Genetic Diversity and Population Structure of Chinese *Corylus heterophylla* and *Corylus kweichowensis* Using Simple Sequence Repeat Markers

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ABSTRACT. *Corylus heterophylla* and *Corylus kweichowensis* are economically and ecologically important nut-producing woody shrubs that are distributed across northern and southern regions of China. However, few studies have examined the genetic diversity and genetic relationships between *C. heterophylla* and *C. kweichowensis*, and their taxonomic relationships have been questioned. In this study, 796 individuals collected from 34 natural populations (21 *C. heterophylla* and 13 *C. kweichowensis* populations) were investigated to assess the genetic diversity and population structure using 11 microsatellite loci. Analysis of molecular variance revealed that genetic differentiation of *C. heterophylla* and *C. kweichowensis* within populations accounted for 93.57% and 88.91% of total variation, respectively. The *C. heterophylla* and *C. kweichowensis* populations as a whole group were analyzed by multiple programs, which showed that the 34 populations were divided into two genetic clusters. One cluster included 21 *C. heterophylla* populations, and the second cluster contained 13 *C. kweichowensis* populations. We conclude from these results that *C. heterophylla* and *C. kweichowensis* are distinct species. The Mantel test showed that the genetic distance was significantly correlated with the geographic distance ($r = 0.580$, $P < 0.001$). The populations of *C. heterophylla* [e.g., populations WC (Weichang), MS (Mishan), and WA (Wu'an)] and *C. kweichowensis* [e.g., populations YX (Yuxi), ZP (Zhenping), LA (Lin'an), and TB (Taibai)] with high allelic richness are considered suitable for in situ conservation. Our study provides valuable information for breeding and conservation of genetic resources of *C. heterophylla*, *C. kweichowensis*, and related species.

Hazelnuts, of the family Betulaceae, are deciduous wind-pollinated, monoecious, self-incompatible nut-bearing shrubs (Gürçan et al., 2010a). More than 20 species have been described that are widely distributed throughout temperate regions of the Northern Hemisphere (Bassil et al., 2005). Hazelnut is one of the most important nut crops in the world and its production was 1,006,178 t in 2017 (Food and Agriculture Organization of the United Nations, 2017). Eight species and two botanical varieties of *Corylus* are reported to be native to China (Zhang et al., 2005), where their nuts have been collected as food for more than 5000 years (Martins et al., 2014). *C. heterophylla* grows throughout Korea, Japan, China,

and the Russian Far East. However, *C. kweichowensis* is native only to parts of China. There are significant differences between *C. heterophylla* and *C. kweichowensis* with regard to their distribution and habitat in China (Supplemental Table 1) (Ma et al., 2014). *Corylus heterophylla* is mainly distributed in northern areas of China, growing in locations with cold and dry environments, whereas *C. kweichowensis* grows in warm and moist environments, primarily in southern China (Liang, 1988). Among the different hazelnut species, *C. heterophylla* and *C. kweichowensis* share a number of similar traits, and there is not a consensus among taxonomists that these two are distinct species. Based on morphological characteristics (Fig. 1), some taxonomists considered *C. kweichowensis* a botanical variety (var. *sutchuensis*) of *C. heterophylla* (Chen, 1953; Zheng, 1985), but other researchers thought that *C. kweichowensis* should be an independent species (Supplemental Table 2) (Qi, 1996; Zhang et al., 2005). Morphological descriptors have traditionally been used to identify hazelnut species, accessions, and cultivars; however, expression of such factors can be influenced by the environment. In recent years, molecular markers have been widely applied to study the genetic diversity and population structure of hazelnut, such as random amplified

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C. heterophylla

C. kweichowensis

C. yunnanensis

Fig. 1. Trees, leaves, and nuts with husk of *Corylus heterophylla*, *Corylus kweichowensis*, and *Corylus yunnanensis*.

polymorphic DNA [RAPD (Galderisi et al., 1999; Miaja et al., 2001)], simple sequence repeat [SSR (Bocacci et al., 2006, 2008; Gökirmak et al., 2009; Gürçan et al., 2010b; Zong et al., 2015)], intersimple sequence repeat [ISSR (Kafkas et al., 2009)], and amplified fragment length polymorphism [AFLP (Ferrari et al., 2005; Martins et al., 2014)], owing to their high discriminatory power and relatively low cost. Among the various kinds of markers, microsatellite or SSR has been demonstrated to be extremely effective for estimating genetic diversity for *Corylus avellana* and related species (Bassil et al., 2005, 2013). SSR markers, which are widely present in eukaryotic genomes (Sharma et al., 2007), are simple tandemly repeated DNA sequences. The advantages of SSR markers include codominant inheritance, high polymorphism, and suitability for sharing among laboratories, as well as cross-species transferability (Barbará et al., 2007). More recently, chloroplast genome and whole genome sequencing have been applied to plant classification and genetic evolution research (Rowley et al., 2018; Yang et al., 2019), but the evolutionary rate of the chloroplast genome sequence is slow, which is not suitable for the study of genetic relationships of related species, whereas whole genome sequencing is less

applied to the study of genetic diversity of large-scale populations because of its high cost and workload. In this study, we investigated the genetic diversity and population structure of 21 populations of *C. heterophylla* and 13 populations of *C. kweichowensis* to clarify their taxonomic relationship and develop a strategy for conservation.

Materials and Methods

PLANT MATERIALS AND DNA EXTRACTION. A total of 796 wild samples, representing 34 populations, were collected throughout their native distributions in China. A map of the sampling locations was drawn using ArcGIS 10.0 (Esri, Redlands, CA) (Fig. 2). Of the 796 samples, 509 individuals representing 21 populations of *C. heterophylla* were collected from the northeast and north of China, and 287 samples were collected from 13 populations of *C. kweichowensis* across the south of China (Table 1). The distance between any two individuals at each location was more than 100 m. Young and healthy fresh leaves were only collected from one independent shoot per plant and dried with silica gel, and then transported to the laboratory for subsequent DNA extraction using a modified CTAB procedure (Doyle and Doyle, 1987).

Fig. 2. Geographic distributions of 34 *Corylus heterophylla* and *Corylus kweichowensis* populations sampled in mainland China. The map of the sampling locations was drawn using ArcGIS 10.0 (Esri, Redlands, CA). The population codes are listed in Table 1.

MICROSATELLITE DNA ANALYSIS. Eleven microsatellite loci were amplified and the results used to study the genetic diversity of *C. heterophylla* and *C. kweichowensis*. Sixty-five SSR markers, previously developed for hazelnut, were tested for their ability to produce amplification products in the two *Corylus* species. Then, 11 microsatellite loci with clear amplicons were finally selected to validate genetic diversity (Supplemental Table 3) (Bassil et al., 2005; Sathuvalli and Mehlenbacher, 2013). Polymerase chain reaction (PCR) amplifications were carried out in 20.0- μ L reactions. Each reaction contained 2.0 μ L DNA, 0.3 μ L of the forward primer (20 μ M), 0.3 μ L of the reverse primer (20 μ M), 0.4 μ L dNTPs, 2.0 μ L PCR buffer, 0.2 μ L Taq polymerase, and 14.8 μ L doubled distilled water. PCR amplification was performed under the following conditions: 94 $^{\circ}$ C for 5 min, followed by 35 cycles of 94 $^{\circ}$ C for 30 s, annealing at 48 to 60 $^{\circ}$ C for 40 s, 72 $^{\circ}$ C for 40 s,

with a final extension at 72 $^{\circ}$ C for 3 min. The forward primers were labeled with a fluorochrome (FAM or HEX). The products were analyzed using a capillary electrophoresis instrument (Applied Biosystems 3730xl; Thermo Fisher Scientific, Waltham, MA) and separated along with an internal size standard (Applied Biosystems GeneScan-500 LIZ, Thermo Fisher Scientific). The amplified fragments were sized using GENEMAPPER 4.0 software (Applied Biosystems, Thermo Fisher Scientific).

DATA ANALYSIS. The level of genetic diversity was calculated using Popgene 32 software (Yeh et al., 1999) with the following statistics: number of alleles (N_a), number of rare alleles (N_r), number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), coefficient of inbreeding (F_{is}), genetic differentiation

Table 1. *Corylus heterophylla* and *Corylus kweichowensis* populations surveyed in China and their sample size, location, and geographical parameters.

Population code	<i>Corylus</i> species	Individuals (no.)	Collection location	Latitude (°N)	Longitude (°E)
NA	<i>C. heterophylla</i>	29	Ning'an, Heilongjiang Province	44°18'	129°40'
TY	<i>C. heterophylla</i>	30	Tangyuan, Heilongjiang Province	46°44'	129°54'
MJG	<i>C. heterophylla</i>	20	Mengjiagang, Heilongjiang Province	46°26'	130°36'
MS	<i>C. heterophylla</i>	29	Mishan, Heilongjiang Province	45°28'	131°56'
DL	<i>C. heterophylla</i>	30	Dailing, Heilongjiang Province	47°03'	129°02'
JL	<i>C. heterophylla</i>	25	Jilin, Jilin Province	43°51'	126°34'
DF	<i>C. heterophylla</i>	12	Dongfeng, Jilin Province	42°40'	125°31'
LJ	<i>C. heterophylla</i>	25	Linjiang, Jilin Province	41°49'	126°54'
TL	<i>C. heterophylla</i>	29	Tieling, Liaoning Province	42°12'	123°46'
MLDW	<i>C. heterophylla</i>	25	Molidawa, Inner Mongolia	48°28'	124°30'
WC	<i>C. heterophylla</i>	30	Weichang, Hebei Province	41°56'	117°44'
LH	<i>C. heterophylla</i>	26	Longhua, Hebei Province	41°18'	117°45'
CC	<i>C. heterophylla</i>	20	Chicheng, Hebei Province	40°51'	115°44'
PS	<i>C. heterophylla</i>	25	Pingshan, Hebei Province	38°15'	114°11'
WA	<i>C. heterophylla</i>	25	Wu'an, Hebei Province	36°41'	114°11'
HR	<i>C. heterophylla</i>	17	Huairou, Beijing	40°19'	116°35'
LQ	<i>C. heterophylla</i>	20	Linqiu, Shanxi Province	39°19'	114°17'
ZY	<i>C. heterophylla</i>	10	Zhongyang, Shanxi Province	37°20'	111°11'
GJ	<i>C. heterophylla</i>	30	Gujiao, Shanxi Province	37°54'	112°09'
QS	<i>C. heterophylla</i>	22	Qinshui, Shanxi Province	35°41'	112°10'
YCX	<i>C. heterophylla</i>	30	Yangcheng, Shanxi Province	35°29'	112°25'
ZP	<i>C. kweichowensis</i>	21	Zhenping, Shaanxi Province	31°53'	109°31'
TB	<i>C. kweichowensis</i>	18	Taibai, Shaanxi Province	34°04'	107°18'
ES	<i>C. kweichowensis</i>	19	Enshi, Hubei Province	30°16'	109°29'
XE	<i>C. kweichowensis</i>	19	Xuan'en, Hubei Province	29°59'	109°29'
LA	<i>C. kweichowensis</i>	25	Lin'an, Zhejiang Province	30°14'	119°43'
TT	<i>C. kweichowensis</i>	25	Tiantai, Zhejiang Province	29°08'	121°02'
PJ	<i>C. kweichowensis</i>	24	Pingjiang, Hunan Province	28°43'	113°35'
HS	<i>C. kweichowensis</i>	24	Huogshan, Anhui Province	31°24'	116°19'
YX	<i>C. kweichowensis</i>	25	Yuexi, Anhui Province	30°51'	116°21'
HSS	<i>C. kweichowensis</i>	24	Huangshan, Anhui Province	30°18'	118°08'
XW	<i>C. kweichowensis</i>	24	Xiwen, Guizhou Province	26°51'	106°36'
WX	<i>C. kweichowensis</i>	25	Wuxi, Sichuan Province	31°24'	109°37'
LD	<i>C. kweichowensis</i>	14	Luding, Sichuan Province	29°55'	102°14'

coefficient (F_{st}), gene flow (Nm) and Nei's genetic distance. Nm was estimated as $Nm = 0.25 (1 - F_{st}) / F_{st}$ (Nei, 1972). The frequency of null alleles were calculated by CERVUS program version 3.07 (Kalinowski et al., 2007). Cluster analysis conducted using the unweighted pair group method with arithmetic mean (UPGMA) generated a Nei's genetic distance matrix, and a dendrogram was constructed using MEGA version 6 (Tamura et al., 2013). The number of homogeneous gene pools (K) was evaluated with the software package STRUCTURE 2.3.4 (Evanno et al., 2005). The length of the burn-in period and number of Markov chain Monte Carlo repetitions after burn-in were both set to 100,000. The algorithm was run 10 times for each K value, from 1 to 30 for the *C. heterophylla* and *C. kweichowensis* populations. Using an ad hoc quantity constructed from the second-order rate of change of the likelihood function with respect to K , the distribution of ΔK , where $\Delta K = \text{mean}(|L'(K)|) / s[L(K)]$, showed a clear peak at the true value of K (Earl and Vonholdt, 2012). The observed genetic variation among and within populations was characterized by analysis of molecular variance (AMOVA) using ARLEQUIN version 3.5 (Hamrick and Godt, 1996). In addition, Nei's genetic distance and geographic distance (kilome-

ters) were tested for correlation using the Mantel test using GENALEX 6.5 (Peakall and Smouse, 2012).

Results

GENETIC DIVERSITY AMONG THE LOCI. A total of 159 alleles were found among the *C. heterophylla* individuals, 92 of which were rare alleles with a frequency less than 1% (Table 2). N_a per locus ranged from 8 to 24, with an average of 14. N_e varied from 2.47 to 10.39, with an average of 6.55. The average of I was 2.05, varied from 1.24 to 2.60. H_o and H_e ranged from 0.57 to 0.84, and 0.60 to 0.90, with average values of 0.74 and 0.82, respectively. The average PIC value was 0.821. Similarly, a total of 162 alleles were found in the 287 *C. kweichowensis* individuals, 92 of which were rare alleles (Table 3). N_a varied from 9 to 23, with an average of 15. N_e ranged from 2.59 to 10.56, with an average of 6.42. The average value of I was 2.06, ranging from 1.36 to 2.59. The H_o in the total individuals was 0.67, which deviated from the H_e (0.82), whereas H_o and H_e ranged from 0.43 to 0.84, and 0.61 to 0.91, respectively. The average PIC value for *C. kweichowensis* was 0.802. The mean value of the frequency of null alleles of *C. kweichowensis* was

Table 2. List of 11 simple sequence repeat (SSR) loci used in the current study with number of alleles (N_a), number of rare alleles (N_r), number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), coefficient of inbreeding (F_{is}), genetic differentiation coefficient (F_{st}), gene flow (Nm), and frequency of null alleles from 509 *Corylus heterophylla* individuals.

Locus	N_a	N_r	N_e	I	H_o	H_e	PIC	F_{is}	F_{st}	Nm	Null alleles (frequency)
CAC-B005 ^z	18	12	6.74	2.26	0.78	0.85	0.838	0.041	0.055	4.312	0.049
CAC-B105 ^z	18	11	10.39	2.56	0.76	0.90	0.897	0.099	0.048	4.983	0.086
CAC-B001 ^z	14	7	7.28	2.21	0.84	0.86	0.849	-0.029	0.052	4.609	0.015
CAC-B028 ^z	15	10	5.68	1.94	0.81	0.82	0.801	-0.045	0.052	4.569	0.007
CAC-B113 ^z	15	9	7.62	2.30	0.73	0.87	0.857	0.096	0.054	4.406	0.089
CAC-C003 ^z	8	3	4.14	1.56	0.84	0.76	0.897	0.048	0.076	3.044	0.070
CAC-C028 ^z	9	5	2.47	1.24	0.57	0.60	0.557	0.024	0.034	7.135	0.020
LG612 ^y	11	5	6.14	1.95	0.68	0.84	0.817	0.128	0.067	3.481	0.100
LG628 ^y	11	6	4.24	1.71	0.71	0.77	0.734	0.027	0.048	4.996	0.032
LG637 ^y	16	9	7.22	2.26	0.57	0.86	0.894	0.275	0.097	2.336	0.207
LG639 ^y	24	15	10.10	2.60	0.83	0.90	0.894	0.025	0.059	4.027	0.046
Mean	14	8	6.55	2.05	0.74	0.82	0.821	0.063	0.058	4.354	0.066

^zSSR primers developed by Bassil et al. (2005).

^ySSR primers developed by Sathuvalli and Mehlenbacher (2013).

Table 3. List of 11 simple sequence repeat (SSR) loci used in the current study with number of alleles (N_a), number of rare alleles (N_r), number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphism information content (PIC), coefficient of inbreeding (F_{is}), genetic differentiation coefficient (F_{st}), gene flow (Nm), and frequency of null alleles from 287 *Corylus kweichowensis* individuals.

Locus	N_a	N_r	N_e	I	H_o	H_e	PIC	F_{is}	F_{st}	Nm	Null alleles (frequency)
CAC-B005 ^z	18	10	6.32	2.22	0.72	0.84	0.828	0.021	0.132	1.639	0.077
CAC-B105 ^z	18	11	8.53	2.37	0.63	0.88	0.872	0.223	0.085	2.677	0.172
CAC-B001 ^z	17	12	6.17	2.15	0.78	0.84	0.822	-0.010	0.086	2.674	0.037
CAC-B028 ^z	17	10	5.78	2.11	0.77	0.83	0.811	-0.030	0.091	2.513	0.041
CAC-B113 ^z	15	8	7.03	2.17	0.57	0.86	0.843	0.272	0.079	2.933	0.202
CAC-C003 ^z	9	4	4.22	1.64	0.68	0.76	0.729	0.034	0.076	3.028	0.056
CAC-C028 ^z	10	6	2.59	1.36	0.58	0.61	0.584	-0.033	0.081	2.835	0.018
LG612 ^y	10	6	4.29	1.68	0.68	0.77	0.732	0.028	0.076	3.038	0.060
LG628 ^y	10	3	6.35	1.98	0.68	0.84	0.824	0.132	0.078	2.945	0.111
LG637 ^y	15	7	8.76	2.36	0.43	0.89	0.875	0.444	0.114	1.953	0.343
LG639 ^y	23	14	10.56	2.59	0.84	0.91	0.898	-0.005	0.088	2.578	0.038
Mean	15	8	6.42	2.06	0.67	0.82	0.802	0.098	0.090	2.619	0.105

^zSSR primers developed by Bassil et al. (2005).

^ySSR primers developed by Sathuvalli and Mehlenbacher (2013).

0.105, whereas that of *C. heterophylla* was 0.066. The highest frequency values of null alleles at Locus LG637 for *C. heterophylla* and *C. kweichowensis* were 0.207 and 0.343, respectively (Tables 2 and 3). Thirteen and 20 unique alleles were found in the *C. heterophylla* and *C. kweichowensis* populations, respectively (Supplemental Table 4). In *C. heterophylla*, the number of unique alleles per locus ranged from one to three with the highest number at locus CAC-B028. In *C. kweichowensis*, the highest number of unique alleles (five) was at locus CAC-B001. In *C. heterophylla*, no unique alleles were detected at CAC-B105, CAC-B001, and LG612, whereas in *C. kweichowensis* no unique alleles were detected at LG628 (Supplemental Table 4).

GENETIC DIVERSITY AMONG THE POPULATIONS. Among the 21 *C. heterophylla* populations, the higher degree of genetic diversity occurred in WC (Weichang, Hebei Province), MS (Mishan, Heilongjiang Province), and WA (Wu'an, Hebei Province), whereas the lowest level of diversity was observed

in ZY (Zhongyang, Shanxi Province) (Table 4). Among the 13 *C. kweichowensis* populations, YX (Yuexi, Anhui Province), ZP (Zhenping, Shaanxi Province), LA (Lin'an, Zhejiang Province), and TB (Taibai, Shaanxi Province) showed relatively higher genetic diversity than other populations, whereas the lowest diversity was in XE (Xuan'en, Hubei Province) (Table 4). H_o and H_e values in *C. heterophylla* populations were slightly larger than those in *C. kweichowensis* populations (Table 4). However, regardless of whether they belonged to *C. heterophylla* populations or *C. kweichowensis* populations, the H_o values were significantly lower than the H_e values except for the ZY and JL (Jilin, Jilin Province) populations.

GENETIC DIFFERENTIATION. The inbreeding coefficient (F_{is}) values per locus of the *C. heterophylla* populations ranged from -0.045 to 0.275, with an average of 0.063 (Table 2). The average F_{st} value was 0.058, and varied from 0.034 to 0.097, indicating that genetic variation within populations (94.13%) was higher than that of among populations (5.87%). AMOVA

revealed that genetic variation within *C. heterophylla* populations accounted for 93.57% of the total variation (Table 5). The average N_m value was 4.354 (Table 2). Among the 13 *C. kweichowensis* populations, the F_{is} value ranged from -0.033 to 0.444, with an average of 0.098. F_{st} value varied from 0.076 to 0.132, with an average of 0.090 (Table 3). As was the case with *C. heterophylla*, we detected genetic variation within the *C.*

kweichowensis populations, which in this case accounted for 91.51% of the total variation. AMOVA showed that variation within populations was 88.91% of the total variation, while variation among populations was 11.09% of the total variation (Table 5). The variation within populations is higher than that among populations, possibly because of the relatively high gene flow of *C. heterophylla* populations ($N_m = 4.354$) and *C. kweichowensis* populations ($N_m = 2.619$).

GENETIC STRUCTURE AMONG POPULATIONS. Based on Nei's genetic distance using UPGMA, the 21 *C. heterophylla* populations and 13 *C. kweichowensis* populations were grouped and analyzed as a whole (Fig. 3). The populations were divided into four groups. Group I contained 21 populations of *C. heterophylla*; group II consisted of 11 *C. kweichowensis* populations, and both group III and group IV contained a single population each, where the LD (Luding, Sichuan Province) population and the XE population belonged to *C. kweichowensis*. The results showed distinct genetic differentiation between *C. heterophylla* and *C. kweichowensis*, reflecting to their different geographical distribution area.

The total populations were evaluated by Bayesian-based population assignment analysis using STRUCTURE 2.3.4 (Fig. 4). LnP (D) showed substantial fluctuation during $K = 1$ and $K = 2$. The ΔK showed a clear maximum for $K = 2$ ($\Delta K = 113.94$), indicating that the 34 populations were classified into two groups: Group I included 21 populations of *C. heterophylla*, and group II contained 13 populations of *C. kweichowensis* (Fig. 4). These results suggested differentiation between the two groups, and grouped them by species.

The 34 populations were also investigated by principal coordinate analysis (PCoA) using GenALEX 6.5. This gave a similar result to the UPGMA and STRUCTURE analyses. The populations were divided into two groups, with the *C. heterophylla* populations in one group and the *C. kweichowensis* populations in the other. The first main coordinates and the second main coordinates explained 41.99% and 18.02% of the total variance, respectively (Fig. 5). An analysis based on the Mantel test (Fig. 6) showed that the genetic distance was significantly correlated with the geographic distance ($r = 0.580$, $P < 0.001$), but the correlation was relatively low, suggesting that geographic distance influences genetic differentiation between *C. heterophylla* and *C. kweichowensis*.

Discussion

GENETIC DIVERSITY. The mean N_a of *C. kweichowensis* and *C. heterophylla* are close to that of *Corylus mandshurica* (Zong et al., 2015), but significantly higher than that of *C. avellana* (Bassil et al., 2005). Perhaps because it compared with 13 unique alleles found in the population of *C. heterophylla*, 20

Table 4. Genetic diversity within 21 *Corylus heterophylla* populations and 13 *Corylus kweichowensis* populations.

Population code ^z	<i>Corylus</i> species	N_a^y	N_e^y	H_o^y	H_e^y	I^y
NA	<i>C. heterophylla</i>	8	4.97	0.70	0.78	1.76
TY	<i>C. heterophylla</i>	8	4.59	0.70	0.76	1.72
MJG	<i>C. heterophylla</i>	7	4.06	0.67	0.71	1.57
MS	<i>C. heterophylla</i>	10	5.89	0.72	0.80	1.88
DL	<i>C. heterophylla</i>	9	4.91	0.67	0.77	1.74
TL	<i>C. heterophylla</i>	9	5.34	0.68	0.79	1.84
WC	<i>C. heterophylla</i>	10	5.93	0.75	0.80	1.89
LH	<i>C. heterophylla</i>	9	5.10	0.75	0.79	1.79
CC	<i>C. heterophylla</i>	9	5.89	0.73	0.79	1.86
HR	<i>C. heterophylla</i>	8	5.01	0.72	0.78	1.77
LQ	<i>C. heterophylla</i>	9	5.38	0.76	0.78	1.81
ZY	<i>C. heterophylla</i>	5	3.59	0.75	0.70	1.40
GJ	<i>C. heterophylla</i>	10	5.70	0.72	0.79	1.87
QS	<i>C. heterophylla</i>	9	5.03	0.70	0.77	1.77
YCX	<i>C. heterophylla</i>	9	5.06	0.75	0.77	1.75
DF	<i>C. heterophylla</i>	8	4.97	0.75	0.78	1.75
MLDW	<i>C. heterophylla</i>	8	5.51	0.75	0.79	1.80
JL	<i>C. heterophylla</i>	8	4.38	0.74	0.72	1.59
LJ	<i>C. heterophylla</i>	9	5.65	0.76	0.79	1.86
PS	<i>C. heterophylla</i>	9	5.02	0.74	0.77	1.75
WA	<i>C. heterophylla</i>	9	5.76	0.68	0.80	1.87
ZP	<i>C. kweichowensis</i>	9	5.22	0.76	0.78	1.81
TB	<i>C. kweichowensis</i>	8	5.59	0.72	0.78	1.81
ES	<i>C. kweichowensis</i>	8	5.01	0.67	0.78	1.76
XE	<i>C. kweichowensis</i>	5	3.08	0.65	0.65	1.25
LA	<i>C. kweichowensis</i>	9	5.31	0.63	0.78	1.82
TT	<i>C. kweichowensis</i>	7	3.78	0.61	0.70	1.49
PJ	<i>C. kweichowensis</i>	7	4.54	0.63	0.76	1.64
HS	<i>C. kweichowensis</i>	8	4.53	0.69	0.77	1.71
YX	<i>C. kweichowensis</i>	9	5.54	0.65	0.79	1.87
HSS	<i>C. kweichowensis</i>	8	4.76	0.71	0.76	1.68
XW	<i>C. kweichowensis</i>	9	5.07	0.69	0.77	1.77
WX	<i>C. kweichowensis</i>	8	4.58	0.64	0.72	1.62
LD	<i>C. kweichowensis</i>	6	3.68	0.68	0.69	1.44

^zThe population codes are listed in Table 1.

^y N_a = number of alleles; N_e = number of effective alleles; H_o = observed heterozygosity; H_e = expected heterozygosity; I = Shannon's information index.

Table 5. Analysis of molecular variance (AMOVA) of genetic diversity of populations.

<i>Corylus</i> species	Source of variation	df	Sum of squares	Variance components	Proportion of variation (%)	P
<i>Corylus heterophylla</i>	Among populations	20	568.02	25.52	6.43	<0.001
	Within populations	488	4562.27	9.59	93.57	<0.001
<i>Corylus kweichowensis</i>	Among populations	12	436.63	36.39	11.09	<0.001
	Within populations	274	2660.83	9.71	88.91	<0.001
<i>Corylus heterophylla</i> and <i>Corylus kweichowensis</i>	Among populations	33	1174.41	35.59	10.33	<0.001
	Within populations	762	7342.87	9.64	89.67	<0.001

Fig. 3. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram of 34 populations of *Corylus heterophylla* and *Corylus kweichowensis* based on Nei's genetic distance. The population codes are listed in Table 1.

Fig. 4. Bayesian STRUCTURE bar plots of membership for 34 *Corylus heterophylla* and *Corylus kweichowensis* populations ($K = 2$) based on simple sequence repeat (SSR) markers. The population codes are listed in Table 1; K = number of homogeneous gene pools, $\Delta K = \text{mean}(|L''(K)|)/s[L(K)]$.

unique alleles were found in the *C. kweichowensis* populations with fewer samples (Supplemental Table 4). The results showed that the population of *C. kweichowensis* had higher

reveal that both *C. heterophylla* and *C. kweichowensis* show substantial genetic variation: the H_o values were lower than the H_e values at the microsatellite loci, with the only exception

allele richness. Heterozygosity is an important parameter in determining genetic diversity. In a previous study of *C. heterophylla* and *C. kweichowensis* collected from Hebei and Henan Provinces of China, Wang et al. (2008) reported H_o and H_e values for *C. heterophylla* and *C. kweichowensis* of 0.52 and 0.66, and 0.48 and 0.58, respectively. For *C. mandshurica* growing in China, Zong et al. (2015) reported H_o and H_e values of 0.67 and 0.78, respectively. Polymorphism of 20 *C. avellana* genotypes and seven *Corylus* species, including *Corylus colurna*, *Corylus chinensis*, *Corylus heterophylla*, *Corylus americana*, *Corylus californica*, *Corylus fargesii*, and *Corylus ferox*, were investigated by Bassil et al. (2005), where the average values for *C. avellana* were H_o (0.62) and H_e (0.68). When the eight species genotypes were included, the average H_o and H_e values were 0.59 and 0.78, respectively. Sathuvalli and Mehlenbacher (2012) studied the genetic diversity of 87 *C. americana* populations using SSR markers and observed a high level of genetic diversity ($H_o = 0.68$, $H_e = 0.74$). Demchik et al. (2018) found a similar result that there was a highly heterozygous for 25 *C. americana* accessions, with 0.69 (H_o) and 0.78 (H_e), in the upper midwestern United States. Compared with these related studies, our results indicate a relatively higher level of genetic diversity, as the mean H_o and H_e values for *C. heterophylla* were 0.74 and 0.82, respectively, and 0.67 and 0.82 for *C. kweichowensis*, respectively. We suggest that this difference in the apparent degree of diversity is due to our samples being collected from wider ranges in China. Moreover, it was also important to use appropriate SSR markers in the study. After all, different markers may give different values in the same plant accessions. Michalski and Durka (2007) reported the mean values for H_o and H_e in *Juncus atratus* as 0.48 and 0.58, respectively. The lower value for H_o was attributed to the frequency of self-pollination in this species. Our results further

Fig. 5. Principal coordinate analysis (PCoA) based on simple sequence repeat (SSR) markers using the genetic distance matrix for distribution of the 34 *Corylus heterophylla* and *Corylus kweichowensis* populations in China. The population codes are listed in Table 1.

Fig. 6. Mantel test for matrix correlation between Nei's genetic distance and geographic distance for 34 *Corylus heterophylla* and *Corylus kweichowensis* populations.

being CAC-C003 in *C. heterophylla* (Table 2), indicating low heterozygosity at these loci. These results indicated a low level of inbreeding. The diversity level expressed an uneven and irregular distribution among *C. heterophylla* and *C. kweichowensis* populations. For instance, adjacent populations such as DL, TY, MJG, and MS from Heilongjiang Province of *C. heterophylla* and HS, YX, and HSS from Anhui Province of *C. kweichowensis* exhibited significant differences in genetic diversity, whereas four allopatric populations of *C. heterophylla* (NA from Heilongjiang Province, DF from Jilin Province, PS from Hebei Province, and YCX from Shanxi Province) and two populations of *C. kweichowensis* (ZP from Shaaxi Province, and LA from Zhejiang Province) showed nearly identical levels. The reason for this phenomenon may largely be attrib-

uted to spatially different ecological environments which facilitate the genetic variation at different levels.

GENETIC DIFFERENTIATION AND GENE FLOW. The F_{st} values of *C. heterophylla* and *C. kweichowensis* indicated that most of the total genetic variation resided within populations, rather than among populations (Tables 2 and 3). Compared with other *Corylus* species, the results agreed with those for *C. mandshurica* (Zong et al., 2015) and for *C. avellana* (Gürçan et al., 2010b). F_{st} values of 25 *C. americana* accessions were evaluated by Demchik et al. (2018), and varied from 0.02 to 0.19, with a mean value of 0.12, which further supported high levels of genetic diversity within populations. Wright (1978) suggested that F_{st} values of between 0.05 and 0.15 were indicative of moderate genetic differentiation. Our results indicated that the genetic differentiation of *C. heterophylla* was slightly lower than that of *C. kweichowensis*, while overall the two species showed a moderate level of genetic differentiation. Gene flow occurring within a species results in the movement of genes from one population to another (Slatkin, 1985; Slatkin and Barton, 1989), and is important in maintaining the genetic and phenotypic homogeneity of a species (Mayr, 1999). Slatkin (1985) proposed that if $Nm > 1$, the effect of genetic drift on population differentiation was reduced. In this study, the mean gene flow values of *C. heterophylla* and *C. kweichowensis* were 4.354 and 2.619, respectively, which suggests extensive gene flow in the different populations. The high level of gene flow for these two species may be related to wind-borne pollen and seed dispersal by birds and animals (Zhao et al., 2016). In addition, *C. heterophylla* has been used for thousands of years, whereas *C. kweichowensis* remains underused in China. Such factors may explain why the gene flow mean value of *C. heterophylla* is higher than that of *C. kweichowensis*. In a previous study, Zhao et al. (2016) evaluated the gene flow of *C. heterophylla* in Tieling, in Liaoning Province of China, and obtained a higher gene flow value up to 7.599. It may have been that the sampling range was less than the range used in our study, leading to a higher estimation of gene flow. The Mantel test showed that the genetic distance was significantly correlated with the geographic distance, and we observed that the gene flow among populations played a key role in genetic differentiation.

GENETIC STRUCTURE AND RELATIONSHIPS BETWEEN *C. HETEROPHYLLA* AND *C. KWEICHOWENSIS*. An UPGMA dendrogram based on Nei's genetic distance showed that 34 populations were divided into two primary groups (Fig. 3). All 21 populations of *C. heterophylla* were clustered into one group, and 11 of the 13 populations of *C. kweichowensis* clustered together, whereas populations LD and XE were placed basal to all the other populations in the study (Fig. 3). We propose that the LD and XE populations may have encountered a barrier or have experienced habitat fragmentation, resulting in their deviation from the other 11 populations of *C. kweichowensis*. The STRUCTURE and PCoA analyses indicated that *C. heterophylla* and *C. kweichowensis* can be classified to two separate groups (Figs. 3 and 4), although they have many similar traits, such as nut size and shape. Based on our genetic diversity and structure analyses, we conclude that they are distinct species. Qi (1996) suggested that *C. kweichowensis* is a separate species based on a comparison of leaf and nut characteristics with those of related species, whereas Ma et al. (2014) also concluded that *C. heterophylla* and *C. kweichowensis* are distinct species based on geographic distribution and

growth in different ecological conditions. Both are distributed across a wide range of climates and soils in China, with the former mainly distributed in northern regions of China, whereas the latter is mostly found in southern China, and the only intersecting region is located in the Qinling Mountains.

A better understanding of the genetic diversity of *C. heterophylla* and *C. kweichowensis* is important for wild resource conservation, breeding programs, and their utilization. Little is known about the genetic diversity and genetic erosion of wild *Corylus* species, with the exception *C. mandshurica* (Zong et al., 2015) and *C. heterophylla* (Zhao et al., 2016), and most studies have focused on cultivated *C. avellana* (Molnar, 2011). The loss of resources due to genetic erosion is intensifying as a consequence of overdevelopment and deforestation, as well as other causes. Ex situ and in situ conservation can be used to preserve germplasm resources, but as wild *C. heterophylla* and *C. kweichowensis* are widely distributed in China, thus, in situ conservation will likely be needed, requiring substantial investment and management. We suggest building a germplasm nursery or core collections for ex situ conservation where the resources will be collected from different distributed regions based on phenotype characteristics and molecular markers. There are examples of ex situ field collections of *Corylus* species, including ≈ 320 named cultivars, selections, and rootstocks that have been preserved at the U.S. Department of Agriculture, Agricultural Research Service National Clonal Germplasm Repository in Corvallis, OR (Hummer, 2001). Furthermore, we have started to construct a core collection of *C. heterophylla* to better use the resource. Compared with *C. avellana*, *C. heterophylla* is adapted to regions with low temperature (-30 to -40 °C) and drought conditions. As is well known, most *C. avellana* cultivars are susceptible to eastern filbert blight, but the causal fungus *Anisogramma anomala* is not present in China. Chinese scientists made controlled pollinations between selected plants of *C. heterophylla* as female parents and *C. avellana* as the male parents (Wang et al., 2018). The first generation hybrids of *C. heterophylla* $\times$ *C. avellana* are called Ping'ou hybrid hazelnuts and ≈ 14 cultivars have been selected in China. In view of the successful hybridization of *C. heterophylla* $\times$ *C. avellana*, more potential resources of *C. heterophylla* and *C. kweichowensis* need further hybrid utilization. In addition, we can preserve high genetic diversity populations in situ. Based on our data (Table 4), the WC, MS, and WA populations of *C. heterophylla*, and YX, ZP, LA, and TB populations of *C. kweichowensis* with high allelic richness should be preserved in situ.

In summary, our studies showed a relatively high level of genetic diversity among *C. heterophylla* and *C. kweichowensis* populations in China. According to their morphological characteristics, geographic distributions, habitat differences, and reproductive isolation of *C. heterophylla* and *C. kweichowensis*, they are considered as independent species (Ma et al., 2014; Qi, 1996; Zhang et al., 2005). The UPGMA, STRUCTURE, and PCoA analysis gave a similar result, showing that *C. heterophylla* and *C. kweichowensis* populations were clearly separated, which better clarifies their taxonomic relationships. To maximize the protection of the diversity of these two hazelnut species, the WC, MS, and WA populations of *C. heterophylla*, and YX, ZP, LA, and TB populations of *C. kweichowensis* should be priority preserved in situ. In addition, the results will provide valuable information for conservation strategy, breeding, and utilization.

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Supplemental Table 1. Geographical parameters and climate types of *Corylus heterophylla*, *Corylus kweichowensis*, and *Corylus yunnanensis*.

<i>Corylus</i> species	Latitude (°N)	Longitude (°E)	Climate type
<i>C. heterophylla</i>	36.78–51.73	100.57–132.20	Temperate
<i>C. kweichowensis</i>	26.48–36.97	102.10–121.43	Warm temperate, subtropical
<i>C. yunnanensis</i>	21.13–29.25	98.50–104.87	Subtropical

Supplemental Table 2. Phenotypic characteristics of *Corylus heterophylla*, *Corylus kweichowensis*, and *Corylus yunnanensis*.

Phenotypic characteristics ^z	<i>C. heterophylla</i>	<i>C. kweichowensis</i>	<i>C. yunnanensis</i>
Tree height (m)	3	9	3
Pubescence on young branches	Sparse	Sparse	Dense
Leaf index	0.84	0.70	0.82
Petiole length (cm)	1.38	1.02	0.64
Pubescence on the adaxial side of the leaf	Sparse	Sparse	Dense
Leaf vein number	6.0	8.6	7.9
Length index of husk	1.81	1.07	1.38
Nut shape index	0.93	1.07	0.83
Bud tip shape	Obtuse	Obtuse	Obtuse

^zPhenotypic characteristics described by Qi (1996).Supplemental Table 3. Characterization of 11 simple sequence repeat (SSR) markers including locus, primer sequence, repeat motif, melting temperature (Tm), and product size among *Corylus* species.

SSR locus	Primer sequence (5' to 3')	Repeat motif	Tm (°C)	Size (base pairs)
CAC-B005 ^z	CAAACCTTATGATAGGCATGCAA TGTCACCTTTGGAAGACAAGAGA	(GA)22	51	270–280
CAC-B105 ^z	AAAGGAGCAAGCATGTTAGG GTTTGTACGGATGATCCACTGAG	(GA)16	51	130–160
CAC-B001 ^z	CCAAATCAAAACAACCAAAACC CCGCAGCCATCACATCTA	(GA)18	50	98–120
CAC-B028 ^z	ATGGACGAGGAATATTTTCAGC CCTGTTTCTCTTTGTTTTCGAG	(AG)16	51	260–280
CAC-B113 ^z	TTGAGGAAGTCCAGGAAAAT GCCAGAGAGAGCAAGAGTTAG	(GA)14	51	148–180
CAC-C003 ^z	CCGCTAAGCTGGTAGGTAGT CGGTGCATCAAGTTGTACTT	(AGG)9	48	100–130
CAC-C028 ^z	CTACCCCATCGCTTGACAC GGAGACTTGTTGCCACAGA	(GAA)10	51	120–150
LG612 ^y	GCGAGTGTTTTAGAAGGAAGGA TTGCACAATATAGCTGACACCC	(GA)8	60	227–243
LG628 ^y	GTTGCTTACTCCCGCTTTTATC TGTCACGTCTTGCTTGCTCT	(GA)10	60	211–221
LG637 ^y	GCTTAATTGATTGCCTTGGGTA GTTTCCATGTTTGGGTTGTAGG	(TC)18	60	230–252
LG639 ^y	ATAAACCCAACTTCAGCGTCAT CACTCTCACAGATCCACCAAAG	(ATGT)6	60	217–235

^zSSR primers developed by Bassil et al. (2005).^ySSR primers developed by Sathuvalli and Mehlenbacher (2013).

Supplemental Table 4. Unique alleles at 11 simple sequence repeat (SSR) loci between 21 *Corylus heterophylla* and 13 *Corylus kweichowensis* populations.

Locus	<i>C. heterophylla</i>		<i>C. kweichowensis</i>	
	Unique alleles (base pairs)	Population code ^z	Unique alleles (base pairs)	Population code ^z
CAC-B005 ^y	270	TL	268	LD
			272	TB
CAC-B105 ^y			148	ZP
			178	ZP
			168	LA
CAC-B001 ^y			90	ES
			118	ES
			120	LA
			84	HSS
			88	HSS
CAC-B028 ^y	274	GJ	252	ZP
	280	GJ		
	250	WA		
CAC-B113 ^y	154	DL	154	PJ
			182	HSS
CAC-C003 ^y	127	PS	123	TB
CAC-C028 ^y	151	NA	126	ZP
	123	WC	123	ES
			151	LD
LG612 ^x			243	XW
LG628 ^x	236	HR		
	226	LQ		
LG637 ^x	226	LQ	230	XE
LG63 ^x	247	MS	251	HS
	201	LQ		

^zThe population codes are listed in Table 1.

^ySSR primers developed by Bassil et al. (2005).

^xSSR primers developed by Sathuvalli and Mehlenbacher (2013).