance mainly existed within populations (*J. regia* 79%, $p < 0.001$; *J. sigillata* 88%, $p < 0.001$; *J. cathayensis* 88%, $p < 0.001$) (Table 2). The Migraten analysis produced θ and M values greater than zero. The θ -value and the size of the immigration rate (M) revealed a highly asymmetric historical gene flow among the three species. The results of the historical gene flow analysis indicate that *J. regia* and *J. sigillata* have no genetic barriers, and the directional gene flow is mainly from *J. regia* to *J. sigillata* (4.63 versus 2.19) (Table 3). *J. regia* and *J. cathayensis* populations in the Qingling Mountains showed lower historical gene flow (0.85 versus 0.93) (Table 3).

Figure 2. Inverse distance weighted (IDW) interpolation of the expected heterozygosity (H_E), the allelic richness (R_s), and the private allele richness (P_{AR}) for the 24 populations (black dots; 12 pairs of the sympatric *J. regia* and *J. cathayensis* populations) from the the Qingling Mountains using 17 expressed sequence tag simple sequence repeat (EST-SSR) markers. The numbers of walnut populations are shown in Table 1. IDW interpolation of (a,c,e) expected heterozygosity (H_E), allelic richness (R_s), and private allele richness (P_{AR}) values of *J. regia*; (b,d,f) expected heterozygosity (H_E), allelic richness (R_s), and private allele richness (P_{AR}) values of *J. cathayensis*.

Figure 3. IDW interpolation of the expected heterozygosity (H_E), the allelic richness (R_s), and the private allele richness (P_{AR}) for the eight populations (black dots; four pairs of the sympatric *J. regia* and *J. sigillata* populations) from Yunnan province using 17 EST-SSR markers. The numbers of walnut populations are shown in Table 1. IDW interpolation of (a,c,e) expected heterozygosity (H_E), allelic richness (R_s), and private allele richness (P_{AR}) values of *J. regia*; (b,d,f) expected heterozygosity (H_E), allelic richness (R_s), and private allele richness (P_{AR}) values of *J. sigillata*.

Table 2. Hierarchical analyses of molecular variance (AMOVA) of walnut samples based on 17 EST-SSR loci.

Species	Source of Variation	d.f.	SS	Variance	% Vari.	Fixation Indices
<i>JR-JS-JC</i>	Between species	2	2008.942	2.34272	34.76	$F_{ST} = 0.45360$ ***
	Among populations	37	984.098	0.71572	10.61	$F_{SC} = 0.16267$ ***
	Within species	1272	4686.325	3.68422	54.64	$F_{CT} = 0.34745$ ***
	within populations					
	Total	1311	7679.365	6.74266		
<i>JR-JS-Yunnan</i>	Between species	1	244.793	1.35585	26.53	$F_{ST} = 0.40647$ ***
	Among populations	6	208.591	0.72137	14.12	$F_{SC} = 0.19213$ ***
	Within species	348	1055.561	3.03322	59.35	$F_{CT} = 0.26531$ ***
	within populations					
	Total	355	1508.944	5.11044		
<i>JR-JC-Qingling</i>	Between species	1	870.176	2.29901	33.77	$F_{ST} = 0.41257$ ***
	Among populations	22	428.294	0.50963	7.49	$F_{SC} = 0.11303$ ***
	Within species	714	2855.330	3.99906	58.74	$F_{CT} = 0.33771$ ***
	within populations					
	Total	737	4153.799	6.80770		
<i>JR</i>	Among populations	19	562.123	0.90989	21.15	$F_{ST} = 0.21148$ ***
	within populations	560	1899.880	3.39264	78.85	
	Total	579	2462.003	4.30253		
<i>JS</i>	Among populations	3	84.600	0.41880	12.00	$F_{ST} = 0.11997$ ***
	Within populations	236	725.017	3.07210	88.00	
	Total	239	809.617	3.49090		
<i>JC</i>	Among populations	15	337.374	0.59536	12.09	$F_{ST} = 0.12086$ ***
	Within populations	476	2061.428	4.33073	87.91	
	Total	491	2398.803	4.92609		

JR—*J. regia*, *JS*—*J. sigillata*, and *JC*—*J. cathayensis*; d.f., degrees of freedom; SS, sum of squares; Variance, variance components; % Vari., percentage of variation; F_{ST} , variance among populations; F_{SC} , variance within populations within groups; F_{CT} , variance among groups relative to total variance. *** $p < 0.001$.

Table 3. Estimated gene flow between sympatric *J. regia* and *J. sigillata* populations in Yunnan Province and *J. regia* and *J. cathayensis* populations in Qinling Mountains by Migrate 3.6.4.

<i>M</i> (m/μ)			
	θ	<i>J. Regia</i> →	<i>J. Sigillata</i> →
<i>J. regia</i>	1.85 (1.69–2.04)		2.19 (1.86–2.57)
<i>J. sigillata</i>	1.03 (0.96–1.10)	4.63 (4.03–5.30)	
<i>M</i> (m/μ)			
	θ	<i>J. Regia</i> →	<i>J. Cathayensis</i> →
<i>J. regia</i>	1.99 (1.89–2.09)		0.93 (0.78–1.10)
<i>J. cathayensis</i>	4.18 (3.98–4.39)	0.85 (0.74–0.98)	

θ is four times the effective population size multiplied by mutation rate per site per generation; *M* is the immigration rate divided by the mutation rate.

3.3. Population Structure of *J. regia*, *J. cathayensis* and *J. sigillata*

According to the whole-range STRUCTURE analysis using three datasets (based on all three walnut species, only sympatric *J. regia* versus *J. cathayensis* populations and *J. regia* versus *J. sigillata* populations), the values of the log-likelihood of the data, loge Pr (K), increased progressively with an increase in K, but delta K indicated that the optimal values for K were all 2 [55,62] (Figure S3a–c). The proportions of each individual in each population that were assigned into two clusters (clusters I and II) are shown in Figure 4. When all samples (from all three walnut species) were included in the STRUCTURE analysis (which was based on the EST-SSR data), the highest mean posterior probability value was detected for K = 2 (Figure 4b). At K = 2, all *J. regia* and *J. sigillata* populations were clustered into one group, and the *J. cathayensis* populations into the other. According to Nei's genetic distances, a phylogenetic tree was established by the unweighted pair-group method with arithmetic means (UPGMA) methods (Figure 4c). Figure 3c shows that three *Juglans* species were clustered into two branches. Cluster I included all *J. regia* and *J. sigillata* populations, in which the *J. sigillata* populations were classified as *J. regia* populations; the other consisted of *J. cathayensis* populations. In addition, the PCoA (Figure 4e), based on genetic distance, showed a similar genetic structure to that detected by the STRUCTURE analysis (Figure 4b). For three species of *Juglans*, all the above results indicate that gene introgression is common among the same groups of *Juglans*, as only rare and low-level gene introgression appeared in distinct groups.

For sixteen pairs with sympatric distributions of *J. regia* and *J. cathayensis* from all of sampling regions, the ΔK values [62] computed for all K classes indicated a strong signal for K = 2 (Figure 4a and Figure S4b). The Bayesian clustering approach revealed that cluster I was the *J. regia* populations across 16 populations (1–12 populations from the Qinling Mountains and 13–16 populations from the four other provinces, Sichuan, Yunnan, Gansu, and Henan), while cluster II was the *J. cathayensis* populations (25–36 pop from the Qinling Mountains and 37–40 pop from the four other provinces). The results of the STRUCTURE analysis using twelve pairs of sympatric distribution of *J. regia* and *J. cathayensis* from the Qinling Mountains were in accordance with the genetic structures of all the populations and indicated little gene introgression or hybridization (Figure 5b). The negative correlation between pollen dispersal distance and population density has been reported in many wind-driven forest species [63,64]. Therefore, the high-density distribution of *Juglans* may, to some extent, limit the pollen dispersion distance. Subsequently, we used the four pairs of sympatric *J. regia* and *J. cathayensis* populations (1-QL-r versus 25-BY-c; 5-NS-r versus 29-NS-c; 8-LT-r versus 32-LT-c; 12-SL-r versus 36-SL-c), with relatively greater gene flow, to carry out the structure analysis, and the optimal K value was 2 (Figure 5d–g). The overall proportions of hybrids estimated by the four results of the STRUCTURE analysis were 4.08% (2/49), 5.41% (2/37), and 6.66% (1/15), respectively. In summary, only rare and low-level gene introgression occurred between sympatric *J. regia* and *J. cathayensis* populations.

Figure 4. Genetic clustering of all individuals of *J. regia*, *J. cathayensis* and *J. sigillata*. (a,d) Population cluster analysis of the sympatric *J. regia* versus *J. cathayensis* populations and *J. regia* versus *J. sigillata* populations using STRUCTURE ($K = 2$) based on the EST-SSR datasets. (b) Population cluster analysis of three walnut species using STRUCTURE ($K = 2$) based on the EST-SSR datasets. (c) The UPGMA tree of all individuals from the three *Juglans* species based on Nei's genetic distance. (e) Principal coordinate analyses (PCoA) of all individuals of 40 *Juglans* populations resolved into three genotype groups based on the EST-SSR datasets.

Figure 5. Genetic structure and geographical distribution of 32 sympatric walnut (*Juglans*) populations based on 17 EST-SSRs in the Qinling Mountains and Yunnan Province. (a) The map of sample collection information for the three walnuts (*J. regia*, *J. cathayensis*, and *J. sigillata*). (b) Results of the STRUCTURE 2.3.4 analysis showing $K = 2$. A total of 24 sympatric *J. regia* versus *J. cathayensis* populations based on 17 EST-SSRs in the Qinling Mountains. (c) Geographic origin of the 12 pairs of sympatric *J. regia* versus *J. cathayensis* populations and their color-coded grouping at the most likely $K = 2$, as determined using the deltaK method of Evanno et al. (2005) [62]. The location of the pie indicates the sampled locations. (d–g). Results of the four pairs of sympatric *J. regia* versus *J. cathayensis* populations (1-QL-r versus 25-BY-c; 5-NS-r versus 29-NS-c; 8-LT-r versus 32-LT-c; 12-SL-r versus 36-SL-c) of STRUCTURE 2.3.4 analysis, shown from $K = 2$. (h) Genetic structure and geographical distribution of eight sympatric *J. regia* versus *J. sigillata* populations based on 17 EST-SSRs in Yunnan province. (i) Results of the sympatric *J. regia* versus *J. sigillata* populations from the Yunnan province via the STRUCTURE 2.3.4 analysis, shown from $K = 2$.

The genetic structures of the four pairs of *J. regia* and *J. sigillata* populations with sympatric distribution from Yunnan province were evaluated by Bayesian cluster analysis using STRUCTURE. The log-likelihood values reached three peaks when $K > 2$, and the values of delta K were highest when K was 2 [55,62] (Figure S3c). At $K = 2$, cluster I (west group) included four *J. regia* populations found in Yunnan province, and cluster II (east group) was comprised of four *J. sigillata* populations from Yunnan province (Figure 5h,i). Two populations (17-QZ-Y-r, 20-BS-Y-r) between the west and the east cluster were identified as admixed. Consistently, the number of subclusters for each larger K was much lower than the number of sampled populations. The results of the STRUCTURE analysis inevitably indicate extensive gene flow, either historical or ongoing (Figure 5i).

4. Discussion

4.1. Genetic Diversity and the Spatial Genetic Structure of *J. regia*, *J. sigillata*, and *J. cathayensis*

Landscape genetics can be regarded as a self-explanatory tool, which can transmit information about the spatial distribution of genetic variation into practice [65]. However, few scholars have combined landscape genetics and genetic diversity studies to analyze the biogeographical history of a species [65,66]. Previous studies have suggested that R_s and H_E are very useful indicators to measure genetic diversity and are considered ideal indicators to determine conservation priorities [67]. Here, we compare the spatial manifestations of H_e , R_s , and P_{AR} , so as to vividly infer the biogeographic history of walnut-related species in the two regions (Figures 2 and 3). Interestingly, the high H_e and R_s values of *J. regia* populations were observed in the eastern and western of Qinling Mountains, whereas the low values of P_{AR} appeared in the same regions. This may be due to the fact that *J. regia* populations, as important nut trees in the eastern and western regions of the Qinling Mountains, experienced more frequent artificial disturbance, which increased genetic diversity in these areas and erased ancient signs of refugia. Unlike the *J. cathayensis* populations in Qinling Mountains, *J. regia* and the *J. sigillata* populations in Yunnan are an economic species, and domesticated species tend to become more scattered and exhibit relatively lower abundance due to human selection and human-mediated dispersal of selected genotypes, which also explains why the genetic diversity of *J. cathayensis* populations is higher than that of *J. regia* and *J. sigillata* populations. The same reason also explains the opposite distribution of the R_s and P_{AR} values of *J. regia* populations in Yunnan.

This study is the first to explore the interspecific introgression of Chinese *Juglans* plants and provides a theoretical basis for the genetic evolution of the genus *Juglans*. In this study, we estimated the genetic diversity and genetic structure of 40 walnut populations sampled across its sympatric range using 17 microsatellite markers (nine neutral EST-SSRs and eight non-neutral EST-SSRs, Figure S2). For our analyses, eight of the seventeen loci were detected to be F_{ST} outliers: JC3412, JM5969, JC7329, JR4964, JR4616, JM78331, JR6160, and JR1817 (Figure S2). The microsatellite loci screened in this study may be located within functional genes. All loci were annotated using Pfam, the GO database, and blastx of NCBI [68]. EST-SSR stands for expressed sequence tags, in coding regions and potentially under selection [43,68]. The STRUCTURE results based on the neutral loci and selected loci were not significantly and consistently the same among the three *Juglans* species (Figure 3). The structure analysis of the two groups of *Juglans* based on the EST-SSR data indicated that *J. regia* and *J. cathayensis* have strong reproductive isolation and rarely exhibit gene introgression phenomena, while *J. regia* and *J. sigillata* have no obvious genetic boundaries and frequently exhibit gene introgression phenomena (Figures 4 and 5). These findings are consistent with those of the UPGMA tree (Figure 4c), PCoA (Figure 4e), and the historical gene flow analysis (Table 3). Because of the limitation of pollen diffusion, we used the four pairs of sympatric *J. regia* versus *J. cathayensis* populations (1-QL-r versus 25-BY-c; 5-NS-r versus 29-NS-c; 8-LT-r versus 32-LT-c; 12-SL-r versus 36-SL-c) with relatively more gene flow to carry out structure analysis (Figure 5d–g). In summary, strong reproductive isolation and low-level gene introgression occurred between sympatric *J. regia* and *J. cathayensis* populations. Our study provides an additional study on the three species of *Juglans* to provide theoretical support for the

genetic conservation of intergeneric plants. The experimental objects of this study are important economic tree species, which have experienced too much human interference. To further study the gene flow between plant genera, more forest tree species need to be studied.

4.2. Species Classification of *J. regia* and *J. sigillata*

A species in the center of its habitat may present some minor adaptive changes from time to time, but they are only varieties and retain a certain geographical distribution. Previous studies on the morphology, molecular biology, phenological, and fossils of *Juglans* have shown that there are five species of *Juglans* in China, which are classified into two groups: section *Juglans* and section *Cardiocaryon*. Section *Juglans* includes the two cultivated walnuts, *J. regia* and *J. sigillata*. However, there is a long-standing controversy about whether *J. sigillata* is a variety or an independent species. Although there are obvious morphological differences between *J. regia* and *J. sigillata*, many studies have indicated that these two species have no genetic barriers. Structural analysis and gene flow analysis based on four pairs of sympatric populations in Yunnan showed that *J. regia* and *J. sigillata* have obvious gene flow, and directional gene flow has been mainly from *J. regia* to *J. sigillata*. Combined with previous research results, we prefer to support the view that *J. sigillata* is a sub-species or, perhaps, a landrace of *J. regia*. Zhao et al. [29] and Yuan et al. [43] have also provided powerful evidence for the establishment of this hypothesis through other molecular techniques. However, some studies have shown that, although there is a high level of gene flow between oak (*Quercus*) and pine (*Pinus*) tree species [69–71], they both still maintain the integrity of their species, which also reflects the complex phenomenon of gene introgression and hybridization between species.

4.3. Influencing Factors of Distinct Gene Introgression among Three *Juglans* Species

Hybridization may have played an important role in the genetic differentiation of *Juglans*, and the phenomenon of interspecific hybridization has been strongly confirmed [36–43]. Accordingly, we selected two groups of walnut species to study. The two cultivated walnuts of section *Juglans* (*J. regia* and *J. sigillata*) were from Yunnan province, while *J. regia* of section *Juglans* and *J. cathayensis* of section *Cardiocaryon* were from the Qingling Mountains. It is noteworthy that the natural coexistence of *J. regia* and *J. sigillata* is very narrow. The results of the STRUCTURE analysis (Figures 4 and 5), the UPGMA tree (Figure 4c), PCoA (Figure 4e), and the historical gene flow analysis (Table 3) indicate that gene introgression is common among the same groups of *Juglans*, as only rare and low-level gene introgression appears in distinct groups.

In plants, interspecific gene introgression often occurs due to the hybridization of closely-related species distributed in the same region. The analysis results of the genetic diversity and genetic structure of *J. regia* and *J. sigillata* showed that *J. regia* and *J. sigillata* have frequent gene flow, which might be due to the dispersal of pollen or seeds to achieve gene introgression among populations, further reducing the genetic divergence of different species. The persion walnut and the iron walnut, which are important economic tree species in China, have been seriously disturbed by human activities. This disturbance may also interfere with the two-way direction of gene introgression between the two closely-related species. However, compared with the frequent gene flow between two closely related species (*J. regia* and *J. sigillata*), the STRUCTURE results showed that there was a strong reproductive isolation mechanism between the distant species, *J. regia* and *J. cathayensis*, in different groups, and hybridization is a rare event. We speculated that the possible reasons for this rarity are related to the reproductive isolation mechanism. A reproductive isolation mechanism among some species is sufficient to maintain the integrity of the species, but relevant studies have shown that multiple isolation mechanisms need to work together among two related species, as an isolation mechanism can almost completely block the gene flow between the two species [72]. However, even if the probability of hybridization is very low, it is normal to occasionally find individual hybridization events. Natural hybridization and gene introgression are ubiquitous among and within genera of plants, thereby providing the main impetus for plant evolution. However, the overwhelming majority

of species retain species stability, even at a high levels of gene flow. This also indicates that there may be complex mechanisms for maintaining stability among species, or, possibly, genes controlling species' divergence may not change due to gene flow among species, thereby maintaining the independence of the species.

5. Conclusions

In this study, 656 individuals from 40 Persian walnut, Chinese walnut, and iron walnut populations were genotyped with 17 EST-SSRs to analyze the gene introgression among *J. regia* and *J. cathayensis*, *J. regia*, and *J. sigillata*. In summary, *J. regia* and *J. cathayensis* showed strong reproductive isolation and rare gene introgression phenomena, and *J. regia* and *J. sigillata* had no obvious genetic boundaries and frequent gene introgression phenomena. For the three species of *Juglans*, all of the above results indicate that gene introgression is common among species in the same group of *Juglans*, and only rare and low-level gene introgression appears in distinct groups. Our study provides information on three species of *Juglans* to provide theoretical support for the genetic conservation of intergeneric plants. The experimental object of this study was to analyze important economic tree species which have experienced too much human interference. To further study the gene flow between plant genera, more forest tree species need to be studied.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/10/11/965/s1>. Figure S1. Genetic clustering of all individuals of *J. regia*, *J. cathayensis*, and *J. sigillata*. (a) Population cluster analysis of three walnut species using STRUCTURE ($K = 2$) based on 17 EST-SSR datasets. (b) Population cluster analysis of three walnut species using STRUCTURE ($K = 2$) based on eight non-neutral EST-SSR datasets. (c) Population cluster analysis of three walnut species using STRUCTURE ($K = 2$) based on nine neutral EST-SSR datasets. Figure S2. Detection of loci under the selection from genome scans based on F_{ST} . Eight of seventeen loci were detected to be the F_{ST} outlier: JC3412, JM5969, JC7329, JR4964, JR4616, JM78331, JR6160, and JR1817. Figure S3. (a–d) According to the whole-range STRUCTURE analysis using three datasets (based on all three walnut species, only sympatric with all *J. regia* versus *J. cathayensis* populations, all *J. regia* versus *J. sigillata* populations, and *J. regia* versus *J. cathayensis* populations in the Qinling Mountains), the values of the log-likelihood of the data, $\log_e Pr(K)$, increased progressively with an increase in K , but ΔK indicated that the optimal values for K were all 2. Table S1. Information of primers and the genetic Characteristics of 17 EST-SSR primers for the 40 walnut populations used in this study. Table S2. Percentage of variation explained by the first three axes using the principal coordinate analysis model approach. Table S3. Description of private alleles by 17 populations. Table S4. Test results of the null alleles in 17 loci.

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