

# Natural Hybridization between Persian Walnut and Chinese Walnut Revealed by Simple Sequence Repeat Markers

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**ABSTRACT.** Hybridization between species of the genus *Juglans* is common because of weak reproductive isolation mechanisms between closely related species with sympatric distributions. In this research, we investigated the possibility of naturally occurring interspecific hybrids between two species in the genus *Juglans*: persian walnut (*Juglans regia*) and chinese walnut (*Juglans cathayensis*). We used 12 pairs of microsatellite markers to analyze introgression between the two species. All amplified microsatellites were polymorphic in the two species. The result of Bayesian admixture analyses showed that introgression between the two species is rare; only three of nine individuals tentatively identified as hybrids, based on intermediate morphological characteristics, were defined as mixed genotypes. The other six putative hybrids and 156 morphologically pure individuals showed no sign of introgression.

Natural hybridization between sympatric forest tree species is common in natural populations; it can influence the genetic diversity within species and promote new species formation (Arnold, 2006; Lexer et al., 2005). Loss of diversity from widespread hybridization between a predominantly cultivated species and an indigenous species may also raise concerns among not only conservation geneticists but also breeders (Zhao and Woeste, 2011).

The genus *Juglans* includes ≈20 species that are distributed over a wide geographical range including southern Europe, east Asia, and the Americas. The species within this genus display differentiation in morphology, particularly in nut characteristics (Krüssmann et al., 1985). Hybridization between species of the genus *Juglans* is common because of weak reproductive isolation mechanisms between phylogenetically close species (Gunn et al., 2010; Hoban et al., 2009; Ross-Davis et al., 2008; Wang et al., 2015; Zhao and Woeste, 2011). *Juglans regia* is grown locally in the Balkans, Iran, Turkey, central Asia, the Himalayas, and China. It is the predominant cultivated species that distributes in most area of China (from Liaoning Province to Yunnan Province) because of its economically valuable nut. Although China is considered one of the origin places of *J. regia*, the natural population is rare in China at present, most of them are landrace populations. Chinese walnut is one of the most widely distributed temperate deciduous native tree species in southern China. It occurs primarily throughout the hilly midelevation area that ranges between the Qinling Mountains–Huai River line and the tropical South (Bai et al., 2014). This region is typical of a subtropical climate with a complex

topography. *Juglans cathayensis* generally grows on mountain slopes or in valleys with 500- to 2800-m elevations. In Hubei Province, China, these two species are frequently sympatric in many regions and hybridization may occur. The nut of *J. cathayensis* is thick shelled. Its commercial importance is not as significant as that of *J. regia*; its fruit typically fall in the vicinity of parental plants without collection or dispersal by humans (Ma et al., 2005). For this reason, it may be subject to different evolutionary and ecological processes than *J. regia*. Thus, some important characteristics of *J. cathayensis* genes could have an evolutionary and ecological impact on *J. regia* genetic resources if the hybrids produce successful descendants. Furthermore, new germplasm (e.g., specifically for resistance to diseases and characteristics of fruiting) can also be created. Whether hybrids of the two species persist in natural settings remains unknown.

Morphology may provide some information regarding hybrid identification, but the wide variability of leaf and nut morphology within species limits the utility of morphological characteristics for hybrid verification (Ross-Davis et al., 2008). Furthermore, many hybrid trees may be the result of more complex introgression as backcrosses, F<sub>2</sub>s, etc. (Hoban et al., 2009). For these reasons, hybrids are often difficult to distinguish by silvicultural characters, and identification using DNA markers is essential. Simple sequence repeat (SSR) markers exhibit hypervariability and codominance and are, therefore, highly informative. Hundreds of specific SSR primer pairs are currently available for *Juglans*; they have been demonstrated to be useful in hybrid identification and introgression analyses in the genus *Juglans* (Hoban et al., 2009; Pollegioni et al., 2009; Ross-Davis et al., 2008; Wang et al., 2015).

To investigate the possibility of genetic invasion, we used 12 microsatellite markers to identify the hybrids between *J. regia* and *J. cathayensis* in three regions where these two species co-occur. The objectives of this study were to assess whether interspecific hybrids occur and reproduce in natural populations.

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## Materials and Methods

**PLANT MATERIALS.** We randomly sampled the plants in three different sites from Hubei Province of China. In Jianshi and Baokang, the relative abundance of *J. cathayensis* is higher than that of *J. regia*. In Shennongjia, the two species are in mixed forest with almost the same frequency. We also observed flowering overlap occurred between the two species in the three sampling sites during 2014–15 (personal observations). Nearly 30 individual of each species were sampled in each site (see Supplemental Table 1 in the electronic supplementary material). The individuals of *J. cathayensis* and *J. regia* were distinguished based on leaflet numbers (9–17 in *J. cathayensis* and 5–9 in *J. regia*), leaf pubescence (the leaves of *J. cathayensis* are densely covered with persistent hairs, whereas those of *J. regia* are completely glabrous) and nut morphology (*J. cathayensis* has deep grooves and several ridges on the nut surface, whereas *J. regia* has a wrinkled nut surface) (Pei and Lu, 2011). In Shennongjia, we found several individuals ( $n = 9$ ) with intermediate morphology [the leaves were slightly hairy; grooves of the nut surface were shallower than those of *J. cathayensis* (Fig. 1)]. We classified these individuals as putative hybrids for further genetic analysis. The leaf material collected from each tree was preserved in silica gel and taken to the laboratory for storage at  $-80\text{ }^{\circ}\text{C}$  until DNA extraction and molecular marker analyses.

**DNA EXTRACTION.** Genomic DNA was isolated from leaves dried in silica gel using a Plant DNA Isolation Kit (Foregene, Chengdu, China) according to the manufacturer's instructions.

**MICROSATELLITE AMPLIFICATION.** DNA was amplified using 12 microsatellite primer pairs selected from previous studies on *J. regia* and *Juglans nigra* (Chen, 2014; Dangl et al., 2005;

Woeste et al., 2002), namely WGA1, WGA4, WGA72, WGA76, WJR31, WJR41, WJR65, WJR69, WJR115, WJR226, WJR281, and WJR291. SSR reactions were conducted according to the protocol of Victory et al. (2006) with some modifications concerning reaction volumes and numbers of cycles. Amplification reactions were performed in a 15- $\mu\text{L}$  volume containing  $1 \times$  reaction buffer (TaKaRa Biotechnology, Dalian, China), 30 ng of genomic DNA, 0.2 mM of each dNTP (Promega, Beijing, China), 0.4 mM of each primer, and 0.9 unit of Taq DNA polymerase (TaKaRa Biotechnology). For DNA amplifications, a GeneAmp PCR System (9700; PerkinElmer, Waltham, MA) was programmed as follows: an initial 3 min incubation at  $94\text{ }^{\circ}\text{C}$ ; followed by 30 cycles of 45 s at  $94\text{ }^{\circ}\text{C}$ , 30 s at the annealing temperature (Chen, 2014; Dangl et al., 2005; Woeste et al., 2002), 45 s at  $72\text{ }^{\circ}\text{C}$ , and a final incubation at  $72\text{ }^{\circ}\text{C}$  for 5 min. Amplified DNA fragments were separated using capillary electrophoresis (ABI 377 genetic analyzer; Yafan Biological Technology, Suzhou, China). Data were collected with the associated data collection software (PerkinElmer). The fragment analyses were performed with GeneScan 3.1 (Thermo Fisher Scientific, Shanghai, China), and data were assembled as multilocus microsatellite genotypes across the 12 loci. Microsatellite data were reformatted from an excel file to an Arlequin (.arp) file using the program Convert (Glaubitz, 2004). In all cases, polymerase chain reactions were performed at least twice to ensure that absence of bands was not due to a failed reaction.

**DATA ANALYSIS.** We used Arlequin 3.1 to test for linkage equilibrium within each pair of loci and population/species using a likelihood ratio statistic, whose distribution was obtained by a permutation (1000) procedure (Excoffier et al., 2005).  $F_{ST}$  and  $R_{ST}$  values for each species reference set were calculated using GenAlEx version 6.5 software (Peakall and

Table 1. Polymorphism characteristics of 12 microsatellite loci amplified from 165 walnut genotypes, *Juglans regia* (R) and *Juglans cathayensis* (C).

Locus	Species	K <sup>z</sup>	Kp	F <sub>ST</sub>	R <sub>ST</sub>	Range	H <sub>E</sub>	H <sub>O</sub>
WGA1	R	5	2	0.208	0.184	177–193	0.634	0.75
	C	7	4			177–199	0.673	0.608
WGA4	R	7	6	0.18	0.595	224–246	0.693	0.632
	C	6	5			232–258	0.737	0.713
WGA72	R	3	1	0.35	0.412	137–141	0.425	0.368
	C	5	4			137–151	0.523	0.525
WGA76	R	4	0	0.343	0.792	227–247	0.501	0.394
	C	3	0			229–233	0.523	0.488
WJR31	R	5	5	0.681	0.986	206–222	0.253	0.197
	C	4	4			142–172	0.164	0.1
WJR41	R	5	4	0.18	0.267	142–166	0.547	0.421
	C	17	16			128–192	0.9	0.9
WJR65	R	4	3	0.381	0.226	195–203	0.380	0.316
	C	3	2			191–219	0.438	0.347
WJR69	R	9	4	0.093	0.136	133–161	0.779	0.421
	C	14	9			131–169	0.847	0.688
WJR115	R	4	3	0.572	0.96	96–108	0.618	0.974
	C	2	1			94–96	0.013	0.013
WJR226	R	3	1	0.436	0.072	152–160	0.659	0.632
	C	2	0			156–160	0.037	0.038
WJR281	R	6	3	0.445	0.042	140–160	0.470	0.5
	C	3	0			148–158	0.328	0.213
WJR291	R	6	4	0.223	0.653	96–112	0.677	0.724
	C	9	6			90–114	0.644	0.544

<sup>z</sup>K = number of alleles; Kp = number of private alleles; F<sub>ST</sub>, R<sub>ST</sub> = differentiation among populations; H<sub>E</sub> = expected heterozygosity; H<sub>O</sub> = observed heterozygosity.

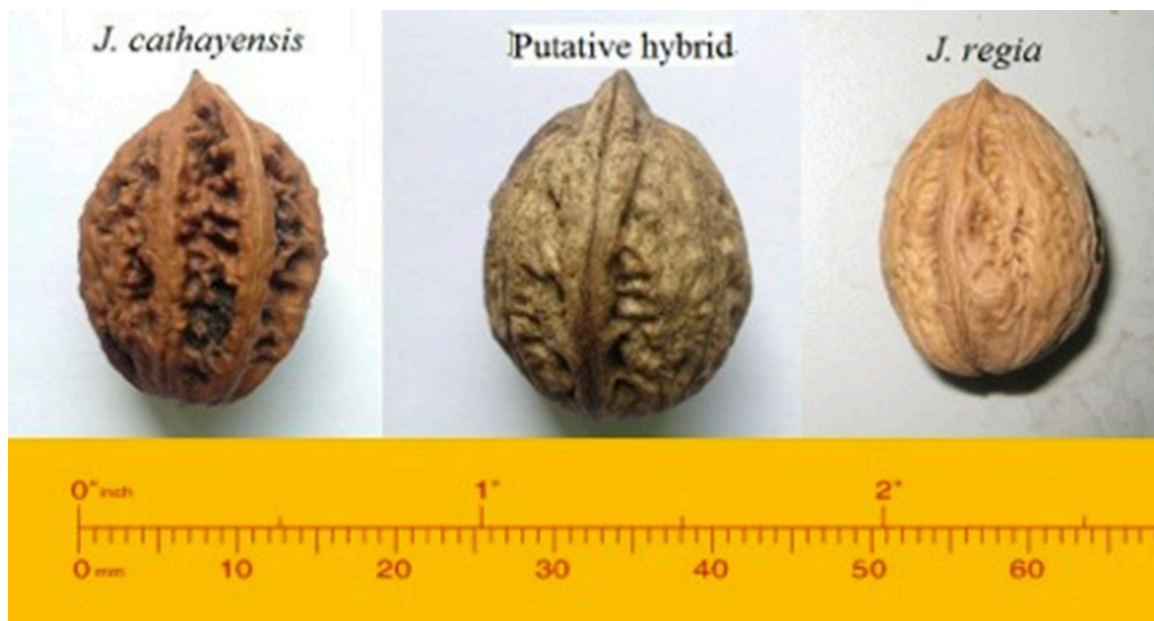


Fig. 1. Nut morphology of *Juglans cathayensis*, *Juglans regia*, and putative hybrid. Grooves of the putative hybrids nut surface found in Shennongjia, an area in Hubei Province of China, were much more shallow compared with *J. cathayensis* and *J. regia*.

Smouse, 2012). The Bayesian model-based clustering algorithm implemented in the program Structure (Pritchard et al., 2000) was used to determine whether individuals from the same species or putative hybrids grouped together. This method allowed us to analyze the correspondence between morphologically based species/hybrids and inferred genetic structure. In this study, the range of possible number of clusters (K) tested ranged from 1 to 10. On the basis of the initial results, a series of 10 independent runs were performed for each K value with a burn-in period of 100,000 steps followed by  $10^6$  Markov chain Monte Carlo replicates. Furthermore, log-likelihood value and the ad hoc statistic  $\Delta K$  defined by Evanno et al. (2005) were used to detect the most likely number of populations. The  $\Delta K$  statistic is based on the second order rate of change of L [K (the posterior probability of the data for a given value of K)] between successive K values over 10 replicates. As demonstrated by Evanno et al. (2005), it is possible to identify the number of clusters corresponding to the uppermost hierarchical level of genetic partitioning between populations. Therefore, the groups inferred by the first Structure analysis were subsequently processed separately to identify possible substructure. For the most likely K (K = 2), we report estimated membership in each cluster for every individual, and 95% posterior probability intervals. We classify individuals with the probability of membership (q) less than 0.90 in one of the two Structure species clusters as probably hybrid genotypes (Hoban et al., 2009; Pollegioni et al., 2013).

## Results and Discussion

All microsatellites tested were previously shown to be useful in *J. regia* and *J. nigra* (Chen, 2014; Dangl et al., 2005; Woeste et al., 2002), and this is the first time they have been used in *J. cathayensis*. In this research, these microsatellites were amplified and found to be polymorphic in *J. cathayensis* (Table 1). After applying Bonferroni correction for multiple comparisons,

we found no consistent pattern of linkage disequilibrium within each pair of loci across species populations.  $F_{ST}$  and  $R_{ST}$  values suggests that the genetic differentiation estimates were much larger than those obtained between other species in *Juglans*, indicating a relatively distant relationship between *J. regia* and *J. cathayensis* (Table 1) (Gunn et al., 2010; Hoban et al., 2009; Wang et al., 2015). Furthermore, because  $F_{ST}$  does not assume a stepwise mutation model, it appears to be a more sensitive measure of intraspecific variation compared with  $R_{ST}$  (Frangois and Nicolas, 2002).

The genetic structures of six walnut populations and putative hybrid clusters were evaluated by Bayesian cluster analysis using Structure (Pritchard et al., 2000). When using the admixture and correlated frequency model, the log-likelihood value [L(K)] determined as a function of K (number of clusters) averaged over 10 replicates increased almost sharply between K = 1 [Ln P(D) = -6468.5] and K = 2 [Ln P(D) = -4295] and that the curve plateaus with only a slight increase between K = 2 and K = 7 [Ln P(D) = -4074.2] (Fig. 2A). The mean log-likelihood value (Fig. 2A) and approach of Evanno et al. (2005) strongly supported K = 2 as the most likely number of clusters because the highest second order of change of the log-likelihood of the data ( $\Delta K$ ), as a function of K, was detected at K = 2 (Fig. 2B).

Using a threshold value of 0.90 to classify each individual as purebred or hybrid, Bayesian admixture analyses revealed that only three individuals of nine tentatively identified as hybrids on the basis of intermediate morphological characteristics actually presented mixed genotypes, and the other six individuals were all purebred *J. cathayensis* (Fig. 3). The other 156 individuals of the two species, which were apparently pure individuals on the basis of morphological criteria, were shown to be pure genotypes, calculated using the assignment scores provided by Structure analyses (Fig. 3). It seems that genetic introgression between the two species is not frequent. Reproductive isolation may be one important factor that accounts

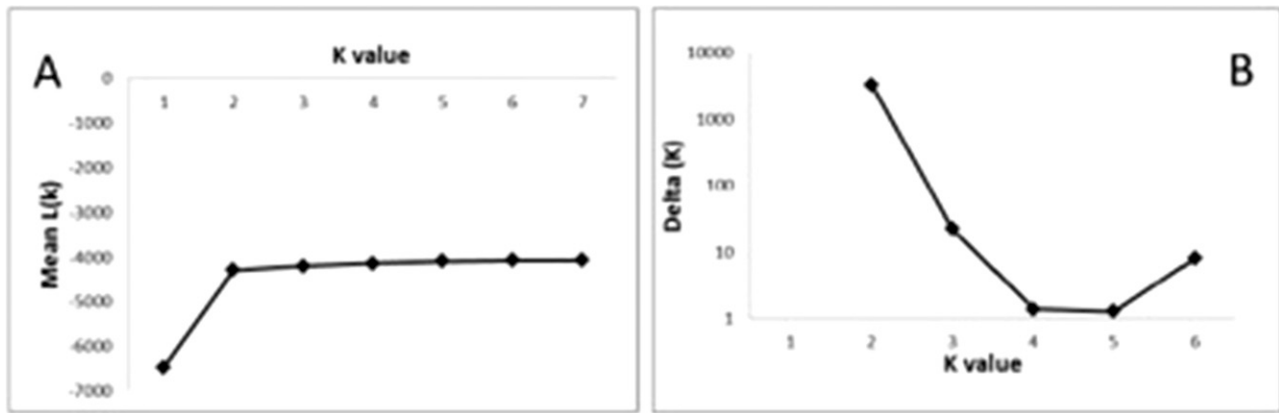


Fig. 2. Inference of the most probable number of clusters (K) using Structure software (Pritchard et al., 2000) based on microsatellite analysis of 165 total walnut samples. (A) Log-likelihood value of data L (K) as a function of K averaged over 10 replicates increased almost linearly from K = 1 [ $\ln P(D) = -6468.5$ ] up to K = 2 [ $\ln P(D) = -4295$ ] and increased slowly from K = 3 to K = 7 [ $\ln P(D) = -4074.2$ ]. (B) The highest second order of change of the log-likelihood of the data ( $\Delta K$ ), as a function of K, was detected at K = 2.

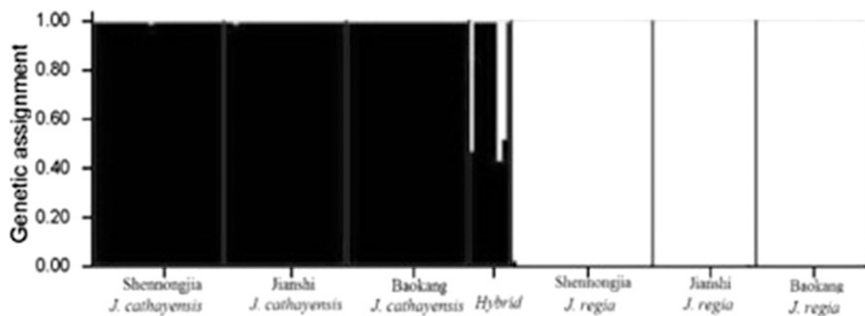


Fig. 3. Results of genetic assignment based on Bayesian clustering implemented in the program Structure (Pritchard et al., 2000) the most probable number of clusters (K) for K = 2. Individuals are grouped in the three study areas and according to their morphological appearance (*Juglans cathayensis*, *Juglans regia*, and hybrids). Each individual is represented by a thin vertical line, which is partitioned into two monochrome segments that represent the individual's proportion of cluster membership coefficients. Shennongjia, Jianshi, and Baokang are three different locations in Hubei Province, China.

for this phenomenon. Although the results of manual controlled crossing between *J. regia*  $\times$  *J. cathayensis* revealed that the two species can produce progeny (Xu et al., 2007), the outcrossing seed-setting rate and field germination rate of the progeny were different (Gunn et al., 2010; Hoban et al., 2009; Ross-Davis et al., 2008; Wang et al., 2015; Zhao and Woeste, 2011). It is possible that the reproductive isolation between *J. regia* and *J. cathayensis* is stronger than between other species pairs in the genus *Juglans*. The observed q of the three hybrids identified by Structure analyses were all close to 0.5 (0.429, 0.521, and 0.547), indicating a high possibility of F<sub>1</sub> hybrid individuals. These results show that natural hybridization between *J. regia*  $\times$  *J. cathayensis* was rare and that crosses between hybrids or backcrosses between hybrids and either of the parental species may not happen.

In this research, putative hybrids were found only in the Shennongjia nature reserve area, where the frequency of the two species is similar and the level of human disturbance is relatively low. This preliminary finding suggests that the relative abundance of *J. regia* individuals and human-mediated disturbance may be additional key factors in interfering with bidirectional introgression of the two species. Because of the significant commercial importance of the nuts, *J. regia* populations

have experienced more human-mediated selection than *J. cathayensis*, often making *J. regia* more scattered and exhibiting relatively lower abundance than *J. cathayensis* (Gunn et al., 2010). Moreover, most of the *J. regia* nuts were collected by humans, making the natural germination of potential F<sub>1</sub> hybrids (i.e., pollination by *J. cathayensis* trees) nearly impossible. These disturbances strongly affected the interspecific gene flow of the two species. Considering this factor, we infer that *J. cathayensis* is the most likely female parent of the three mixed genotypes, whose morphological appearance do have more in common with *J. cathayensis*. Con-

sidering a threshold value of 0.90 to classify each individual as purebred or hybrid, genetic analyses revealed six of the nine apparently morphologically intermediate individuals with signatures of purebred *J. cathayensis*. The most plausible explanation is that the *J. cathayensis* are all wild populations that possess a large number of individuals and abundant genetic diversity (Bai et al., 2014). The variation of morphological characteristics makes some genotypes of *J. cathayensis* show an intermediate appearance.

## Conclusions

In this research, we found that hybridization between *J. regia* and *J. cathayensis* seems to be a rare phenomenon and that backcrosses between hybrids and either of the parental species may be even rarer. Six of the nine putative hybrid individuals, which were identified by phenotypic traits, showed no sign of genetic introgression, suggesting that the analyzed molecular markers were more powerful in identifying hybridization and introgression between *J. regia* and *J. cathayensis* than the phenotypic traits. The main barriers to introgression of the two species may be reproductive isolation and human disturbances. In the future, if we can find more sympatric regions where the relative abundance of *J. regia* is higher, it

should help to explain the impact of human disturbance on the interspecific introgression.

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Supplemental Table 1. *Juglans* populations surveyed, and their geographical parameters.

Location	Landform feature	Altitude (m)	Longitude (E)	Latitude (N)	No. of samples
Jianshi	Alpine and canyon	1,250	109°53'14.3"	30°16'27.4"	22 ( <i>Juglans regia</i> )
	Hillock		111°18'22.5"		26 ( <i>Juglans cathayensis</i> )
Baokang	Alpine and canyon	870	110°27'39.6"	31°47'39.5"	24 ( <i>J. regia</i> )
					26 ( <i>J. cathayensis</i> )
Shennongjia	Alpine and canyon	2,640		31°32'8.6"	30 ( <i>J. regia</i> )
					28 ( <i>J. cathayensis</i> )
					9 (putative hybrids)