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Genetic Diversity and Population Structure of *Corylus yunnanensis* (Franch.) A. Camus Using Microsatellite Markers in Sichuan Province

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Abstract: *Corylus yunnanensis* (Franch.) A. Camus is a deciduous shrub, native to the Hengduan Mountain of Qinghai–Tibetan Plateau, and is an economically and ecologically important woody crop species. In the present study, one hundred and fifty trees sampled from ten populations of *C. yunnanensis* in Sichuan Province were investigated to assess the population genetic variation using nine SSR markers. The results revealed that *C. yunnanensis* has an average value of 12.111 alleles, 3.376 effective alleles, an expected heterozygosity of 0.648, and an observed heterozygosity of 0.630, presenting a relatively high level of genetic diversity. The *C. yunnanensis* populations in Maoxian and Wenchuan of Aba Prefecture expressed the highest value of genetic diversity, whereas the Hanyuan and Muli populations showed the lowest. Moreover, the genetic differentiation of ten *C. yunnanensis* populations averaged to 0.106. Correspondingly, AMOVA revealed that 87% of the total variance was accounted for the variation within populations, and only 13% was among the populations. Both UPGMA and Bayesian STRUCTURE clustering suggested that the ten *C. yunnanensis* populations could fall into three clusters: the Aba Prefecture population, the Ya'an population, and the population of Ganzi and Liangshan Prefecture, indicating a significant geographic distribution, which was also confirmed by the Mantel test. Our study could provide a better understanding of population genetic diversity, and serve valuable information for the genetic improvement of *C. yunnanensis*.

Keywords: *Corylus yunnanensis*; simple sequence repeat; genetic diversity; population structure; Hengduan mountain

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1. Introduction

The genus *Corylus* L. (commonly known as hazel plants), belonging to the family Betulaceae, are deciduous shrubs or small trees, native to the northern temperate zone. The genus contains about twenty species, among which eight species and two varieties are naturally occurring in China, mainly distributed in the north–east, north, and south–west of China [1–3]. Hazelnuts, one of the four major nuts in the world, are abundant in microelements, widely used in food processing and in the manufacture of confectionery products, including chocolate, biscuits, candy, dairy products, and so on. In addition, hazelnuts contain a higher overall proportion of fatty acids (~60% of the hazelnut kernel), mostly oleic acid (~80% of the fatty acids), and other unsaturated fatty acids, which could reduce the risk of cardiovascular disease by reducing blood pressure, thereby inhibiting cholesterogenesis and the atherosclerotic process [4–6]. Therefore, its economic value is growing increasingly of great concern.

Sichuan province is an important distribution region for the genus *Corylus*, and most domestic hazelnut resources in China are distributed here, including *C. yunnanensis*, *C. heterophylla* var. *sutchuenensis* Franch., *C. ferox* Wall., *C. ferox* var. *thibetica* (Batal.) Franch.,

C. mandshurica Maxim., *C. chinensis* Franch., and *C. fargesii* Schneid. [1]. These resources are mainly distributed in Aba Prefecture, Liangshan Prefecture, and Ganzi Prefecture, as well as the Qinba mountains in northern Sichuan. Due to the characteristics of their root systems, the hazelnut plants easily form continuous shrubs, and the hazelnuts are also important food sources for squirrels and other small wild animals, thereby being an important part of the mountain ecosystem. At present, the hazelnut resources in the above-mentioned region have not yet been bred effectively.

To utilize these wild hazelnut resources, it is necessary to clarify their population genetic variation. Microsatellites, or simple sequence repeats (SSRs) markers, are present in eukaryotic genomes extensively, and are suitable for automated allele sizing, exhibit co-dominant inheritance, and are highly polymorphic, and therefore have been widely used in studying population genetic diversity and structure. The SSR markers applied in the study of hazelnut plants were first developed by the laboratory of Mehlenbacher in Oregon State University, which constructed three microsatellite enrichment libraries of *C. avellana* (GAA, CA, and GA), and Bassil et al. (2003) screened fifty-three polymorphic loci from these libraries at first [7]. Thereafter, in 2005, 51 SSR markers in total were identified to dissect the population genetic diversity of *C. avellana*, and interspecific amplification was also successfully carried out [8–10]. So far, more than 200 SSR markers have been screened from the above three libraries, and are widely used in current times [11–14]. In China, SSR markers have been also successfully used in the study of genetic diversity, population structure, and relationship of hazelnut plants in *C. mandshurica*, *C. heterophylla* Fisch. ex Trautv., and *C. heterophylla* var. *sutchuenensis*, and so on, and this guided further hazelnut genetic improvements [15–20].

In this study, the genetic diversity, genetic structure, and variation patterns of the natural populations of *C. yunnanensis* in Sichuan province were investigated using SSRs, with the eventual aim to provide valuable information for the conservation strategy and utilization of *C. yunnanensis*.

2. Materials and Methods

2.1. Population Sampling

A total of 150 trees of *C. yunnanensis* were collected from ten populations in Sichuan province (Figure 1, Table 1). Fresh young leaves from individual trees were collected, promptly dried with silica gel in a non-woven bag, and then transported to the laboratory and stored at −70°C for DNA extraction.

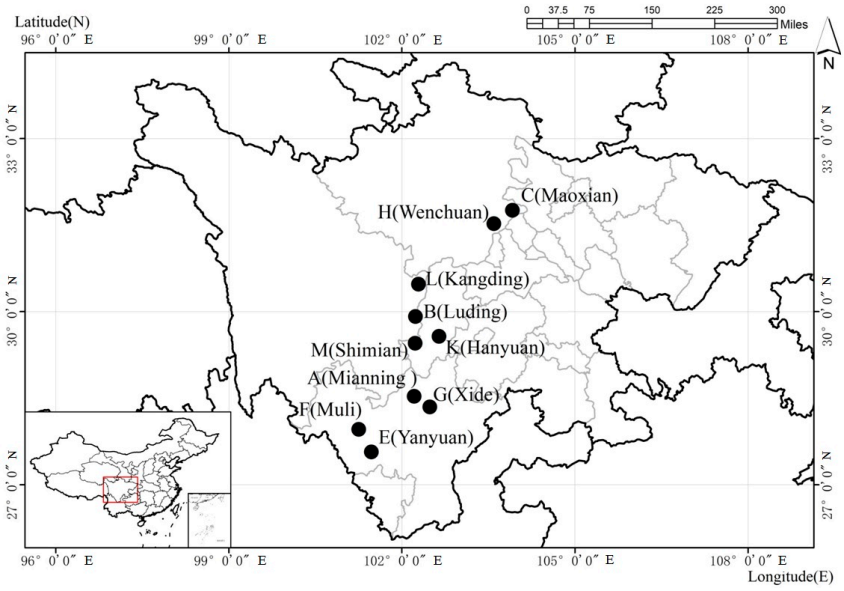

Figure 1. Locations of the 10 sampled populations of *C. yunnanensis* in Sichuan Province.

Table 1. Location and number of trees of the 10 *C. yunnanensis* populations in Sichuan Province.

Population Code	Location	Number of Samples	Longitude (E)	Latitude (N)	Altitude (m)
A	Mianning (Liangshan)	25	28.534	102.214	1768–2224
B	Luding (Ganzi)	14	29.916	102.236	1788–2573
C	Maoxian (Aba)	25	31.756	103.917	1430–2336
E	Yanyuan (Ya'an)	10	27.568	101.470	2563–3184
F	Muli (Liangshan)	16	27.960	101.252	2279–3230
G	Xide (Liangshan)	14	28.349	102.485	2085–2226
H	Wenchuan (Aba)	12	31.523	103.594	2239–2389
K	Hanyuan (Ya'an)	9	29.572	102.644	1700–1920
L	Kangding (Ganzi)	14	30.475	102.288	2220–2388
M	Shimian (Ya'an)	11	29.451	102.230	1254–1593

Note: The text in the parentheses indicates the prefecture for the corresponding population in the location column.

2.2. DNA Extraction and Microsatellite Analysis

Extraction of genomic DNA was performed with a gDNA extraction kit (Tiangen, Beijing, China). A total of 9 primer pairs (Table 2) presenting higher levels of polymorphism were screened from published papers for hazel plants [8,10,21]. The forward primer was fluorescently labelled with 6-FAM, and PCR and genotyping analysis were executed as described previously by Guo et al. [22].

2.3. Data Analysis

Micro-Checker 2.2.3 software was used to check the SSR loci for null alleles and possible misprints [23]. The parameters of genetic diversity (number of different alleles (*Na*), effective number of alleles (*Ne*), Shannon's information index (*I*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), inbreeding coefficient at the population level (*Fis*), inbreeding coefficient at the total sample level (*Fit*), genetic differentiation coefficient (*Fst*), and gene flow (*Nm*)) were calculated using the GenAlEx 6.51 Toolkit [24]. Nei's genetic distance (1978) was calculated using Popgene 1.32 software [25], which was then used to construct the UPGMA tree with the NTSYS-pc 2.10s software [26,27], and to implement the PCoA analysis using GenAlEx 6.51. In addition, Nei's genetic distance and geographic distance were analyzed for correlation with the Mantel test by NTSYS-pc 2.10s using 1000 random permutations [28].

STRUCTURE 2.3.4 was used to analyze the population structure with 10 times and 500,000 Markov Chain Monte Carlo (MCMC) repetitions following a burn-in period of 100,000 interactions for each group number K from 1 to 10 [29]. The optimal K value was determined by the method from Evanno [30,31]. After repeated sampling analysis with Clumpp 1.1.2 [32], the inferred clusters were visualized using Distruct 1.1 [33].

Table 2. Characterization of 9 SSR loci in *C. yunnanensis* based on 150 trees representing 10 populations in Sichuan Province.

Locus	Primers (5′-3′)	Size Range (bp)	Annealing Temperature (°C)	*Na*	*Ne*	*Ho*	*He*	*I*	*Fis*	*Fit*	*Fst*	*Nm*	Primer Source
Ch01	F: CAAACTTATGATAGGCATGCAA R: TGTCACTTTGGAAGACAAGAGA	270–300	55	17	3.396	0.699	0.718	1.436	−0.027	0.091	0.115	1.930	CAC-B005 [10]
Ch03	F: AGCAACAGAGGTTAGGTGTG R: GCCCCATTAGCCTTCTTA	164–185	55	6	2.461	0.595	0.605	0.982	−0.027	0.038	0.064	3.655	CAC-C118 [10]
Ch04	F: GTAGGTGCACTTGATGTGCTTTAC R: ACACCATATTGAGTCTTTCAAAGC	107–161	55	18	4.773	0.792	0.818	1.734	−0.013	0.064	0.077	3.004	CaDCAT28 [21]
Ch05	F: GGTTTGTTACAGAAATTCAGACG R: GCGTGTGGTTAATGTTTTCTTT	208–228	55	10	4.209	0.763	0.790	1.542	−0.011	0.057	0.067	3.496	CAC-A14a [8]
Ch06	F: ATGGACGAGGAATATTTCAGC R: CCTGTTTCTCTTTGTTTTCGAG	254–280	55	14	4.342	0.808	0.779	1.623	−0.085	0.047	0.122	1.802	CAC-B028 [8]
Ch07	F: AAAGGAGCAAGCATGTTAGG R: GTTTGTACGGATGATCCACTGAG	138–166	55	15	5.346	0.751	0.836	1.779	0.059	0.127	0.072	3.201	CAC-B105 [10]
Ch08	F: GGTCTCCTTCGCTAACATAACCAA R: GTTGCCCTCGAGTTGTAGTA	151–175	56	8	1.673	0.351	0.387	0.695	0.051	0.138	0.092	2.474	CaDCAT6 [21]
Ch09	F: CTAAGCTCACCAAGAGGAAGTTGAT R: GCTTCTGGGTCTCCTGCTCA	180–200	55	12	2.133	0.494	0.468	0.902	−0.109	0.122	0.208	0.952	CaDCAT10 [21]
Ch10	F: CTTCCAAGGATGGCTCAG R: TTTCAGGACGAGGACTCTG	179–197	58	9	2.049	0.415	0.429	0.837	−0.013	0.127	0.139	1.553	CAC-B014 [10]
Mean				12.111	3.376	0.630	0.648	1.281	−0.019	0.090	0.106	2.452	

Na, Number of different alleles; *Ne*, Number of effective alleles; *Ho*, Observed heterozygosity; *He*, Expected heterozygosity; *I*, Shannon's information index; *Fis*, inbreeding coefficient at the population level; *Fit*, inbreeding coefficient at the total sample level; *Fst*, genetic differentiation coefficient among populations; *Nm*, gene flow.

3. Results

3.1. Microsatellite Variation andPopulation Genetic Diversity

Micro-Checker analysis indicated that one microsatellite marker (Ch02) presented null alleles at high frequencies, which were therefore excluded from further analysis. Amplification of one hundred and fifty *C. yunnanensis* individuals representing the ten populations with the remaining nine markers generated a total of one hundred and nine alleles, with an average of 12.111 alleles at each locus (Table 2). The observed heterozygosity (*H*o) and expected heterozygosity (*H*e) ranged from 0.351 (Ch08) to 0.808 (Ch06), and from 0.387 (Ch08) to 0.836 (Ch07), and averaged 0.630 and 0.648, respectively (Table 2). Among all nine SSR loci, Ch06 and Ch09 had lower *H*e values than *H*o, whereas the rest had higher *H*e values compared to *H*o.

Among the ten populations, *N*a and *N*e ranged from 4.222 (E) to 8.000 (C), and 2.666 (E) to 4.360 (B), and averaged 5.667 and 3.376, respectively. *H*o ranged from 0.519 (F) to 0.757 (C), and *H*e ranged from 0.549 (E) to 0.758 (C), and averaged 0.630 and 0.758, respectively (Table 3).

Table 3. Genetic diversity within the 10 *C. yunnanensis* populations in Sichuan Province.

Population Code	*Na*	*Ne*	*I*	*Ho*	*He*	*F*
A	7.111	3.243	1.265	0.580	0.586	−0.018
B	7.222	4.360	1.519	0.667	0.702	−0.001
C	8.000	4.242	1.627	0.757	0.758	−0.019
E	4.222	2.666	1.023	0.522	0.549	−0.002
F	4.333	2.989	1.085	0.519	0.577	0.048
G	6.111	2.892	1.220	0.611	0.590	−0.076
H	4.889	3.433	1.356	0.743	0.753	−0.055
K	4.778	3.000	1.195	0.657	0.638	−0.106
L	5.556	3.784	1.322	0.667	0.656	−0.076
M	4.444	3.149	1.197	0.574	0.668	0.067
Mean	5.667	3.376	1.281	0.630	0.648	−0.024

*N*a, Mean number of different alleles; *N*e, Number of effective alleles; *I*, Shannon's information index; *H*o, Observed heterozygosity; *H*e, Expected heterozygosity; *F*,Fixation index.

Thus, the *C. yunnanensis* populations showed a relatively high degree of genetic diversity. In addition, among the ten populations, populations C (Maoxian), B (Luding), and H (Wenchuan) exhibited a higher diversity, while populations E (Hanyuan) and F (Muli) showed a lower level of genetic diversity by comparison, which was also revealed from the Shannon's information index (*I* values are as shown in Table 3).

3.2. Population Genetic Differentiation

As shown in Table 2, the inbreeding coefficient (*F*is) per locus ranged from −0.109 (Ch09) to 0.059 (Ch07), and averaged −0.019 per locus, while the genetic differentiation (*F*st) ranged from 0.064 (Ch03) to 0.208 (Ch09) across the nine loci, and averaged 0.106, indicating that the average genetic differentiation among the ten *C. yunnanensis* populations was 10.6%, and that genetic variation within the populations (89.4%) accounted for the main source of variation. In addition, gene flow (*N*m) ranged from 0.952 (Ch09) to 3.655 (Ch03), and averaged 2.452 (Table 2), indicating that the gene flow among the ten populations of *C. yunnanensis* was relatively frequent. Similarly, the results of the AMOVA analysis showed that genetic variation occurred mostly within the populations (87%), with only 13% being among the populations (Table S1). Additionally, both results indicated that the genetic variation of the *C. yunnanensis* populations mainly resides within the populations.

3.3. Population Genetic Structure

The generated UPGMA tree showed that the *C. yunnanensis* populations were grouped into three clusters (C and H, K and M, and others). Interestingly, populations C and H were from Aba Prefecture, populations K and M were from Ya'an, and the others were

populations from Liangshan Prefecture and Ganzi Prefecture (Figure 2A), indicating that the *C. yunnanensis* populations in Sichuan province exhibited a geographic distribution.

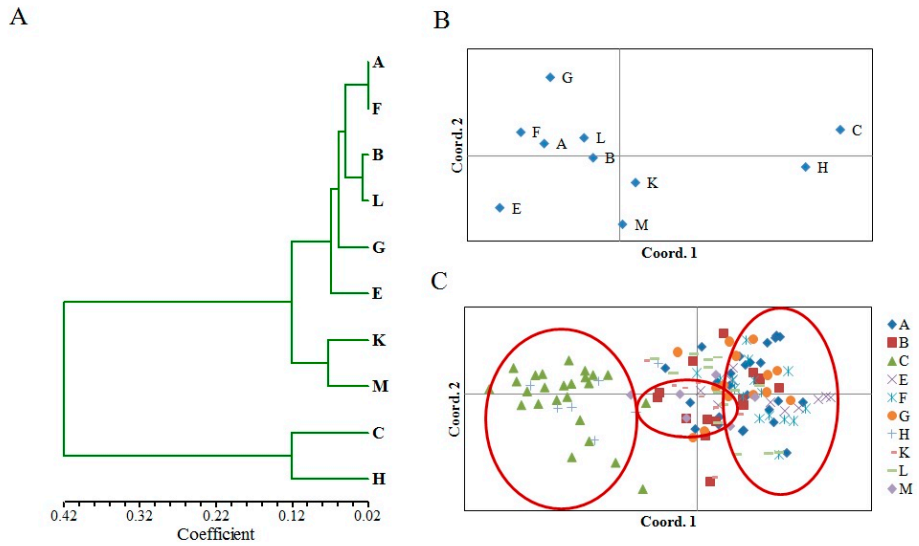

Figure 2. Genetic structure of the 10 *C. yunnanensis* populations. (**A**) UPGMA (Unweighted pair-group method with arithmetic means) cluster analysis based on Nei's genetic distance. (**B**) PCoA (Principal Coordinate Analysis) between populations. (**C**) PCoA between individuals.

The STRUCTURE analysis showed that the ΔK gave a clear maximum for K = 3 (ΔK = 10.843), also suggesting that the ten *C. yunnanensis* populations could be classified into three groups (Supplementary Figure S1). The cluster membership proportions of each individual are presented in Figure 3, and the cluster membership proportions of each population were also graphed (as shown in Figure 4), revealing a clear geographic distribution of the original populations. In the Aba populations, the first cluster (green) had a larger proportion. For populations from the Liangshan and Ganzi, the third cluster (blue) or the second cluster (red) showed a bigger portion, with a very small portion of the first cluster. In comparison with the Liangshan and Ganzi populations, the first cluster (green) was slightly larger for the Ya'an populations. This result is consistent with the UPGMA tree (Figure 2A). Similarly, the PCoA between either populations or individuals also revealed that the ten *C. yunnanensis* populations could be classified into three main groups (Figure 2B,C).

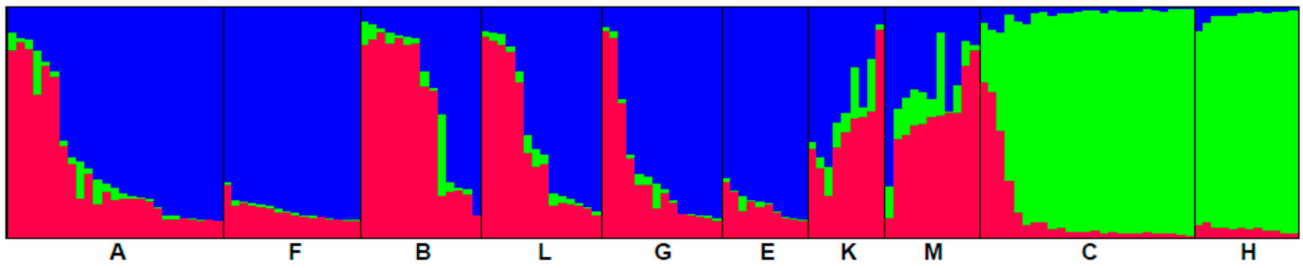

Figure 3. The cluster membership proportions of the 10 *C. yunnanensis* populations at the individual level with STRUCTURE (K = 3). Each bar representing one individual was partitioned into three different colored segments, showing the individual's estimated ancestry proportion of the genetic clusters.

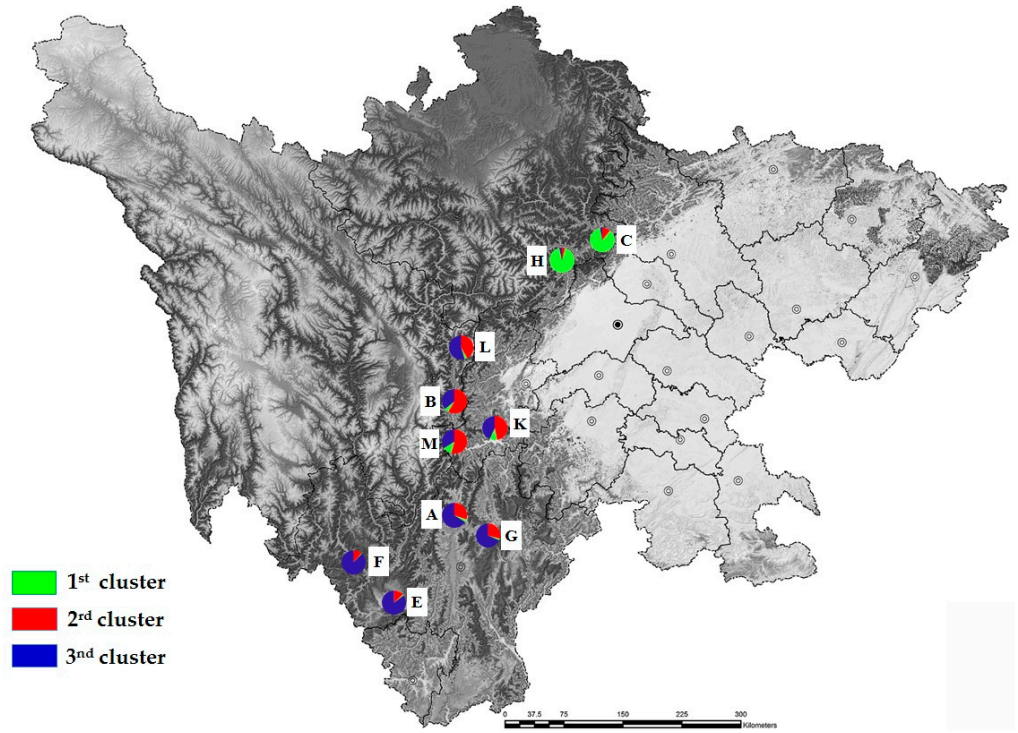

Figure 4. Mean cluster membership proportions of the 10 *C. yunnanensis* populations with STRUCTURE (K = 3).

The Mantel test revealed a significant correlation [r = 0.715 (p = 0.002)] (Figure 5) between Nei's genetic distance among populations and geographic distance, suggesting that the geographic distance presumably gives rise to the genetic differentiation observed of the ten *C. yunnanensis* populations.

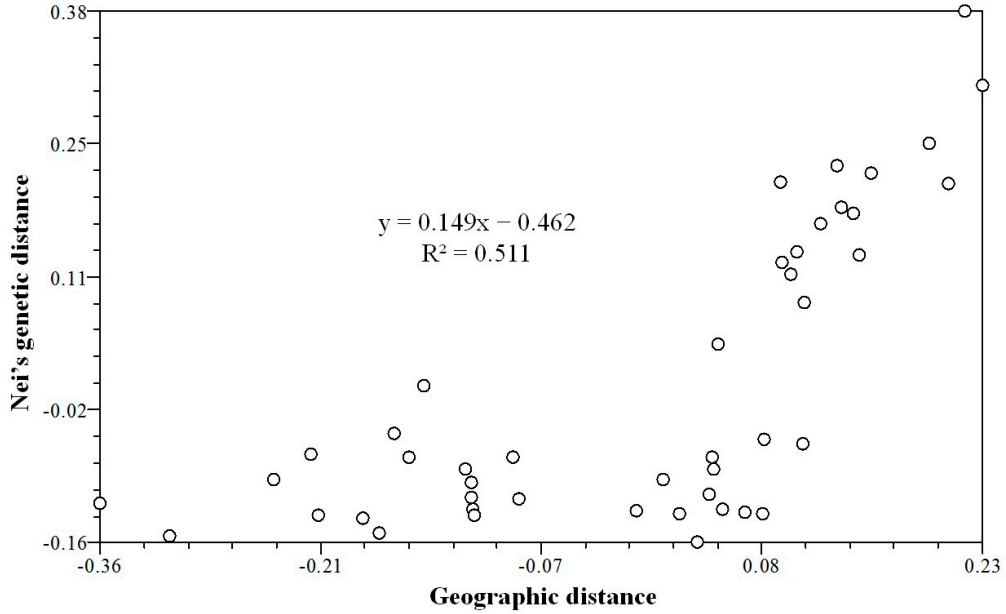

Figure 5. Mantel test for matrix correlation between Nei's genetic distance and geographic distance for the 10 *C. yunnanensis* populations.

4. Discussion

4.1. Population Genetic Diversity in C. yunnanensis

Overall, our SSR data indicates that all ten populations of *C. yunnanensis* have relatively higher genetic diversity, with the highest level of genetic diversity occurring in Aba Prefecture populations (Maoxian, Wenchuan).The genetic diversity of *C. yunnanensis* (*N*a = 12.111, *H*e = 0.648) in this study is higher than that of *C. heterophylla* (*N*a = 5.125, *H*e = 0.553) [17], and *C.cornuta* var. *californica* (*N*a = 6.496, *H*e = 0.619) [34]. Meanwhile, the *C. yunnanensis* genetic diversity is lower than that of *C. mandshurica* (*N*a = 15.3, *H*e = 0.777) [15], *C. avellana* (*N*a = 6.325, *H*e = 0.709) [35], and *C. americana* (*N*a = 10.90, *H*e = 0.74) [36]. This may be related to the sampling range of the hazelnut plant. For example, the *C. mandshurica* populations were sampled from several provinces, including Heilongjiang, Liaoning, Beijing, Hebei, and Shanxi in north and north–east China [15], while the *C. avellana* plants were collected from many countries or regions in Europe [35]. Alternatively, this might be attributed to the different SSR markers used in these studies. In accordance with the biological characteristics of *C. yunnanensis*, its population genetic diversity in this study was similar with what is typical for species that are perennial (*H*e = 0.68), outcrossed, and regionally distributed (*H*e = 0.65), and higher than that of species with a narrow distribution (*H*e = 0.56), early succession (*H*e = 0.42), wind- or water-dispersed (*H*e = 0.61), and endemism (*H*e = 0.46) [37].

4.2. Population Genetic Structure and Geographic Variation in C. yunnanensis

The genetic differentiation coefficient among the *C. yunnanensis* populations was relatively small (*F*st = 0.106), lower than that of *C. mandshurica* (*F*st = 0.122) [15], similar to that of *C. avellana* (*F*st = 0.097) [35], but higher than those of *C. cornuta* var. *californica* (*F*st = 0.012–0.054) [34], *C. heterophylla* (*F*st = 0.058), and *C. heterophylla* var. *sutchuenensis* (*F*st = 0.090) [16]. As according to Wright's consideration [38], the genetic differentiation among *C. yunnanensis* populations was moderate, which suggests that gene flow among the populations of *C. yunnanensis* was relatively frequent, preventing the impact of genetic drift and weakening genetic differentiation among the *C. yunnanensis* populations [39], conforming to the gene flow value (*N*m = 2.452).

Nevertheless, both UPGMA and STRUCTURE analyses showed that the ten *C. yunnanensis* populations could be divided into three clusters: Aba Prefecture populations, Ya'an populations, and Liangshan and Ganzi Prefecture populations, suggesting that the population genetic variation in *C. yunnanensis* presents a clear characteristic geographic distribution. On the other hand, Ya'an populations with the lowest distribution altitude are differentiated from the Liangshan and Ganzi populations, although the Ya'an populations are closer to the Ganzi populations in geographic distance, meaning that the genetic structure of *C. yunnanensis* is affected by the elevation gradient to some extent.

The plants of the genus *Corylus* and the genus *Alnus* Mill., both of which belong to the family Betulaceae, are widely distributed in the Hengduan mountain, but there is a significant difference in Sichuan basin. No wild hazelnut resources have been found in this area, whereas *A. cremastogyne* Burk. is widely distributed here [22]. The two genera were considered to have originated early from southwestern China. The fossil of *A. ferdinandi-coburgii* Schneid. was discovered in Mangkang of Tibet (~34.6Ma) [40,41], and the recent common ancestor of *Corylus* was also considered to occur in southwestern China using genome-wide SNPs (~36.38 Ma) [42], when major parts of Hengduan mountains of the Qinghai–Tibetan Plateau were being established [43]. For their distribution difference, we speculate that there are two possibilities. First, both were only distributed in the Hengduan mountain historically. However, accompanied with the uplift of the Qinghai–Tibetan Plateau, regional drainage systems such as the Daduhe river, Minjiang river, and Fujiang river were established, and the lightweight alder nutlets were easily dispersed into Sichuan Basin. While hazelnuts are large and heavy, they were presumably unaffected by the drainage systems. Second, both were historically distributed in both the Hengduan

mountain and Sichuan Basin. However, with the climatic environmental changes resulting from the uplift of the Qinghai–Tibetan Plateau, hazelnut plants in Sichuan Basin might therefore become extinct due to flower abortion or flowering asynchronism. For example, the catkins of *C. heterophylla* × *C. avellana* introduced into Sichuan Basin tend to abortion (data not shown). Of course, these speculations need further study.

4.3. Genetic Improvement of C. yunnanensis

In Sichuan province, *C. yunnanensis* is mainly distributed in the alpine valley region of western Sichuan, which belongs to parts of the Hengduan mountain of the Qinghai–Tibetan Plateau. Compared to the smaller area, a relatively high genetic variation was accumulated in the populations of *C. yunnanensis*, which could be presumably attributed to the diverse habitats resulting from the historically geological and climatic changes across the Qinghai–Tibetan Plateau [43]. However, ongoing expanding human activities, and increasingly severe climatic changes, are resulting in the loss of germplasm resources of *C. yunnanensis*. Therefore, one effective way to protect the germplasms of *C. yunnanensis* and its population genetic diversity is to screen some core populations for in situ conservation [16,44]. Thus, *C. yunnanensis* populations C, H, and B with the highest genetic diversity should be considered priorities for in situ conservation in future.

In addition, *C. yunnanensis* has a smaller nut and thicker shell, so it could not be directly used as a commercial variety. To cross with *C. heterophylla* × *C. avellana*, or with *C. avellana* for regional suitable varieties, or to directly breed the pollinating varieties, are two ways for their utilization. In this respect, it is necessary to select suitable populations for ex situ conservation, such as a larger hazelnut size, a thin nut shell, sufficient catkins, and appropriate blooming time. These are what we are currently designing and working towards.

In addition to *C. yunnanensis*, *C. heterophylla* var. *sutchuenensis*, *C. ferox*, *C. mandshurica*, *C. chinensis*, and *C. fargesii* are also distributed in the alpine valley region of western Sichuan [1,2]. Among these, some occupy the same ecological niches with *C. yunnanensis*, whereas some show differences with *C. yunnanensis*. The differentiation and evolution of these hazelnut plants deserve further study. Moreover, compared to *C. yunnanensis*, *C. ferox*, with some unique characteristics, such as spiny husks and arbor tree, show a wider distribution range, and also deserves further investigation.

5. Conclusions

In summary, our studies showed that the ten *C. yunnanensis* populations show a relatively high degree of genetic diversity. There was moderate genetic differentiation among the *C. yunnanensis* populations, and the majority of variation occurred within the populations. Gene flow among *C. yunnanensis* populations was relatively frequent, which reduced the population variation. These ten *C. yunnanensis* populations could be classified into three groups, and exhibited a significant geographic distribution. Taken together, the findings of our studies may provide valuable information for conservation management, genetic improvement, and utilization of *C. yunnanensis*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14050932/s1, Figure S1: Estimated population structure (K = 3) by STRUCTURE; Table S1: Molecular variance analysis among and within *C. yunnanensis* populations using 9 SSR loci.

Author Contributions: Conceptualization, experimental, data analysis, and writing—original draft preparation, Z.W.; writing—review and editing, Y.L.; investigation and resources, X.G. and J.D.; experimental, data analysis, and visualization, M.W.; funding acquisition, Z.W. and Y.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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