forests

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Genetic Analysis of the Cultivars of Ping'ou Hybrid Hazelnut (*C. heterophylla* Fisch. × *C. avellana* L.) in China Based on SSR Markers

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Abstract: Ping'ou hybrid hazelnut is one of the most profitable tree nuts in China, but economically important cultivars must first be genetically validated to meet industrial demand. Traditional approaches used for cultivar identification are mainly trait-based and unreliable. Previous approaches at the DNA level, focusing on the identification of species or/and varieties that originated in China, were not used widely in hybrid hazelnut because there was no proper standard sample. In this research, a multiplexed fingerprinting test was conducted to allow for hazelnut cultivar identification using SSR markers derived from European hazelnut. Twenty-seven SSR markers were used to fingerprint 57 genetically unique Ping'ou hybrid hazelnut and related wild species. All markers showed a high level of polymorphism, as indicated by mean values for observed heterozygosity (H_o = 0.84), expected heterozygosity (H_e = 0.80), and polymorphism information content (*PIC* = 0.78). A total of 301 alleles were detected, and the number of effective alleles varied from 6 for KG817 and GB818 to 18 for B654, with an average of 11.2 alleles per locus. Moreover, the Shannon's information index (*I*) ranged from 1.293 for BR215 to 2.385 for B654, with an average of 1.908. The neighbor-joining tree, principal coordinate analysis, and Bayesian analysis revealed clear separation between hybrid cultivars and wild forms (Cluster/group I), as well as the differentiation within hybrid genotypes (Clusters/groups II and III). Additionally, the NJ dendrogram demonstrated a further split within Clusters/group III (III a and III b). Altogether, with the comparable SSR information of the European hazelnut cultivar 'Barcelona', the newly developed marker sets can assist in the germplasm identification of hazelnut cultivars and reproductive materials. Importantly, these combined SSR loci can be applied to characterize the genetic relationships and population structures among wild genotypes and hybrid cultivars, which will then provide information to guide hazelnut breeding based on their genetic background.

Keywords: Ping'ou hybrid hazelnut; SSR markers; germplasm identification; fingerprinting; genetic relationships

Citation: Yang, Z.; Zhao, T.; Liang, L.; Jiang, L.; Wang, L.; Hou, S.; Ma, Q. Genetic Analysis of the Cultivars of Ping'ou Hybrid Hazelnut (*C. heterophylla* Fisch. × *C. avellana* L.) in China Based on SSR Markers. *Forests* **2023**, *14*, 1405. https://doi.org/10.3390/f14071405

Academic Editor: Richard Dodd

Received: 27 May 2023
Revised: 29 June 2023
Accepted: 5 July 2023
Published: 10 July 2023

1. Introduction

Hazelnut (*Corylus* L.) is an important nut crop and woody oil plant with high economic and nutritional value. The genus *Corylus* is a member of the Birch family, Betulaceae, and of the order Fagales. To date, 13 species are commonly recognized by taxonomists around the world. Among these, the European hazelnut, *C. avellana* L., is a species commercially cultivated in Turkey, Italy, the USA, Azerbaijan, Georgia, Chile, Spain, France, and Iran [1,2]. Experts have introduced some European hazelnut to China since the 1970s, but unfortunately, few suitable areas in China have been found for the commercial cultivation

of European hazelnut up to now, due to the climatic condition [3]. In China, there are eight commonly considered hazelnut species, but only one of the species, *C. heterophylla* Fisch. (named Ping hazelnut in Chinese) has been well developed in terms of commercial cultivation, in some limited parts of northeast China. The nuts of Ping hazelnut are not suitable for the commercial kernel market due to their smaller size, thicker shell and lower kernel percentage than European hazelnuts. Ping'ou hybrid hazelnuts (*C. heterophylla* × *C. avellana*) are the products of interspecific hybridization between some excellent maternal trees of Ping hazelnut from northeast China and several paternal cultivars of European hazelnut introduced from Italy [4]. Dozens of cultivars were selected from up to 2000 seedlings after 6 years of cross-breeding, among which, 15 cultivars have been released and widely cultivated in around twenty provinces of China, over about 112,000 h^2, since 2000. The commercial traits of the cultivars are significantly better than those of their maternal parent. Additionally, the cultivars can tolerate low temperatures during the winter, showing a stronger adaptation ability than their paternal parent [4]. According to the genetic background of cultivars of Ping'ou hybrid hazelnuts, some traits of the cultivars vary from each other in morphology and adaptation. On the contrary, however, it is difficult to distinguish between cultivars at the sapling stage. Thus, confusion regarding cultivars is one of the primary problems facing hazelnut production in China. Meanwhile, new cross-breeding works have been carried out by many Chinese researchers in recent years. New cultivars have been released, such as 'Xianda 1' by the Economic Forestry Research Institute of Liaoning Province, and 'Jinzhen 1' from Shanxi Agricultural University. A group of candidate cultivars are about to be released by the Research Institute of Forestry, Chinese Academy of Forestry. Thus, it is important and urgent that we develop a genetic identification method to characterize and distinguish the present cultivars and any possible future cultivars.

DNA markers are highly polymorphic and independent of environmental interactions, which are noted to be the best tools for understanding genetic diversity and relationships. SSR markers have the distinguishing features of multi-allelic, co-dominant inheritance patterns, reproducibility, high polymorphism, locus specificity, and transferability to related species and genera [5]. At present, more than 700 SSR loci have been developed in *Corylus* [6–13], and have been widely used in population structure assessment [14–16], germplasm identification and genetic diversity analysis [17–20], linkage map construction [21–24], and molecular marker-assisted selection [25,26]. In our previous study, we identified some cultivars of Ping'ou hybrid hazelnut by using the EST-SSR markers we developed in a pistil transcriptome [27]. All the present cultivars could be identified by four markers, showing the efficiency of the EST-SSR markers. However, no samples of other species were used in the study, limiting the universal application of the cultivar identification method.

'Barcelona' is a European hazelnut cultivar that is commonly used in genetic relationship research [23,28–30], and its SSR loci information is also available online "www.ars.usda.gov/pacific-west-area/corvallis-or (accessed on 20 May 2023)". In the present study, we collected 46 accessions of Ping'ou hybrid hazelnut, 1 accession of 'Barcelona', 5 accessions of *C. heterophylla*, 3 accessions of *C. mandshurica*, and 2 accessions of *C. kweichowensis* for SSR marker-based genetic analysis. Based on the fingerprinting of 'Barcelona' in this study, the published SSR data of 'Barcelona' was used to adjust the allele sizes. The objective of this study was to identify the present cultivars of the Ping'ou hybrid hazelnut, assess their genetic diversity level and genetic relationships, and develop a universal cultivar-identifying method for *Corylus*.

2. Materials and Methods

2.1. Plant Materials

A total of 57 samples were used in this study (Table 1), including 46 accessions of Ping'ou hybrid hazelnut, 1 accession of 'Barcelona', and 5, 3 and 2 accessions of *C. heterophylla*, *C. mandshurica* and *C. kweichowensis*, respectively. The samples of 42 accessions

of Ping'ou hybrid hazelnut and 10 of wild *Corylus* species were collected from the hazelnut repository of the Chinese Academy of Forestry, Yanqing District, Beijing. Young, tender, and healthy leaves of each selected accession were collected in the early summer of 2020, immediately placed in liquid nitrogen, and brought back to the laboratory. The samples were stored at −80 °C until DNA was extracted. 'Barcelona' was collected from National Clonal Germplasm Repository, Corvallis, Oregon, USA in September 2019. Fresh and healthy leaves were selected, cleaned and dried in a freeze drier, then stored in silica gel until DNA was extracted. Samples of 'Liaozhen 5' and 'Liaozhen 6' were collected from the hazelnut repository of Shenyang Agriculture University in the summer of 2020. The samples of 'Xianda 1' and 'Jinzhen 1' were collected directly from breeders from the Economic Forestry Research Institute of Liaoning Province and Shanxi Agricultural University, respectively. Of those samples, fresh and healthy leaves were selected in the summer of 2020, and stored in silica gel until DNA was extracted.

Table 1. Leaf samples used in this experiment.

Code	Name	Place	Code	Name	Source
1	Dawei	Yanqing, Beijing	30	Ping'ou 90	Yanqing, Beijing
2	Yuzhui *	Yanqing, Beijing	31	Ping'ou 119	Yanqing, Beijing
3	Bokehong	Yanqing, Beijing	32	Ping'ou 124	Yanqing, Beijing
4	Kuixiang	Yanqing, Beijing	33	Ping'ou 127	Yanqing, Beijing
5	Pingdinghuang	Yanqing, Beijing	34	Ping'ou 140	Yanqing, Beijing
6	Liaozhen 1 *	Yanqing, Beijing	35	Ping'ou 162	Yanqing, Beijing
7	Liaozhen 2 *	Yanqing, Beijing	36	Ping'ou 202	Yanqing, Beijing
8	Liaozhen 3 *	Yanqing, Beijing	37	Ping'ou 230	Yanqing, Beijing
9	Liaozhen 4 *	Yanqing, Beijing	38	Ping'ou 237	Yanqing, Beijing
10	Liaozhen 5	Shenyang, Liaoning	39	Ping'ou 360	Yanqing, Beijing
11	Liaozhen 6	Shenyang, Liaoning	40	Ping'ou 376	Yanqing, Beijing
12	Liaozhen 7 *	Yanqing, Beijing	41	Ping'ou 402	Yanqing, Beijing
13	Liaozhen 8 *	Yanqing, Beijing	42	Ping'ou 415	Yanqing, Beijing
14	Liaozhen 9 *	Yanqing, Beijing	43	Ping'ou 460	Yanqing, Beijing
15	Xianda 1 **	Dalian, Liaoning	44	Ping'ou 545	Yanqing, Beijing
16	Jinzhen 1 **	Taigu, Shanxi	45	Ping'ou 572	Yanqing, Beijing
17	Ping'ou 3	Yanqing, Beijing	46	Ping'ou 617	Yanqing, Beijing
18	Ping'ou 10	Yanqing, Beijing	47	Barcelona (*C. avellana*)	Corvallis, OR
19	Ping'ou 14	Yanqing, Beijing	48	P1 (*C. heterophylla*)	Yanqing, Beijing
20	Ping'ou 15	Yanqing, Beijing	49	P2 (*C. heterophylla*)	Yanqing, Beijing
21	Ping'ou 21	Yanqing, Beijing	50	P3 (*C. heterophylla*)	Yanqing, Beijing
22	Ping'ou 28	Yanqing, Beijing	51	P4 (*C. heterophylla*)	Yanqing, Beijing
23	Ping'ou 30	Yanqing, Beijing	52	P5 (*C. heterophylla*)	Yanqing, Beijing
24	Ping'ou 33	Yanqing, Beijing	53	M1 (*C. mandshurica*)	Yanqing, Beijing
25	Ping'ou 40	Yanqing, Beijing	54	M2 (*C. mandshurica*)	Yanqing, Beijing
26	Ping'ou 48	Yanqing, Beijing	55	M3 (*C. mandshurica*)	Yanqing, Beijing
27	Ping'ou 62	Yanqing, Beijing	56	C1 (*C. kweichowensis*)	Yanqing, Beijing
28	Ping'ou 72	Yanqing, Beijing	57	C2 (*C. kweichowensis*)	Yanqing, Beijing
29	Ping'ou 88	Yanqing, Beijing			

Note: Samples 1 to 46 are all Ping'ou hybrid hazelnuts (*C. heterophylla* × *C. avellana*). "*" indicates the main cultivars of Ping'ou hybrid hazelnut, "**" indicates the newly released cultivars.

2.2. DNA Extraction and PCR Amplification

DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction method described by Zong et al. [14]. The purified total DNA was quantified via gel (1.0%) electrophoresis, and its quality verified via spectrophotometry. Then, all the DNA samples were diluted using dd H_2O with a concentration of 10 ng/μL, and were stored at −20 °C for later PCR amplification.

A total of 72 SSR primers that were previously reported to be polymorphic by Oregon State University, USA (Table S1) were used to select the high efficiency SSR primers in this experiment, using the DNA samples of 'Dawei', 'Yuzhui', 'Liaozhen 3', and 'Barcelona'.

The annealing temperatures were used as suggested in the references [11,12,19]. Of the 72 SSR primers, 38 generated the expected polymorphic alleles on PAGE gels, among the five samples that were selected to analyze the genetic polymorphism of all the accessions. The forward and reverse primers were labeled with the fluorochrome of FAM or HEX (Table 2). The most suitable annealing temperatures of these primers were tested individually via the amplification of the DNA of 'Dawei' to ensure the accuracy of the multiplex PCR. Grouping information for the multiplex PCR as well as the post-PCR multiplexing for capillary electrophoresis is shown in Table S2. After the amplification in all accessions, six primers, i.e., B702a, B758, B779, B795, BR359 and GB423, were discarded after genotyping all the 57 hazelnut samples for complicated allele scoring due to large allele bin width, consecutive alleles, stuttering, and split peaks. Five primers, i.e., BR423, GB437, GB673, GB808 and GB867, were discarded for the relatively low PIC values in this experiment, as compared with other primers. The remaining 27 primers were subsequently tested for their ability to detect polymorphisms in 57 accessions.

Table 2. Information of the SSR markers used in the study.

Loci	LG	Tm	Motif	Forward Primer	Reverse Primer
B029b	1	60	(GA)3	CAATTTACACCTCAGGGAAGAG	AAGTTCACCCAAGAAATCCAC
B504	2	60–63	(CT)8	GCCATCTCCATTTCCCAAC	CGGAATGGTTTTCTGCTTCAG
A640	10	63–67	(CT)15(CA)13	TGCCTCTGCAGTTAGTCATCAAATGTAGG	CGCCATATAATTGGGATGCTTGTTG
B606	3	57–60	(ACAT)6Ns(AG)16	TCTTGTGGTTTAGCATACTTCTCG	GAAGAAAGCAAGAAGAGAGGAGA
B613	7	60–63	(CT)16	CGCGTTTTGAGTCCCTTTAG	CTACCCGCCTGCGAGAAC
B619	3	60	(TC)21	AGTCGGCTCCCCTTTTCTC	GCGATCTGACCTCATTTTTG
B654	8	57–60	(GA)9Ns(GA)(GA)9Ns(GA)20Ns(GA)7	TCGCATGGGTAATTTTCTCAC	TCATCATTTGGGTGCTTCAA
B657	11	57–63	(AG)15	GAGAGTGCGTCTTCCTCTGG	AGCCTCACCTCCAACGAAC
B664	10	60–63	(TC)21	CAAAGCCGTCGACAACAG	TTTGCATTTGATGCCGATAA
B702a	4	60–63	(CT)13CG(CT)3Ns	AGTTGGCGCTCGCTCTCT	TTGCAGCTCAGATGGTTCAC
B716	6	57	(CA)4GACAT(GA)13Ns(GGT)4	GAACATTGTCGTATGCGGACT	TCTGTTTGTTGCGCATGATT
B720	5	63	(AG)14	CTCTGTGTCGGCTTTCTGGT	ATAAACCTCACGCCACACCT
B726	8	57–63	(TC)16NNN(TA)9	GGAAATGGCAAATCCGTCTA	AACGTTTTGCCTTCCTTGTG
B733	7	60	(TC)15	CACCCTCTTCACCACCTCAT	CATCCCCTGTTGGAGTTTTC
B734	10	57	(TG)11(GA)10	AAGGTCCTGTTTGTTGGATCTC	TGTTTCTTTGACAACCTGCATT
B751	7	60–63	(GA)15	AGCTGGTTCTTCGACATTCC	AAACTCAAATAAAACCCCTGCTC
B758	2	57–60	(CT)15	TAATTTAAGCTGCCGTGCAA	TGCAAAATTGCATTGCTCAT
B777	9	57–63	(GA)15	AGGGAAGGGTGTAGGACGTT	TCGTTTTCTCCACATCACCA
B779	4	60–63	(CT)18	CGCTCTTGGACTTGGGATAC	TTGCAGCTCAGATGGTTCAC
B791	3	57–60	(AG)14	CACCAGGACCCTGATACCAT	TCCACAATGATTTTGTGAAAAC
B795	7	57–63	(TC)8Ns(CT)7Ns	GACCCACAAACAATAACCTATCTC	TGGGCATCATCCAGGTCTA
KG811	2	60	(GA)17	GAACAACTGAAGACAGCAAAG	AAGGCGGCACTCGCTCAC
KG817	2	60	(AG)11	AAAGTTAGAAGGGTCATTTGT	CAAGGTGGAGATTGTTGG
KG845	9	63–67	(TC)8NS(GT)8	TATAGATGCCATGGGTGCAAACAAAA	ACTATCACTTGACCCACCTTCCCTCTTT
BR215	8	60–63	(CGC) 5	TGAAATCTTCACCTCTTAAAAGATCC	GGAATCTGAGCTGCCAAGTC
BR359	4	63	(TCT)5	TACCTAACACAACAGCCACCAC	TCAGAATGGTAATTGCACCTTG
BR423	4	63	(GAA)6	ACAAACCAAAGGGAGTGTGG	CAAGCTTTCCATCATCGTCA
BR483	11	60–63	(AG)12	TTACCACCACTTTTCAACACCA	GGTACATCAAAGAAGGGAGCAC
GB332	9	60–63	(CAG)5	CCCTTCTACACGCAACACAA	GGGCACTCTCACCAAACAAT
GB410	4	60–63	(ATCC)4ag(CCAT)4	CCTCTACTATCTAGGAAGCCCCA	ACTTTGGCCTTTTGGACTTTG
GB423	6	57–63	(GAGC)5	GTCAAAGCTGAGGAATGGTTTT	TCGGTTGTCACTTGGTCAATTA
GB437	4	60–63	(GTGA)7	GCTTCTTGGAGGGTTCTGC	GCCAGAGCGTAAGAGAGAGAGA
GB673	5	57–63	(TCACCA)5	CAACAATGGGAATGTTGCAG	GGGCCAATAGCAAAAGTTCA
GB808	4	60–63	(CTG)7	GCATAAACCACTCCAACTCCTC	TTTGCTATCCCTACTCAGCTCC
GB818	1	57–63	(GAG)5	GAAGTTGGGTTGGAAGCAGTT	CGTCCTCTGCACACTCTCATAC
GB867	11	57–63	(GGA)6	CTTGGCAAAGCTACCCTCAC	ACGCGTTCTCTCCTAACGAA
GB875	5	63	(GGA)9	ATGATGATGAGGAGGAGGAGAA	CAAAATCAGGCATACAGAACCA
GK6.63	6	60–63	(GA)18	GCAAACTTCCAGAAAACCAA	AATGTTCGTAGGACAACTGCAT

Note: "LG" indicates linkage group. "Tm", annealing temperature. Tm of the primers was tested in this experiment.

PCR amplification was performed in a total of 20.0 µL volume that contained 2.0 µL of plant DNA, 0.4 µL of each dNTP (2.5 mM), 0.3 µL of forward primer (20 µM), 0.3 µL of reverse primer (20 µM), 2.0 µL of 10 × PCR buffer (containing $MgCl_2$), 0.2 µL of Taq polymerase (TaKaRa), and dd H_2O 14.8 µL. PCR amplification was performed with the following cycling parameters: a pre-denaturation step at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, annealing at 57 °C~63 °C for 40 s (a different primer annealing temperature, see Table 2), 72 °C for 40 s, and a final extension at step 72 °C for 3 min.

2.3. Microsatellite Analysis

Amplified fragments of SSRs were analyzed separately with an ABI 3730XL capillary sequencer (Applied Biosystems, Foster City, CA), along with the GeneScan-500 LIZ size standard (Applied Biosystems, Foster City, CA). Post-PCR multiplexing of 4–6 primer pairs in a single well was used according to the fluorescent label and the size ranges of the products. For multiplexing, 2.0 µL of the PCR products from each primer pair were mixed and diluted with water to make a final volume of 150.0 µL. An aliquot of 1.0–1.5 µL of the multiplex was used for the capillary electrophoresis. The SSR allele sizes were called with GENEMAPPER software (version 4.0, Applied Biosystems) for all samples, and entered in a spreadsheet. The allele sizes read by the software were rounded up or down manually according to the reported SSR loci data of 'Barcelona'. PCR amplification and capillary electrophoresis were repeated if the initial PCR failed or the result was ambiguous. the size standard.

2.4. Data Analyses

The codominant SSR data were analyzed using MICROCHECKER software [31] for detecting null alleles at each locus for each population. Population genetic analysis was performed using POPGENE v.1.3.2 [32] to calculate the diversity parameters, including number of alleles (N), effective number of alleles (N_e), Shannon's information index (I), Nei's gene diversity (H), observed heterozygosity (H_o), and expected heterozygosity (H_e). The polymorphism information content (PIC) of each locus was computed using the Excel Microsatellite Toolkit [33].

Based on the presence or absence of a binary data matrix, a pairwise genetic distance matrix was calculated for all individual accessions using GENALEX 6.5 [34]. Then, a genetic clustering analysis based on the genetic distance matrix was carried out to generate a neighbor-joining (NJ) dendrogram representing the genetic relationships among accessions in MEGA 6 [35]. Furthermore, principal coordinate analysis (PCoA) was conducted to identify genetic variation patterns among the hazelnut genotypes in GENALEX.

A separate analysis of population genetic structure was conducted using a Bayesian model-based clustering strategy implemented in STRUCTURE 2.3.4 software [36]. This method uses a Markov Chain Monte Carlo (MCMC) algorithm to cluster individuals into populations on the basis of multi-locus genotype data. For STRUCTURE analysis, all accessions were initially assigned to 49 groups: 45 Ping'ou hybrid hazelnut accessions (1–45), 2 *C. avellana* accessions (46), 5 *C. heterophylla* accessions (47), 3 *C. mandshurica* accessions (48), and 2 *C. kweichowensis* accessions (49). The number of clusters (K) was estimated by performing 10 independent runs for each K (2–20), using 100,000 MCMC repetitions and 50,000 burn-in periods. We used the admixture model with correlated allele frequencies to account for possible ancestral admixture. The most optimal K value was determined using the ΔK method [37], as implemented in STRUCTURE HARVESTER [38].

3. Results

3.1. SSR Polymorphism and Genetic Diversity

Some 57 hazelnut accessions were successfully amplified by all the 27 SSR primer pairs (Table 3), and each genotype displayed unique fragment size at one or several loci (Table S3). This indicated that the combination of these 27 primers could be used well for the cultivar identification of hazelnut accessions in China. Fingerprints of most primers (17 loci) tested in 'Barcelona' had an identical fragment size to data downloaded from the reference or from NCGR website, while the rest of the 10 loci showed 0–4 bp differences. A total of 301 alleles were detected at all 27 loci, and the number of effective alleles (Na) varied from 6 for KG817 and GB818 to 18 for B654, with an average of 11.2. All markers showed a high level of polymorphism, as indicated by mean values for observed heterozygosity (H_o = 0.8415), expected heterozygosity (H_e = 0.8074), and polymorphism information content (PIC = 0.7762). Half of the loci showed high PIC values (>0.80) in the

experiment. Moreover, the Shannon's information index (*I*) ranged from 1.293 for BR215 to 2.385 for B654, with an average of 1.908 (Table 3).

Table 3. Characteristics of the polymorphic SSR markers used in the study.

Name	Na	Ne	I	Ho	He	PIC	Size 1 (bp)	Size 2 (bp)	Size 3 (bp)	Fingerprints 1		Fingerprints 2		Comparison of Fingerprints
B029b	13	7.0401	2.1885	0.9123	0.8655	0.8435	115–141	115–143	115–143	122 *	128 *	121	127	−1 bp
B504	12	6.8544	2.1385	0.9123	0.8617	0.8386	161–187	144–182	144–188	158	182	158	182	identical
A640	13	7.3507	2.1964	0.9825	0.8716	0.8498	354–378	350–374	350–378	354	374	350	370	−4 bp
B606	11	4.2094	1.7911	0.3571	0.7693	0.7330	264–280	258–280	258–280	270	274	270	274	identical
B613	9	3.6338	1.5960	0.6964	0.7313	0.6898	192–216	184–206	184–216	200	202	198	200	−2 bp
B619	13	6.2904	2.1162	0.9123	0.8485	0.8250	146–180	145–177	145–181	156	170	157	171	+1 bp
B654	18	7.2929	2.3851	0.9474	0.8705	0.8526	276–302	250–302	250–302	286	302	286	302	identical
B657	12	5.8966	1.9623	1.0000	0.8378	0.8091	202–234	206–240	202–240	218	222	218	222	identical
B664	15	8.0922	2.2624	0.9649	0.8842	0.8638	186–218	186–216	186–218	206	216	206	216	identical
B716	14	4.0663	1.9001	0.6842	0.7608	0.7345	199–221	191–229	191–229	207	207	207	207	identical
B720	11	4.6514	1.9359	0.8246	0.7920	0.7680	155–191	159–177	155–191	161	167	161	167	identical
B726	16	8.2567	2.3485	0.9474	0.8867	0.8676	199–237	207–241	199–241	213	229	213	231	0/+2 bp
B733	8	4.2113	1.6033	0.5965	0.7693	0.7277	161–185	161–179	161–185	171	173	171	173	identical
B734	16	7.7082	2.2857	0.8947	0.8780	0.8575	231–261	217–259	217–261	255	255	257	257	+2 bp
B751	10	6.1244	1.9548	0.8596	0.8441	0.8160	137–161	135–157	135–161	143	153	141	153	−2/0 bp
B777	10	3.5625	1.6223	0.7719	0.7257	0.6834	202–224	200–224	200–224	202	222	202	222	identical
B791	12	5.7504	1.9966	0.7895	0.8334	0.8046	205–241	219–226	192–242	221	225	222	226	+1 bp
KG811	10	7.7542	2.1243	0.8596	0.8787	0.8571	240–278	240–274	240–278	258 *	264 *	256	262	−2 bp
KG817	6	3.1775	1.3897	0.8421	0.6914	0.6437	351–377	351–371	351–377	353 *	365 *	353	365	identical
KG845	10	4.3669	1.7187	0.9474	0.7778	0.7403	212–246	218–242	212–246	222 *	242 *	222	242	identical
BR215	6	3.1931	1.2932	0.7895	0.6929	0.6293	120–135	117–132	117–135	123	126	123	126	identical
BR483	14	6.9646	2.1839	1.0000	0.8640	0.8419	282–318	280–312	280–318	302	310	302	310	identical
GB332	7	3.6201	1.5023	0.7368	0.7302	0.6831	275–292	275–292	275–292	283	286	283	286	identical
GB410	8	3.0987	1.4053	0.7368	0.6833	0.6379	160–190	147–175	147–191	161	169	159	167	−2 bp
GB818	6	5.2403	1.7177	0.9825	0.8163	0.7816	129–144	129–144	129–144	129	144	129	144	identical
GB875	9	4.4054	1.7731	0.8772	0.7798	0.7475	325–352	334–358	325–358	340	340	340	340	identical
GK6.63	12	6.5504	2.1189	0.8947	0.8548	0.8308	76–116	77–113	76–116	95	101	95	101	identical
Average	11.1481	5.5320	1.9078	0.8415	0.8074	0.7762								

Notes: Size 1 shows the allele size range reported in the references; Size 2 shows the allele size range in this experiment; Size 3 shows the allele size range combined with the previous two columns. Fingerprints 1 shows the allele IDs of 'Barcelona' downloaded from the references or from the NCGR website (with a "*" mark); Fingerprints 2 shows the allele IDs of 'Barcelona' in this experiment.

3.2. Genetic Relationships among Accessions

To interpret the genetic relationships among diverse *Corylus* accessions, an NJ cluster analysis based on the Jaccord's similarity coefficient was performed. The unrooted dendrogram revealed three major clusters (Figure 1). Fourteen accessions, including ten accessions of three wild species (*C. heterophylla*, *C. kwechowensis*, and *C. mandshurica*), one accession of 'Barcelona', and three accessions of Ping'ou hybrid hazelnut ('Pingdinghuang', 'Liaozhen 5', 'Liaozhen 6'), were clearly divergent from the rest and closely clustered into Cluster I. Twelve hybrid accessions constituted Cluster II, among which 'Dawei' and 'Yuzhui' were excellent cultivars widely cultivated in north China. Cluster III comprised all the remaining 31 hybrid accessions, indicating that the majority of the hybrid cultivars had high genetic similarity. Moreover, we found that Cluster III could further be divided into two subclusters, III a (18 accessions) and III b (13 accessions), with accessions in each subcluster showing closer genetic relationships.

PCoA was performed to check the displacement of the accessions and to further confirm the clustering pattern obtained from the dendrogram (Figure 2). The first two PCs explained 47.38% of the cumulative variance among accessions, with PC1 accounting for 34.58% of the variance and PC2 for an additional 12.8%. The two-dimensional projection defined by the first two PCs of 57 hazelnut accessions also showed a similar clustering pattern to that of the NJ dendrogram. All accessions were grouped into three parts according to their genetic distance along the two axes. Apparently, the wild genotypes showed a tendency to separate from the hybrid hazelnut. Additionally, the hybrid accessions were separated into two main groups, corresponding to two clusters (II and III) in the phylogenetic tree. Information on genetic relationships among breeding accessions is essential for plant breeders to efficiently improve species.

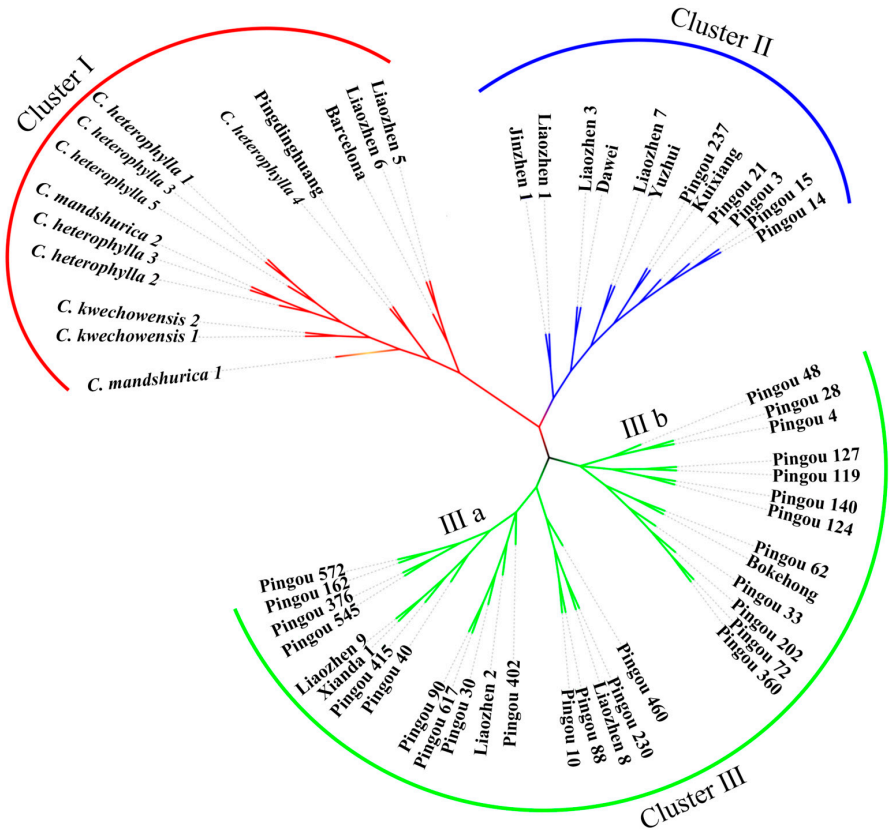

Figure 1. Unrooted neighbor-joining (NJ) dendrogram based on Nei's genetic distance of 57 hazelnut accessions. All accessions were divided into three genetic lineages: Cluster I (red) included 11 accessions of four wild species and 2 accessions of Ping'ou hybrid hazelnut; Cluster II (blue) included 12 accessions of Ping'ou hybrid hazelnut; Cluster III (green) included 31 hybrid accessions of Ping'ou hybrid hazelnut. Cluster III was divided into two sub-clusters III a and III b.

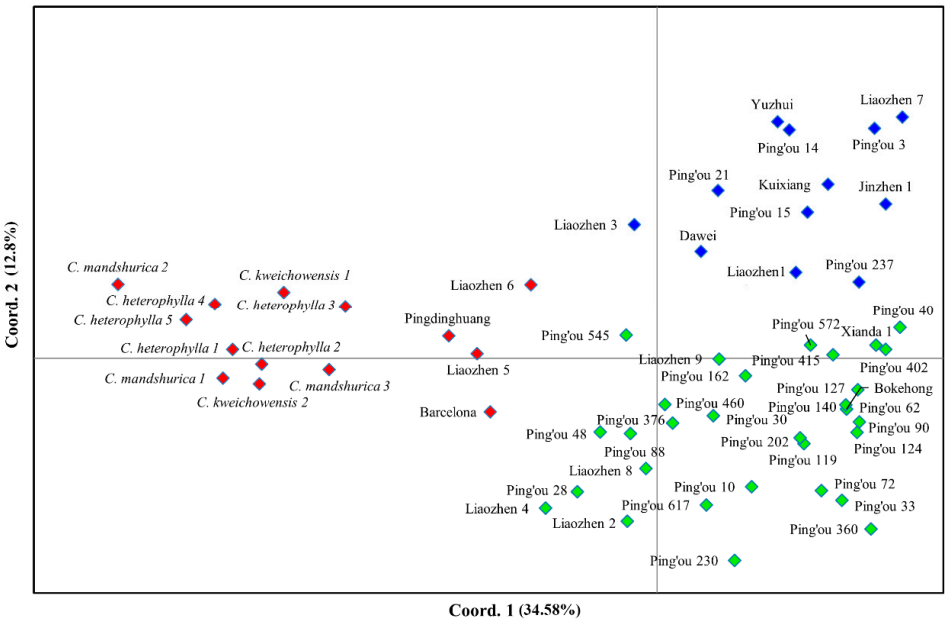

Figure 2. Principal coordinate analysis (PCoA) for the first and second coordinates estimated for 57 hazelnut accessions. Different groups are shown by different colors, and are consistent with that of the NJ dendrogram.

3.3. Population Stratification and Genetic Admixture

The 57 hazelnut accessions were further evaluated for population stratification and admixture analyses using a Bayesian model with STRUCTURE software (Figure 3). SSR data were analyzed, increasing the number of subgroups (*K*) from 2 to 20. The estimation of Δ*K* revealed the highest value for *K* = 3 (Δ*K* = 34.43) (Figure S1), suggesting the existence of three major groups. Group I accounted for 24.5% of all accessions, and it included ten accessions belonging to three wild species, one accession of Barcelona, and three hybrid accessions. Group II contained 12 hybrid accessions, while Group III comprised the remaining 31 hybrid accessions. At *K* = 2, these 57 accessions were divided into two groups, with Groups II and III identified at *K* = 3 assembled into a large group. When *K* = 4, Group II further split into two subgroups. Generally, the Bayesian clustering analysis strongly confirmed the results of the NJ dendrogram (Figure 1) and the PCoA scatterplot (Figure 2). Simultaneously, genetic admixture was observed among accessions, especially within Groups II and III. For instance, Ping'ou 28, Ping'ou 48, Liaozhen 4, and Liaozhen 8 of Group II showed some genetic admixture with accessions of Group I, while Jinzhen 1 of Group III shared similar ancestral components with the accessions of Group II. We believe that the admixture among accessions is due to artificial hybridization in the process of hazelnut breeding in China.

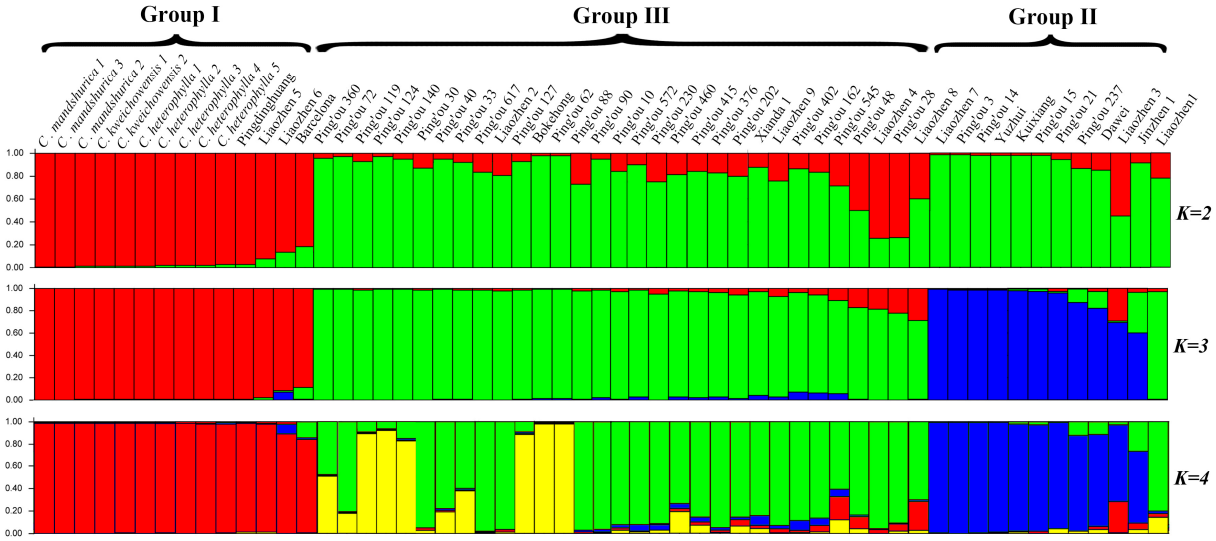

Figure 3. Population stratification and genetic admixture of 57 hazelnut accessions, as inferred by STRUCTURE, with three, four, and five populations (*K* = 2, *K* = 3, and *K* = 4). A single vertical line represents a single accession, and different colors indicate different groups. Segments of each vertical line represent the extent of admixture in an individual.

4. Discussion

Assessment of trueness-to-type through phenotypic observation is very difficult, and mistakes during the several steps of nursery plant propagation are costly. Therefore, developing a reliable DNA fingerprinting set for verifying the identity of hazelnuts would provide a crucial tool for verifying cultivar integrity in propagation systems and in hazelnut collections, and for protecting of breeders' rights. Molecular markers are highly useful in the precise identification of landraces, hybrids, and wild genotypes; this facilitates their planned utilization in hybrid breeding programs. In recent decades, various DNA-based molecular markers (e.g., RAPD, RFLP, AFLP, SSR, ISSR, etc) have been used in crop plants. The amplicon fragment analysis of these markers is gel-dependent, and has a limited ability to rapidly assay large numbers of marker loci. However, recent improvements in molecular marker technology, such as fluorescence-based automated DNA detection and fragment sizing, enable cost-effective genotyping to characterize the germplasm for crop improvement. For example, ITS regions, the *pth*N gene, and CP gene were designed to

detect strains of fungal, bacterial, and viral pathogens of cotton [39]. Multiplex molecular markers (11 RAPD, 11 ISSR, and 12 SSR) revealed a high polymorphism and significant differentiation across 20 commercial banana cultivars [40]. SSR and RAPD primers were used to evaluate the diversity and identify duplicates/misnomers among diverse grape accessions [41,42].

Of the various DNA markers, microsatellites or simple sequence repeats (SSRs) have become the marker of choice because of their advantages over other marker systems. SSRs are tandemly repeated 1–6 bp sequence motifs. They are abundant and dispersed throughout the genome and can be found in both coding and noncoding regions. Valuable characteristics such as high polymorphism, co-dominance, sensitivity (even a small quantity of DNA can be amplified by PCR), transferability to related species and genera, reproducibility, and ease of scoring have led to the extensive use of microsatellite markers for fingerprinting [43]. The exchange of primer sequences allows other labs to work with the same loci. In recent years, microsatellites have been used for various applications in fruit and nut crops, such as cultivar identification, breeding record verification, management of germplasm collections and identification of duplicate accessions, and evaluation of genetic diversity.

Previous studies have indicated that SSR loci developed from European hazelnuts were highly conserved and universal in other *Corylus* species, e.g., *C. heterophylla* and *C. kweichowensis* [15], *C. colurna* [44], and *C. mandshurica* [14]. However, a very limited number of SSRs have been tested in Ping'ou hybrid hazelnut so far. In this study, the transferability of 38 pairs of SSR loci was evaluated for 57 hazelnut accessions, a diverse group containing the most comprehensive breeding germplasm in China. Some 27 primers were successfully shown to have produced amplified products of expected length in all individuals; of these, some SSRs showed low H_o and H_e values, and only a few alleles, such as B606, B733, and BR215 (Table 2). Accordingly, we suggest that a subset of loci be used for future fingerprinting studies, with a preference for loci that are easy to score, have high H_o, H_e, and *PIC* values, and have a low frequency of null alleles [45]. According to the criteria, 11 robust SSRs, i.e., B029b, B504, A640, B619, B654, B664, B726, B734, KG811, BR483, and GK663 were recommended as the prior polymorphic markers for hybrid hazelnuts. These loci generate products of different sizes, and with different florescent tags could be used in three multiplex sets. Based on 26 SSR loci, the average genetic diversity of hybrid hazelnut (H_o = 0.84, H_e = 0.81), averaged over all hybrid accessions, was slightly higher than that of its parents *C. avellana* (H_o = 0.67, H_e = 0.72) and *C. heterophylla* (H_o = 0.74, H_e = 0.82) [15,19], and simultaneously, higher than its congeneric species, *C. kweichowensis* (H_o = 0.67, H_e = 0.82) and *C. mandshurica* (H_o = 0.67, H_e = 0.79) [14,15]. The high levels of heterozygosity may result from the biological features of hazelnut, including sporophyte incompatibility, dichogamy, and vegetative propagation of superior genotypes [11]. In particular, hybrid hazelnuts are destined to have high heterozygosity due to their hybrid origins in *C. heterophylla* × *C. avellana*.

Nowadays, hybrid strains have become the dominant hazelnut resources in China, with more than forty cultivars cultivated successively. However, some critical issues have gradually surfaced with the acceleration of the breeding process. On one hand, the existing hybrid hazel cultivars (strains) are selected from a mixed progeny group of multiple female and male parents, and the genetic relationships among cultivars are quite complicated, making it difficult to distinguish them in morphology. On the other hand, the phenomena of synonym and the confusion of the cultivars is one of the primary problems in hazelnut production in China. Unsuitable or/and mislabeled cultivars are either cannot tolerant the extreme weather, causing some physiological diseases (such as freeze damage and branch shriveling), or are incompatible with the main cultivar and the pollinizer, resulting in different levels of yield loss. In this study, we constructed the first DNA fingerprint for Ping'ou hybrid hazelnuts using highly polymorphic SSR markers, and fragment sizes were subjected to clustering and structural analyses. Our results revealed significant genetic differentiation of the three gene pools, and each group constitutes an

independent source of genetic variability and a valuable resource of hereditary properties for breeders. The clustering analysis showed a clear separation between wild (i.e., *C. heterophylla*, *C. mandshurica*, *C. kwechowensis*, and Barcelona) and cultivated genotypes (i.e., hybrid cultivars), except for 'Liaozhen 5' and 'Liaozhen 6'. Similar phenomena were also discovered in closely related species, for instance, Martins et al. [46], Boccacci et al. [47], and Campa et al. [48] observed a clear separation of wild genotypes from cultivated forms in *C. avellana*, except for a special type Ca24. Particularly, based on chloroplast SSR loci, Martins et al. [49] discovered that most of the wild genotypes had unique haplotypes, whereas Ca24 shared the most common chloroplast haplotype with landraces. Hence, the data reinforce the hypothesis that wild genotypes hold unique genetic variations and can provide valuable genetic resources for hazelnut breeding.

In addition, the population structure of these 57 hazelnut accessions was best depicted through standard structure analysis at $K = 3$, where three possible subgroups were identified. In brief, SSR markers clearly distinguish wild genotypes in a more homogeneous subgroup, while most of the hybrid cultivars displayed some genetic admixture sharing coefficients of ancestry. These results are generally consistent with the hereditary property that hybrid cultivars receive from the artificial hybridization of *C. heterophylla* and *C. avellana*. Nonetheless, multiple analyses supported the status of distinct taxonomic units of hybrid hazelnuts, as shown in the NJ tree, PCoA scatter plot, and STRUCTURE inference. Moreover, our results revealed a high level of differentiation within hybrid hazelnuts, for instance, the two distinct clusters/groups (II and III) and further subclusters (III a and III b).

Altogether, with the comparable SSR information of 'Barcelona', SSR information as well as the genetic background of Ping'ou hybrid hazelnuts in China can be recognized and analyzed by other researchers; these newly developed marker sets will assist in identifying hazelnut cultivars and reproductive materials derived from characterized stands. Importantly, these combined SSR loci can be applied to characterize the genetic relationships and population structures among wild genotypes and hybrid cultivars, which can supply information for guiding hazelnut breeding based on their genetic background.

5. Conclusions and Implications

The present study screened a set of 27 pairs of markers from 72 SSR primers developed in European hazelnut, which was highly polymorphic in Ping'ou hybrid hazelnut and related wild species. It provides evidence for the potential transferability of EST-SSRs tween related hazelnut germplasm, indicating that species-related cross-amplification is a useful method for the application of SSR markers in this genus. Additionally, these 27 primers were verified to be efficient for genetic identification of 46 economically important cultivars of Ping'ou hybrid hazelnut in China. Based on the unique molecular bands of each accession, genetic analysis revealed a clear separation between hybrid cultivars and their wild relatives. In particular, we identified two major genetic lineages within Ping'ou hybrid hazelnut, as well as two sub-lineages within Cluster III, which enabled an SSR-based population structure inference and a hybridity evaluation of the F1 hybrid cultivars.

The knowledge of this genetic background would be useful in designing strategies to improve the utilization of available genetic variation in the context of hazelnut breeding in China. The established genetic identification technique system will help to ensure the uniformity of the saplings in production, and protect newly released cultivars at present and in the future.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f14071405/s1, Figure S1: ΔK values for different numbers of clusters (K) in STRUCTURE analysis; Table S1: Information on the 72 candidate SSR markers used in the study. Table S2: Grouping information for the multiplex PCR and capillary electrophoresis. Table S3: Allele sizes of 57 hazelnut accessions at 27 SSR loci.

Author Contributions: Conceptualization and methodology, Z.Y. and Q.M.; validation, Z.Y. and T.Z.; formal analysis, Z.Y. and L.L.; investigation and resources, S.H. and L.L.; writing—review and editing, Z.Y., L.J., L.W. and Q.M.; experimental, data analysis, and visualization, Z.Y., Q.M. and S.H.; supervision, T.Z. and L.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key R&D Program of China (2022YFD2200400), the Key Research and Development Program of Hebei Province (21326804D) and the Key Science and Technology Program of Anhui Province (202103B06020017).

Data Availability Statement: Data are contained within the article.

Acknowledgments: We appreciate Shawn A. Mehlenbacher and Jacob W. Snelling from Oregon State University for sharing information on the SSR markers. We appreciate the help of Kim E. Hummer, Nahla V. Bassil, Barbara S. Gilmore, and April M. Nyberg from the National Clonal Germplasm Repository, Corvallis, Oregon, USA, for offering the leaf samples of 'Barcelona'. We thank Wenxuan Dong from Shenyang Agriculture University for offering the leaf samples of 'Liaozhen 5′ and 'Liaozhen 6′, Daoming Wang from the Economic Forestry Research Institute of Liaoning Province for offering the leaf samples of 'Xianda 1′, and Suoxing Liang from Shanxi Agricultural University for offering the leaf samples of 'Jinzhen 1'.

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

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