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Development of SSR Markers for and Fingerprinting of Walnut Genetic Resources

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Abstract: Walnut is one of four major nuts in the world. China has abundant walnut germplasm resources, but there are still shortcomings in the identification of germplasm resources. This study used different walnut varieties as materials and developed 14 high-quality SSR molecular markers from 60 pairs of primers based on genome re-sequencing results. This study analyzed the genetic diversity of Chinese walnut genetic resources using 14 selected SSR markers. A total of 64 alleles were detected in 47 walnut resources, with an average of 4.571 alleles per locus. The variation range of polymorphism information content was 0.096~0.711, with an average value of 0.422. Cluster analysis, population genetic structure, and principal coordinate analysis divided 47 walnut resources into ordinary walnuts, *Juglans hopeiensis*, and Liaoyi 1. In addition, core SSR markers (Jr45, Jr40, Jr29, Jr35, and Jr11) were selected from 14 SSR markers, which were sufficient to distinguish 47 walnut resources. At the same time, 47 unique molecular fingerprints of walnuts were constructed using these core SSR markers. This study provides strong scientific support for rapid and efficient identification, germplasm innovation, and a variety of property protection of walnut germplasm.

Keywords: walnut; nut phenotype; simple sequence repeats; breeding

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1. Introduction

The walnut genus (*Juglans* L.) consists of approximately 22 species [1], with species native to China [2], namely *J. regia* L., *J. mandshurica* Maxium, *J. cathayensis* Dode, *J. sigillata* Dode, and *J. hopeiensis* Hu. Among them, ordinary walnuts are the most widely cultivated species both domestically and internationally and are known as one of four major nuts [3]. According to the records of leaf fossils and carbonized nuts from the ^{14}C era, ordinary walnuts have a history of 7335 ± 100 years in the Shandong and Hebei provinces of China [4]. The production of walnuts (including iron walnuts) in China reached approximately 5.4035 million tons in 2021, firmly ranking first in the world [5]. China, one of the centers for genetic diversity of walnuts, provides important germplasm resources for the cultivation of new walnut varieties both domestically and internationally [6]. According to the "Records of Chinese Fruit Trees-Walnut Volume", Chinese farmers and breeders have cultivated over 380 walnut varieties (germplasm resources) through hybridization and other means. The southwest region is the main distribution center of walnut germplasm resources in China, and it has been recognized as having a high level of genetic diversity [4,6]. Therefore, exploring scientific and efficient identification methods has positive guiding significance for the identification, protection, and utilization of walnut germplasm resources.

The traditional methods for identifying walnut varieties mainly rely on morphological detection based on appearance features [7]. However, this method is not only time-consuming and labor-intensive, but also influenced by the external environment. With

the advancement of science and technology, research methods represented by molecular markers have been widely applied in the field of walnut variety identification [8–10]. At present, molecular marker techniques mainly include restriction fragment length polymorphism (RFLP) [11], random amplified polymorphism DNA (RAPD) [12], amplified fragment length polymorphism (AFLP) [13,14], simple sequence repeat (SSR) [15], and single nucleotide polymorphism (SNP) [16]. Among them, SSR is also called microsatellite DNA, and the core sequence of its tandem repeat is usually 1–6 bp [17]. Because of its codominance inheritance, locus specificity, and high polymorphism [18], it has been widely used in the study of genetic diversity and evolutionary relationships of walnuts. The International Union for the Protection of New Varieties of Plants (UPOV) recommends using SSR and SNP markers as the preferred methods for constructing plant DNA fingerprints [19]. In addition, capillary electrophoresis has gradually replaced traditional polyacrylamide gel electrophoresis (PAGE) due to its advantages of rapidity and automation, and has become the mainstream technology for separating and quantifying PCR products [20].

In recent years, many scholars have used SSR markers to study the genetic diversity, population genetic structure, and evolutionary relationship of many walnut germplasm resources and progeny [6,15,21]. At present, the approaches to developing SSR markers include searching EST sequences in public databases such as GenBank [22], genome re-sequencing [23], transcriptome sequencing [24], and chloroplast genome sequencing [25]. Doğan et al. used 25 pairs of RAPD primers, 25 pairs of ISSR primers, and 16 pairs of SSR primers to identify 59 foreign and Turkish walnut varieties. The polymorphism rate of SSR was 99.1%, much higher than those of RAPD (69.1%) and ISSR (71.1%) [26]. Magige et al. used 31 SSR markers to analyze the genetic structure of 12 walnut populations [9]. Jin et al. used WJR265, WGA331, and WJR031 (or WJR281, WGA321, WGA032) to fully distinguish 21 walnut materials under test [27]. Davoodi et al. used WGA001, WGA009, and WGA276 to completely separate 21 high-quality walnut varieties from Iran [10]. With the continuous increase of new walnut germplasm resources, there is an urgent need to develop highly polymorphic SSR markers to quickly and efficiently distinguish the increasing number of germplasm resources. It is imperative to use well-characterized SSR markers to construct DNA molecular fingerprints. This not only has guiding significance for the rapid identification of walnut resources and the establishment of walnut DNA fingerprint databases, but also contributes to the screening of walnut core germplasm and the development of germplasm innovation work.

Based on the genome re-sequencing results of Baokexiang, this study used eight different walnut samples as materials and selected 14 polymorphic SSR markers from 60 pairs of primers. These SSR markers were used to conduct molecular identification of 47 walnut germplasm via capillary electrophoresis detection, analyzing the genetic diversity, genetic relationship, population genetic structure, and principal coordinate analysis among different germplasm. This study seeks to achieve several goals, including molecularly identifying walnut germplasm resources, examining genetic diversity and population structure, creating a fingerprint map, and conducting association analysis between SSR markers and walnut traits. Overall, this study will provide a reference for future breeding of walnut germplasm with excellent traits and lay a theoretical foundation for screening walnut resources with excellent traits.

2. Materials and Methods

2.1. Plant Materials

The test walnut materials for this study were all collected from the Walnut Germplasm Resource Nursery of the Forestry Fruit Tree Research Institute of Beijing Academy of Agriculture and Forestry. Fresh tender leaves of various walnut varieties were collected in the summer of 2022, totaling 47 varieties, including 28 ordinary walnuts, 14 hemp walnuts, and 5 Yunnan walnuts. The detailed information is shown in Table 1. The collected tender leaves were placed in a self-sealing bag and promptly placed in an ice box. After being frozen in liquid nitrogen, they were stored in a refrigerator at −80 °C.

Table 1. Information sheet of the 47 tested materials.

Number	Cultivar Name	Origin	Species
1	Santai	Selected from progenies of Yunnan *Juglans sigllata*	*Juglans sigllata* Dode
2	Yangbipao	Selected from progenies of Yunnan *Juglans sigllata*	*Juglans sigllata* Dode
3	Yunxin 301	Selection of 'Santai' × 'Xinjiang Xinzao 13'	*Juglans regia* L. × *Juglans sigllata* Dode
4	Yunxin 303	Selection of 'Santai' × 'Xinjiang Xinzao 13'	*Juglans regia* L. × *Juglans sigllata* Dode
5	Yunxin 306	Selection of 'Santai' × 'Xinjiang Xinzao 13'	*Juglans regia* L. × *Juglans sigllata* Dode
6	Liaoyi 1	Selection of 'Hebei Changli Big Thin Skin 10103' × 'Xinjiang paper-shell walnut 11011'	*Juglans regia* L.
7	Liaoning 1	Selection of 'Hebei Changli Big Thin Skin 10103' × 'Xinjiang paper-shell walnut 11001'	*Juglans regia* L.
8	Liaoning 4	Selection of 'Damahetao' × 'Xinjiang paper-shell walnut'	*Juglans regia* L.
9	Liaoning 5	Selection of 'Xinjiang baoke' × 'Xinjiang luren'	*Juglans regia* L.
10	Liaoning 6	Selection of 'Hebei Changli Big Thin Skin 10301' × 'Xinjiang paper-shell walnut 11001'	*Juglans regia* L.
11	Liaoning 7	Selection of 'Damahetao' × 'Xinjiang paper-shell walnut 21102'	*Juglans regia* L.
12	Liaoning 10	Selection of 'Xinjiang Boke5 60502' × 'Xinjiang paper-shell walnut 11004'	*Juglans regia* L.
13	Jingxiang 1	Selected from progenies of Beijing walnuts	*Juglans regia* L.
14	Jingxiang 2	Selected from progenies of Beijing walnuts	*Juglans regia* L.
15	Jingxiang 3	Selected from progenies of Beijing walnuts	*Juglans regia* L.
16	Baokexiang	Selected from progenies of Xinjiang walnuts	*Juglans regia* L.
17	Beijing 861	Selected from progenies of Xinjiang walnuts	*Juglans regia* L.
18	Lvbo	Selected from progenies of Xinjiang walnuts	*Juglans regia* L.
19	Fengxiang	Selected from progenies of Beijing walnuts	*Juglans regia* L.
20	Qingxiang	Selected from progenies of Japan walnuts	*Juglans regia* L.
21	Meixiang	Selection of 'Xiangling' × 'Yunxin 34'	*Juglans regia* L.
22	Luguang	Selection of 'Kakazi' × 'Shangsong 6'	*Juglans regia* L.
23	Xinjufeng	Selected from progenies of 'Xinjiang Hechun 4'	*Juglans regia* L.
24	Xiangling	Selection of 'Shangsong 5' × 'Aksu 9'	*Juglans regia* L.
25	Lipin 1	Selected from progenies of Xinjiang walnut 'A2'	*Juglans regia* L.
26	Lipin 2	Selected from progenies of Xinjiang walnut 'A2'	*Juglans regia* L.
27	Zhonglin 1	Selection of 'Jian 9-7-3' × 'Fenyangchuanzi'	*Juglans regia* L.
28	Zhonglin 5	Selection of 'Jian 9-11-12' × 'Jian 9-11-15'	*Juglans regia* L.
29	Zijing	Selected from progenies of Beijing walnuts	*Juglans regia* L.
30	Robert Livermore	Selection of 'Howard' × 'RA 1088'	*Juglans regia* L.
31	Jinghong 1	Selected from progenies of Beijing walnuts	*Juglans regia* L.
32	Jinghong 2	Selected from progenies of Beijing walnuts	*Juglans regia* L.
33	D2-1	Selected from progenies of Beijing walnuts	*Juglans regia* L.
34	Jingyi 1	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
35	Jingyi 2	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
36	Jingyi 5	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*

Table 1. *Cont.*

Number	Cultivar Name	Origin	Species
37	Jingyi 6	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
38	Jingyi 7	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
39	Jingyi 8	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
40	Jingyi 8-2	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
41	Huayi 1	Selected from progenies of Shanxi *Juglans hopeiensis*	*Juglans hopeiensis*
42	Huayi 2	Selected from progenies of Shanxi *Juglans hopeiensis*	*Juglans hopeiensis*
43	Huayi 7	Selected from progenies of Shanxi *Juglans hopeiensis*	*Juglans hopeiensis*
44	Yihe 1	Selected from progenies of Hebei *Juglans hopeiensis*	*Juglans hopeiensis*
45	Qianlongguanmao	Selected from progenies of Shanxi *Juglans hopeiensis*	*Juglans hopeiensis*
46	Nanjiangshi	Selected from progenies of Hebei *Juglans hopeiensis*	*Juglans hopeiensis*
47	Mantianxing	Selected from progenies of Beijing *Juglans hopeiensis*	*Juglans hopeiensis*

2.2. Walnut Fruits

The determination of phenotypic traits of walnut nuts was based on the GB/T 26909-2011 "Guidelines for testing the specificity, consistency, and stability of new plant varieties: walnut genus". Walnut fruits were randomly selected, and the fresh weight of the green fruit, fresh fruit, green skin, seed kernel, seed meat, and seed coat and the dry weight of the seed kernel, seed meat, seed coat, and nut were measured and recorded using an electronic balance and a vernier scale. Referring to Zhang et al.'s method [28], the green skin rate, fruiting rate, fresh seed coat and kernel rate, nut coat and kernel rate, and nut kernel rate were calculated. The calculation formula was as follows: Green skin rate (%) = Green skin weight/Green fruit weight × 100%, Fruiting rate (%) = Fresh fruit weight/Green fruit weight × 100%, Fresh seed coat and kernel rate (%) = Fresh weight of seed coat/Fresh weight of seed kernel × 100%, Nut coat and kernel rate (%) = Dry weight of seed coat/Dry weight of seed kernel × 100%, Nut kernel rate (%) = Dry weight of seed kernel/Nut dry weight × 100%.

2.3. Extraction and Detection of Genomic DNA

The plant DNA extraction kit (QIAGEN) was used to extract the genomic DNA of the tested walnuts. After the extraction of DNA, the quality and concentration of DNA were tested using 1% agarose gel electrophoresis and a NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) ultra microspectrophotometer. The concentration of each DNA was diluted to 25 ng/μL. Samples were placed in a −20 °C refrigerator for PCR amplification.

2.4. Development of SSR Markers

The walnut leaf material was sent to Shanghai Yuanshen Biomedical Technology Co., Ltd. (Shanghai, China) for genomic re-sequencing. We conducted quality control on the original sequencing data and filtered out low-quality read data. Minia splicing v3.2.6 software was used to splice the filtered paired-end sequence, and MISA [29] and Primer 3 (https://www.primer3plus.com, accessed on 1 October 2023) were used to screen microsatellite sites and design EST-SSR primers. Based on the difference in Tm values between the upstream and downstream primers and the size of the products, 60 pairs of primers were screened and synthesized (Table S1). They were synthesized by Shanghai Shenggong Biotechnology Co., Ltd. (Beijing, China).

To confirm the effectiveness of these 60 pairs of primers in different genera of walnut, eight walnut varieties (Table 2) were selected for the development of SSR molecular markers. Based on the PCR amplification results of each pair of primers, primers with good specificity

and polymorphism were selected as the SSR markers for constructing the walnut population fingerprint map.

Table 2. Information sheet of the 8 walnut materials for developing SSR markers.

Number	Cultivar	Origin	Species
1	Baokexiang	Selected from progenies of Xinjiang walnuts	*Juglans regia* L.
2	Jinghong 1	Selected from progenies of Beijing walnuts	*Juglans regia* L.
3	Jinghong 2	Selected from progenies of Beijing walnuts	*Juglans regia* L.
4	Black walnut	Selected from America walnut	*Juglans nigra*
5	Jingyi 2	Selected from progenies of Beijing wild *Juglans hopeiensis*	*Juglans hopeiensis*
6	Xiaoxiaoqiu	Selected from progenies of Beijing *Juglans mandshurica* Maxim	*Juglans mandshurica* Maxim
7	Yangbipao	Selected from progenies of Yunnan *Juglans sigllata*	*Juglans sigllata* Dode
8	Changshan 99-11	Selected from progenies of *Carya cathayensis* Sarg.	*Carya cathayensis* Sarg.

2.5. SSR Amplification

The PCR amplification was carried out using a 20 μL reaction system: 8 μL ddH_2O, 10 μL 2 × Taq Mixture enzyme (Shanghai Shenggong Biotechnology Co., Ltd.), with 1 μL upstream and 1 μL downstream primer, and 1 μL template DNA. The PCR reaction procedure was as follows: pre-denaturation at 94 °C for 3 min. Denatured at 94 °C for 30 s, annealing at around 55 °C for 30 s (specific annealing temperature depended on different SSR primers), extended at 72 °C for 1 min, 30 cycles. Finally, extended at 72 °C for 5 min and stored at 12 °C (SCILOGEX TC1000-G, USA). After the reaction, preliminary detection was carried out on agarose gel electrophoresis.

2.6. Capillary Electrophoresis Detection

We selected differentially stable and specific SSR primers from the selected primers and performed capillary electrophoresis detection using Fragment Analyzer (Advanced Analytical). After obtaining the peak map, we used PROSize 3.0 software [30] to read the SSR site information and input it into Excel.

2.7. Data Analysis

Genetic diversity indicators were calculated using PowerMarker 3.25 [31] and GenAlEx 6.503 [32] software, including polymorphism information content (PIC), major allele frequency (MAF), number of alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), Shannon index (I), genetic diversity (GD), population differentiation index (Fst), identification probability (PI), and identification probability after combination (Pisibs). The Nei's genetic distances among 47 walnut varieties were calculated using PowerMarker 3.25, and the UPGMA cluster tree was constructed using MEGA11 [33]. The construction of the phylogenetic tree used the neighbor-joining method, with the Bootstrap parameter set to 1000. The Bayesian model of Structure 2.3.4 was used for population structure analysis. The parameter iteration number and the Markov chain iteration number were set to 10,000 and 100,000, respectively. Principal coordinate analysis (PCoA) was conducted on 47 walnut varieties using GenAlEx 6.503. The strip data were arranged in series in the order Jr45, Jr40, Jr29, Jr35, and Jr11, and the fingerprint was imaged using GraphPad Prism 9.0. The SPSS 26.0 [34] single-factor ANOVA method was used to analyze the correlation between SSR molecular markers and phenotypic traits, and the LSD method was used for multiple comparisons of SSR markers with significant differences.

3. Results

3.1. Distribution Characteristics of SSR Loci in Baokexiang

Based on the genomic re-sequencing results of Baokexiang (Tables 3 and 4), a total of 3,509,660 sequences with a total length of 529,208,302 bp were detected, and a total of 943,186 SSR loci were screened. Among them, dinucleotide repeat locus accounted for the

largest proportion (350,229, 37.13%), followed by single nucleotide repeat (26.06%), tetranucleotide repeat (18.86%), trinucleotide repeat (12.54%), pentanucleotide repeat (3.40%), and hexanucleotide repeat (2.01%), respectively (Table S2). In dinucleotide repeats, the majority (66,349, 18.94%) of SSR loci repeated six times. As the number of repeats increased, the frequency of distributed repeat bases gradually decreased, and similar phenomena were also observed in other repeat base types. Analysis of base repeat types (Table S2) showed that A/T base types accounted for the highest proportion (233,978, 24.81%) in the microsatellite sites of Baokexiang, followed by AA/TT (139,372, 14.78%), AT/AT (118,186, 12.53%), and AAA/TTT (87,664, 9.29%).

Table 3. Search Results for EST-SSR Loci in Baokexiang.

Total Number of Sequences Examined	3,509,660
Total size of examined sequences (bp)	529,208,302
Total number of identified SSRs	943,186
Number of SSR containing sequences	382,500
Number of sequences containing more than 1 SSR	269,491
Number of SSRs present in compound formation	513,771

Table 4. The distribution of different repeat type classes in Baokexiang.

Unit Size	Number of SSRs	Proportion (%)
1	245,791	26.06
2	350,229	37.13
3	118,290	12.54
4	177,899	18.86
5	32,038	3.40
6	18,939	2.01
In total	943,186	100.00

3.2. Development and Genetic Diversity Analysis of SSR Primers in Walnuts

By conducting PCR pre-experiments on eight walnut varieties, including Baokexiang, Jinghong 1, Jinghong 2, Black walnut, Jingyi 2, Xiaoxiaoqiu, Yangbipao, and Changshan 99-11, 60 pairs of primers were designed to amplify bands, accounting for 100% of the total. However, only 23 pairs of primers amplified bands without primer dimers, accounting for 38.33%. The amplification results of SSR primers within walnut species, black walnut, and hickory showed that these 23 pairs of SSR primers could amplify the target product bands within walnut species, black walnut, and hickory, accounting for 100%. That was to say, these 23 pairs of SSR primers had good generality. However, the polymorphism of Jr18, Jr19, Jr24, Jr25, and Jr51 was poor and would not be considered in subsequent analysis. Therefore, the remaining 18 pairs of SSR primers with good specificity and clear bands were selected for capillary electrophoresis detection. The genetic diversity of walnut germplasm is shown in Table 5. The variation range of Na in experimental samples with 18 pairs of polymorphic primers ranged from 2 to 6, with an average value of 3.556. The variation range of Ne was 1.882~4.741, with an average value of 2.611. The variation range of MAF was 0.250~0.688, with an average value of 0.538. The variation range of Ho ranged from 0 to 0.875, with an average value of 0.167. The variation range of He ranged from 0.469 to 0.789, with an average value of 0.590. The variation range of uHe was 0.500~0.842, with an average value of 0.629. The variation range of PIC was 0.359~0.757, with an average value of 0.526. The variation range of I ranged from 0.662 to 1.646, with an average value of 1.048. The variation range of GD was 0.456~0.789, with an average value of 0.590. The variation range of Fst ranged from −0.043 to 1.000, with an average value of 0.762. However, Jr09, Jr22, and Jr48 amplified more hetero-bands besides the target site, which were not suitable for constructing the fingerprint of 47 walnut resources. In addition, Jr60 was not considered in the construction of 47 walnut resource fingerprints due

to the low MAF value. Other SSR markers had rich polymorphism, and eight experimental samples tested had high heterozygosity, which was more suitable for molecular markers in subsequent walnut populations.

Table 5. Key genetic data of 18 SSR molecular markers in 8 walnut varieties.

Marker	MAF	Na	Ne	Ho	He	uHe	I	GD	PIC	Fst
Jr05	0.500	3 (2,1,1)	2.462	0.000	0.594	0.633	0.974	0.594	0.511	1.000
Jr09	0.625	3 (2,1,1)	2.133	0.000	0.531	0.567	0.900	0.531	0.468	1.000
Jr11	0.625	4 (3,1,1)	2.286	0.000	0.563	0.600	1.074	0.563	0.524	1.000
Jr12	0.625	3 (3,1,1)	2.133	0.000	0.531	0.567	0.900	0.531	0.468	1.000
Jr15	0.625	3 (3,1,1)	2.133	0.000	0.531	0.567	0.900	0.531	0.468	1.000
Jr22	0.375	3 (3,1,1)	2.909	0.000	0.656	0.700	1.082	0.656	0.582	1.000
Jr29	0.438	5 (3,1,2)	3.459	0.875	0.711	0.758	1.401	0.711	0.668	−0.167
Jr35	0.438	6 (2,1,1)	3.879	0.125	0.742	0.792	1.575	0.742	0.713	0.851
Jr40	0.500	4 (3,1,1)	2.909	0.000	0.656	0.700	1.213	0.656	0.605	1.000
Jr44	0.688	3 (3,1,1)	1.910	0.125	0.477	0.508	0.831	0.477	0.427	0.767
Jr45	0.563	5 (4,1,1)	2.723	0.125	0.633	0.675	1.277	0.633	0.599	0.825
Jr48	0.625	2 (2,1,1)	1.882	0.000	0.469	0.500	0.662	0.469	0.359	1.000
Jr50	0.625	3 (2,1,1)	2.133	0.000	0.531	0.567	0.900	0.531	0.468	1.000
Jr52	0.625	2 (2,1,1)	1.882	0.000	0.469	0.500	0.662	0.469	0.359	1.000
Jr53	0.563	3 (3,1,1)	2.415	0.125	0.586	0.625	0.984	0.586	0.520	0.811
Jr55	0.625	2 (2,1,1)	1.882	0.500	0.469	0.500	0.662	0.469	0.359	0.000
Jr56	0.375	4 (3,1,1)	3.122	0.250	0.680	0.725	1.223	0.680	0.618	0.671
Jr60	0.250	6 (4,2,1)	4.741	0.875	0.789	0.842	1.646	0.789	0.757	−0.043
Mean	0.538	3.556	2.611	0.167	0.590	0.629	1.048	0.590	0.526	0.762

Note: The numbers in parentheses represent the number of alleles amplified by the primer in walnut species, black walnut, and hickory.

3.3. Cluster Analysis

Based on the amplification of 18 SSR markers to eight walnut varieties, the genetic distance between eight samples was calculated. The results showed that Jr05 + Jr29 + Jr35 + Jr50 + Jr55 + Jr60 could effectively classify these eight walnut varieties (Figure 1B). The genetic distance between Changshan 99-11 and the other seven samples of walnuts was large, which meant these could be separated. Within the walnut genus, black walnut had a distant genetic relationship with other samples, so it was clustered on different branches from the other six samples. In addition, both Jinghong 1 and Jinghong 2 were descendants of *J. regia* 'Robert Livermore'. Jingyi 2 was a hybrid offspring of *J. regia* and *J. mandshurica*. The above three belonged to the ecological type of Beijing walnut. Baokexiang was the offspring of Xinjiang's early fruiting walnut, but these four samples were all of the northern walnut ecological type. Therefore, Jinghong 1, Jinghong 2, and Jingyi 2 first gathered together and then formed a group with Baokexiang.

3.4. Genetic Diversity

Capillary electrophoresis was performed on 47 walnut resources, and a total of 64 allele loci were obtained from 14 SSR markers (Table 6). The variation range of Na ranged from 2 (Jr52) to 9 (Jr45), with an average of 4.571. The variation range of MAF ranged from 0.468 (Jr15) to 0.947 (Jr52), with an average of 0.687. The variation range of Ho ranged from 0 (Jr05, Jr11, and Jr12) to 0.681 (Jr55), with an average of 0.202. The variation range of He was 0.101 (Jr52) to 0.731 (Jr45), with an average of 0.456. The variation range of uHe ranged from 0.102 (Jr52) to 0.739 (Jr45), with an average of 0.461. Among them, He of all SSR markers was higher than Ho. The variation range of GD ranged from 0.101 (Jr52) to 0.731 (Jr45), with an average value of 0.458. The variation range of I ranged from 0.208 (Jr52) to 1.723 (Jr45), with an average value of 0.897. The variation range of PIC ranged from 0.096 (Jr52) to 0.711 (Jr45), with an average value of 0.422. Among them, there were 2 SSR markers with low polymorphism (<0.25), 6 SSR markers with moderate polymorphism (0.25~0.5), and 6 SSR markers with high polymorphism (>0.5). The variation range of Fst

ranged from −0.312 (Jr53) to 1.000 (Jr05, Jr11, Jr12, and Jr50), with an average value of 0.581. In summary, the 14 selected SSR markers exhibited rich polymorphism, and the tested walnut resources exhibited high heterozygosity and rich genetic diversity.

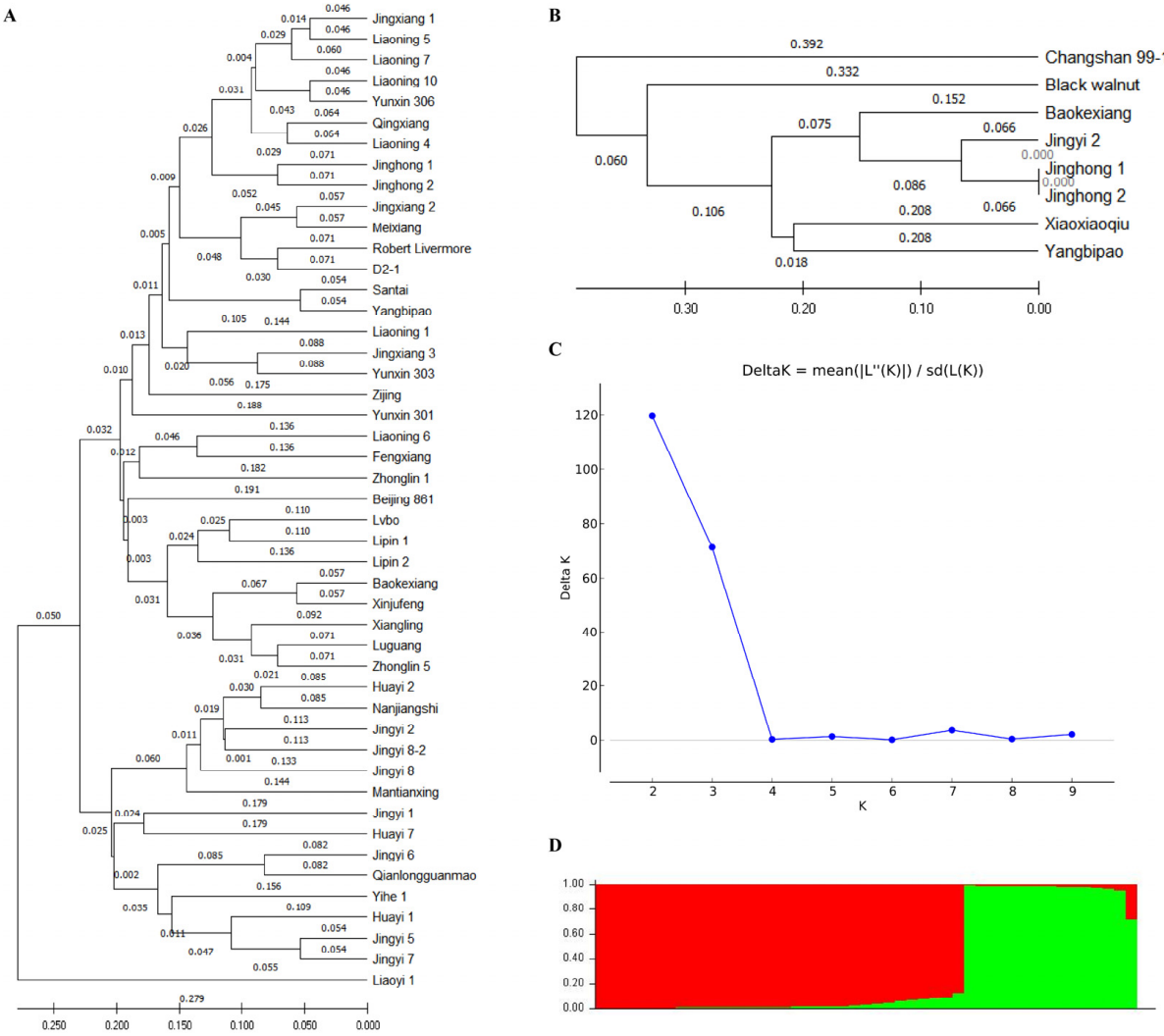

Figure 1. Phylogenetic tree and population structure of the test walnut. Note: (**A**) Phylogenetic trees of 47 walnuts; (**B**) Phylogenetic tree of eight different types of walnuts; (**C**) The relationship between ΔK and K-values; (**D**) Population structure of 47 walnut samples tested.

Table 6. Genetic data of 14 SSR molecular markers in 47 walnuts.

Marker	MAF	Na	Ne	Ho	He	uHe	I	GD	PIC	Fst	PI	PISibs
Jr05	0.809	3.000	1.476	0.000	0.322	0.326	0.590	0.322	0.291	1.000	0.491	0.712
Jr11	0.596	5.000	2.363	0.000	0.577	0.583	1.103	0.577	0.527	1.000	0.229	0.519
Jr12	0.787	4.000	1.552	0.000	0.356	0.360	0.688	0.356	0.325	1.000	0.445	0.683
Jr15	0.468	4.000	2.834	0.043	0.647	0.654	1.161	0.647	0.583	0.934	0.189	0.474
Jr29	0.574	7.000	2.499	0.362	0.600	0.606	1.187	0.600	0.551	0.397	0.209	0.502
Jr35	0.564	5.000	2.524	0.362	0.604	0.610	1.130	0.620	0.578	0.388	0.202	0.499
Jr40	0.500	8.000	3.299	0.468	0.697	0.704	1.557	0.697	0.669	0.328	0.120	0.432
Jr44	0.702	4.000	1.908	0.277	0.476	0.481	0.929	0.476	0.445	0.419	0.306	0.588
Jr45	0.479	9.000	3.713	0.234	0.731	0.739	1.723	0.731	0.711	0.680	0.092	0.408
Jr50	0.830	3.000	1.406	0.000	0.289	0.292	0.520	0.289	0.258	1.000	0.537	0.740
Jr52	0.947	2.000	1.112	0.021	0.101	0.102	0.208	0.101	0.096	0.789	0.814	0.903
Jr53	0.809	3.000	1.457	0.298	0.313	0.317	0.529	0.313	0.271	0.050	0.514	0.722

Table 6. *Cont.*

Marker	MAF	Na	Ne	Ho	He	uHe	I	GD	PIC	Fst	PI	PISibs
Jr55	0.638	3.000	2.079	0.681	0.519	0.525	0.882	0.519	0.457	−0.312	0.293	0.564
Jr56	0.915	4.000	1.189	0.085	0.159	0.160	0.354	0.159	0.151	0.464	0.715	0.849
Mean	0.687	4.571	2.101	0.202	0.456	0.461	0.897	0.458	0.422	0.581	0.368	0.614
Combined	-	-	-	-	-	-	-	-	-	-	6.4×10^{-8}	7.2×10^{-4}

Note: - indicate that there is no such value.

3.5. Genetic Relationships and Clustering Characteristics of 47 Walnut Resources

To understand the genetic relationships between members of the walnut population, cluster analysis was conducted on 47 walnut resources based on their genetic similarity coefficients (Figure 1A). The first branch could be further divided into two sub-branches. The first sub-branch of branch 1 consisted of ordinary walnuts, iron walnuts, and their hybrid offspring, including Jingxiang 1, Liaoning 5, Liaoni ng 7, Liaoning 10, Yunxin 306, Qingxiang, Liaoning 4, Jinghong 1, Jinghong 2, Jingxiang 2, Meixiang, Robert Livermore, D2-1, Santai, Yangbipao, Liaoning 1, Jingxiang 3, Yunxin 303, Zijing, Yunxin 301, Liaoning 6, Fengxiang, Zhonglin 1, Beijing 861, Lvbo, Lipin 1, Lipin 2, Baokexiang, Xinjufeng, Xiangling, Luguang, and Zhonglin 5. The second sub-branch of branch 1 was composed of *J. hopeiensis*, including Huayi 2, Nanjiangshi, Jingyi 2, Jingyi 8-2, Jingyi 8, Mantianxing, Jingyi 1, Huayi 7, Jingyi 6, Qianlongguanmao, Yihe 1, Huayi 1, Jingyi 5, and Jingyi 7. The second branch only had one member, Liaoyi 1, which was the actual offspring of *J. cordiformis* in the *J. mandshurica* group.

3.6. Population Genetic Structure Analysis and Principal Coordinate Analysis of 47 Walnut Cultivars

To further evaluate the genetic characteristics of these 47 walnut varieties, population genetic structure analysis and principal coordinate analysis were conducted for walnut populations (Figure 1C,D and Figure 2). When K = 2, ΔK had a maximum value, indicating that 47 walnut resources could be best divided into two groups (Table S3). Group I consisted of 32 varieties, including Santai, Yangbipao, Yunxin 301, Yunxin 303, Yunxin 306, Liaoning 1, Liaoning 4, Liaoning 5, Liaoning 6, Liaoning 7, Liaoning 10, Jingxiang 1, Jingxiang 2, Jingxiang 3, Baokexiang, Beijing 861, Lvbo, Fengxiang, Qingxiang, Meixiang, Luguang, Xinjufeng, Xiangling, Lipin 1, Lipin 2, Zhonglin 1, Zhonglin 5, Zijing, Robert Livermore, Jinghong 1, Jinghong 2, and D2-1. Among them, the Qi values of all varieties in group I were greater than 0.8. In addition, group I included two iron walnuts, three hybrid offspring of ordinary walnuts and iron walnuts, and 20 ordinary walnuts. Group II consisted of 15 varieties, namely Liaoyi 1, Jingyi 1, Jingyi 2, Jingyi 5, Jingyi 6, Jingyi 7, Jingyi 8, Jingyi 8-2, Huayi 1, Huayi 2, Huayi 7, Yihe 1, Qianlongguanmao, Nanjiangshi, and Mantianxing. Among them, the Qi value of 14 varieties in group II was greater than 0.8, while the Qi value of Liaoyi 1 was less than 0.8, which was 0.712. In addition, group II was composed of both *J. hopeiensis* and *J. mandshurica*. The genetic sources within each group were single, and the similarity between varieties was high. Therefore, Group I and II were divided into ordinary walnuts and *J. hopeiensis*, respectively.

PCoA analysis (Figure 2) showed that 47 walnut resources could be roughly divided into two groups. Group I was mainly composed of ordinary walnuts and iron waluts, including Santai, Yangbipao, Yunxin 301, Yunxin 303, Yunxin 306, Liaoning 1, Liaoning 4, Liaoning 5, Liaoning 6, Liaoning 7, Liaoning 10, Jingxiang 1, Jingxiang 2, Jingxiang 3, Baokexiang, Beijing 861, Lvbo, Fengxiang, Qingxiang, Meixiang, Luguang, Xinjufeng, Xiangling, Lipin 1, Lipin 2, Zhonglin 1, Zhonglin 5, Zijing, Robert Livermore, Jinghong 1, Jinghong 2, and D2-1. Group II was mainly composed of *J. hopeiensis*, including Jingyi 1, Jingyi 2, Jingyi 5, Jingyi 6, Jingyi 7, Jingyi 8, Jingyi 8-2, Huayi 1, Huayi 2, Huayi 7, Yihe 1, Qianlongguanmao, Nanjiangshi, and Mantianxing, all of which were *J. hopeiensis*. However, Liaoyi 1 had a certain distance from both Group I and Group II, which was consistent

with the results of the cluster analysis. Therefore, Group I and Group II were divided into ordinary walnuts and *J. hopeiensis*, respectively.

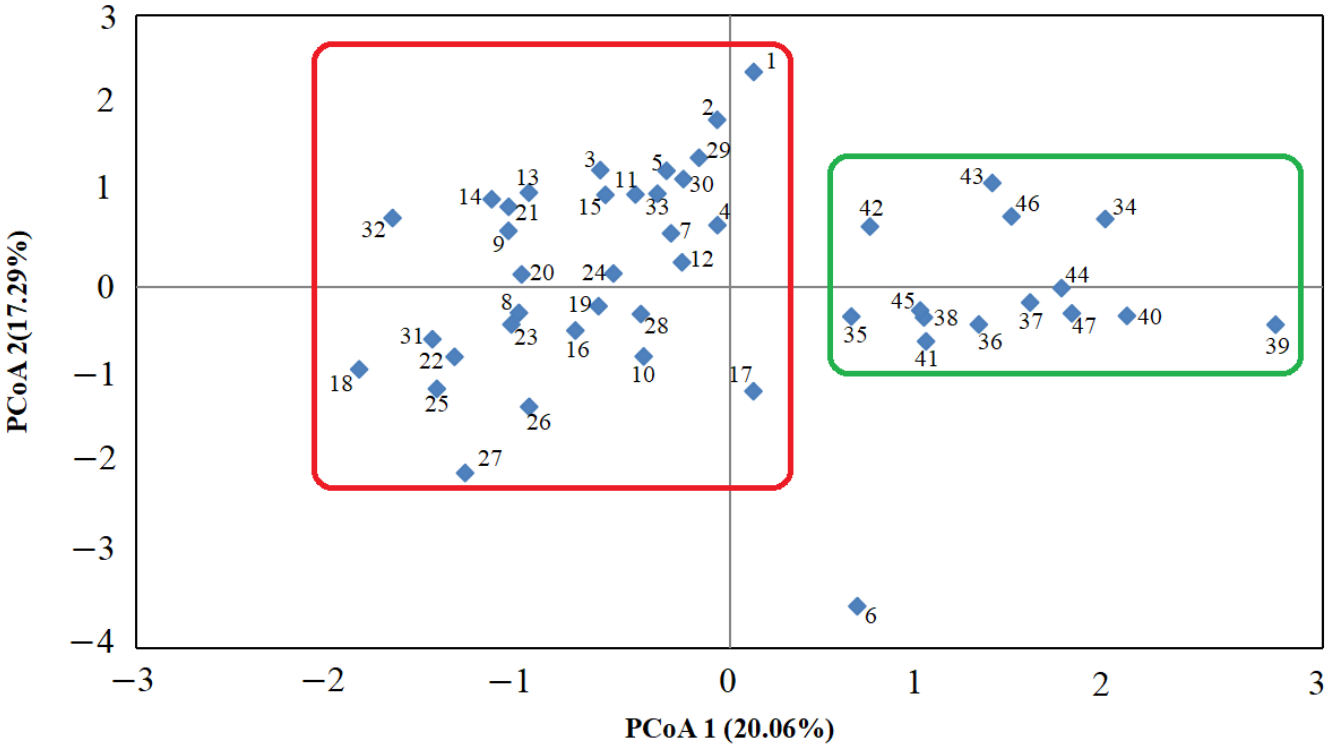

Figure 2. Principal coordinate analysis of walnut cultivars.

3.7. The Identification Ability of SSR Markers and the Construction of DNA Fingerprints

To evaluate the fingerprint recognition ability of these 14 SSR markers, PI and Pisibs were calculated separately (Table 6). The PI variation range of each molecular marker was 0.092 (Jr45) to 0.814 (Jr52), with an average value of 0.368. Assuming that all marker loci were independently separated, the probability of two random individuals having identical multi-locus genotypes among 14 SSR markers was estimated to be 6.4×10^{-8}. Usually, Pisibs was defined as the upper limit of PI, with a variation range of 0.408 (Jr45) to 0.903 (Jr53) for the 14 SSR markers, with an average value of 0.614. In addition, the combined Pisibs was 7.2×10^{-4}.

Based on the data of gene locus combinations, the recognition ability of 14 random combinations of SSR markers was calculated (Table 6) to evaluate the number of markers required to fully distinguish 47 walnut varieties. The SSR markers with strong identification ability included Jr29, Jr40, Jr45, and Jr56, which could identify five, five, five, and three different varieties, respectively (Table 7). Jr29 could identify Yunxin 301, Fengxiang, Baokexiang, Liaoyi 1, and Xinjufeng. Jr40 could identify Liaoyi 1, Liaoning 1, Huayi 1, Robert Livermore, and Zijing. Jr45 could identify Robert Livermore, Jingyi 6, Jingyi 8, Huayi 7, and Mantianxing. Jr56 could distinguish between Jingyi 5, Huayi 7, and Mantianxing. Furthermore, Jr11, Jr12, Jr35, Jr44, Jr50, Jr52, Jr53, and Jr55 could only identify one variety, namely Yunxin 301, Lipin 1, Beijing 861, Liaoning 6, Yihe 1, Yunxin 306, Yunxin 303, and Mantianxing, respectively. However, Jr05 and Jr15 could not independently identify any walnut varieties.

Table 7. Identification ability of 14 SSR markers.

Primer	Total Numbers	Cultivars
Jr05	0	None
Jr11	1	Yunxin 301
Jr12	1	Lipin 1
Jr15	0	None
Jr29	5	Yunxin 301, Fengxiang, Baokexiang, Liaoyi 1, Xinjufeng
Jr35	1	Beijing 861
Jr40	5	Liaoyi 1, Liaoning 1, Robert Livermore, Zijing, Huayi 1
Jr44	1	Liaoning 6
Jr45	5	Robert Livermore, Jingyi 6, Jingyi 8, Huayi 7, Mantianxing
Jr50	1	Yihe 1
Jr52	1	Yunxin 306
Jr53	1	Yunxin 303
Jr55	1	Mantianxing
Jr56	3	Jingyi 5, Huayi 7, Mantianxing

When five SSR markers were combined, the PI value approached 0, and 47 walnut varieties could be independently identified (Figure 3, Table S4). Based on the principle of core marker selection and genotype data results, five SSR markers were selected as core markers. Due to its high Na, Ne, He, GD, I, and PIC values, as well as lower PI and Pisibs values, Jr45 was selected as one of the core markers. This marker could separate five varieties independently from all varieties. When the second SSR marker (Jr40) was added, 24 out of 47 varieties could be identified, accounting for 51.06%. When the third SSR marker (Jr29) was added, 32 varieties could be identified, accounting for 68.09%. When the fourth SSR marker (Jr35) was added, 43 varieties could be identified, accounting for 91.49%. When adding the fifth SSR marker (Jr11), 47 walnut varieties could be completely distinguished.

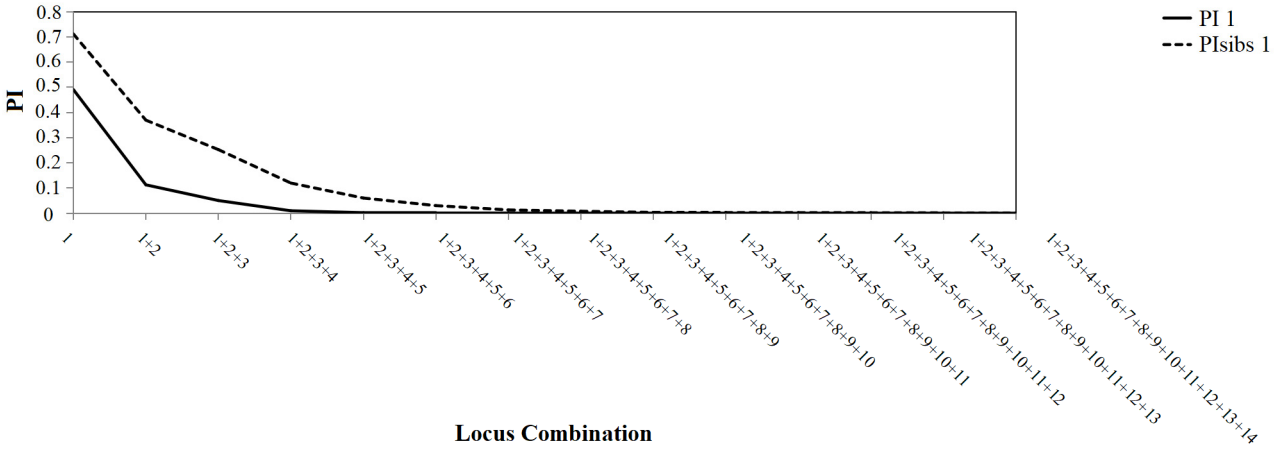

Figure 3. Evaluation of the fingerprinting power of 14 SSR markers in walnut cultivars.

The allele sizes and frequencies of the five core markers Jr45, Jr40, Jr29, Jr35, and Jr11 are shown in Figure 4. Jr45 had nine alleles, with the highest allele frequency of 200 bp (0.479) and the lowest allele frequency of 272 bp (0.021). Jr40 had eight alleles, with the highest frequency of 138 bp (0.500) and the lowest frequency of 146, 154, and 158 bp (0.032). Jr29 had seven alleles, with the highest frequency of 195 bp (0.574), and the lowest frequency of 204 and 228 bp (0.011). Jr35 had four alleles, with the highest allele frequency of 136 bp (0.174) and the lowest allele frequency of 127 bp (0.087). Jr11 had five alleles, with the highest allele frequency of 155 bp (0.596) and the lowest allele frequency of 147 bp (0.021). The amplified alleles of 47 walnut varieties were arranged in the order of core markers Jr45, Jr40, Jr29, Jr35, and Jr11 to obtain a unique genotype. The DNA fingerprint data of 47 walnut genetic resources were visualized in the form of heat maps (Figure 5).

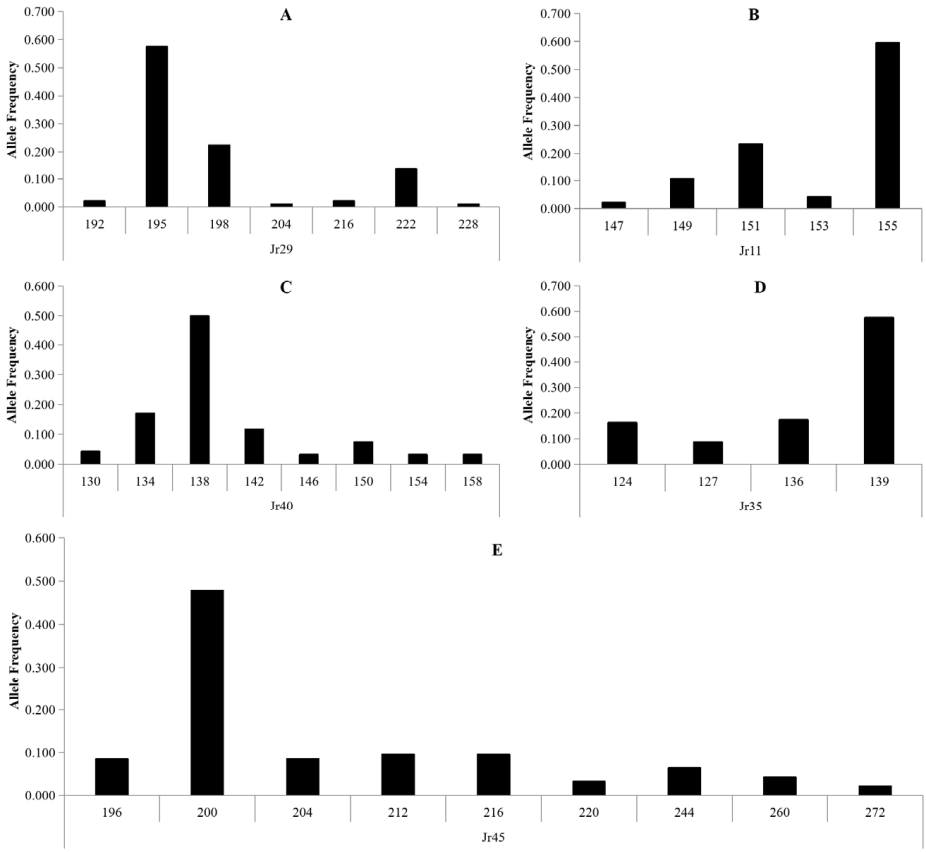

Figure 4. Allelic frequency of 5 core SSR primers. Note: (**A**–**E**) represent the allele frequencies of SSR markers, Jr29, Jr11, Jr40, Jr35, and Jr45, respectively.

3.8. Correlation Analysis between SSR Markers and Phenotypic Traits of Walnuts

Based on the correlation analysis of 14 SSR markers with 14 morphological traits of walnuts (Table 8 and Table S5), four SSR markers (Jr35, Jr50, Jr52, and Jr53) were significantly associated with phenotypic traits of walnuts ($p < 0.05$). Jr35 was only significantly associated with dry weight of nuts. Jr50 was significantly associated with green fruit weight, fresh fruit weight, fresh weight of seed kernel, fresh weight of seed meat, and nut kernel rate. Jr52 was significantly associated with green skin rate and fruiting rate ($p < 0.05$). Jr53 was significantly correlated with fresh weight of seed kernel, fresh fruit kernel rate, fresh weight of seed meat, dry weight of seed kernel, dry weight of seed meat, dry weight of nut, and nut kernel rate ($p < 0.01$), and significantly correlated with green fruit weight, fresh fruit weight, green skin weight, green skin rate, fruiting rate, fresh weight of seed coat, and dry weight of seed coat ($p < 0.05$).

There were significant differences in the dry weight of nuts among different genotypes of nuts in Jr35, with AB (124, 136) > AC (124, 139) > CD (127, 139) > CC (139, 139) > AA (124, 124) > BB (136, 136). There were significant differences in green fruit weight, fresh fruit weight, fresh weight of seed kernel, fresh weight of seed meat, and nut kernel rate among different genotypes of Jr50, with AA (170, 170) > BB (165, 165). There were significant differences in the green skin rate and fruiting rate among different genotypes of Jr52, with AB (130, 140) > BB (130, 130) > AA (140, 140), AA (140, 140) > BB (130, 130) > AB (130, 140), respectively. There were significant differences in the green fruit weight, fresh fruit weight, green skin weight, green skin rate, fruiting rate, fresh weight of seed kernel, fresh seed kernel rate, fresh weight of seed meat, fresh weight of seed coat, dry weight of seed kernel, dry weight of seed meat, dry weight of seed coat, dry weight of nut, and nut kernel rate among different genotypes of Jr53, with AA (140, 140) > BB (175, 175).

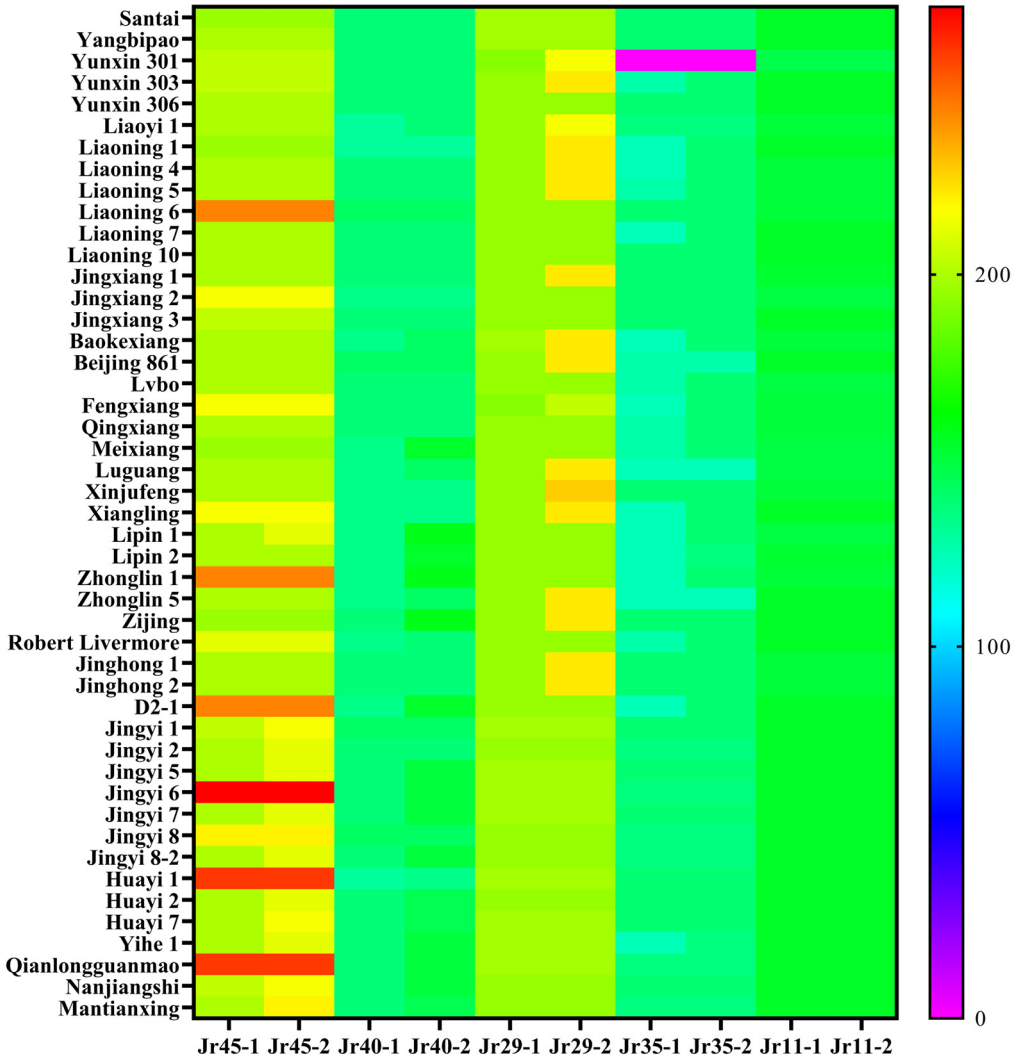

Figure 5. The fingerprint of 47 cultivars constructed by three core primer pairs. Note: Each color represents an allele site. In addition, the horizontal axis is the core SSR marker, and the vertical axis is the name of the walnut variety.

Table 8. Correlation analysis between 14 SSR markers and walnut traits.

Primer	Jr05	Jr11	Jr12	Jr15	Jr29	Jr35	Jr40	Jr44	Jr45	Jr50	Jr52	Jr53	Jr55	Jr56
Green fruit weight	0.057	0.052	0.126	0.178	0.02	0.014	0.021	0.114	0.075	0.529 *	0.272	0.581 **	-	-
Fresh fruit weight	0.039	0.117	0.247	0.132	0.149	0.222	0.061	0.123	0.072	0.557 *	0.165	0.623 **	-	-
Green skin weight	0.095	0.127	0.045	0.302	0.101	0.129	0.059	0.212	0.064	0.427	0.444	0.463 *	-	-
Green skin rate	0.035	0.164	0.197	0.284	0.197	0.38	0.094	0.305	0.007	0.311	0.486 *	0.468 *	-	-
Fruiting rate	0.035	0.164	0.197	0.284	0.197	0.38	0.094	0.305	0.007	0.311	0.486 *	0.468 *	-	-
Fresh weight of seed kernel	0.045	0.136	0.162	0.193	0.153	0.213	0.002	0.103	0.02	0.473 *	0.247	0.626 **	-	-
Fresh fruit kernel rate	0.363	0.043	0.091	0.092	0.025	0.236	0.406	0.098	0.036	0.139	0.195	0.663 **	-	-
Fresh weight of seed meat	0.036	0.156	0.147	0.184	0.136	0.217	0.021	0.102	0.012	0.477 *	0.232	0.625 **	-	-
Fresh weight of seed coat	0.025	0.014	0.266	0.158	0.22	0.096	0.008	0.061	0.042	0.417	0.206	0.484 *	-	-
Fresh seed coat and kernel rate	0.085	0.143	0.352	0.069	0.207	0.001	0.203	0.102	0.091	0.31	0.014	0.392	-	-

Table 8. *Cont.*

Primer	Jr05	Jr11	Jr12	Jr15	Jr29	Jr35	Jr40	Jr44	Jr45	Jr50	Jr52	Jr53	Jr55	Jr56
Dry weight of seed kernel	0.038	0.175	0.123	0.216	0.102	0.286	0.006	0.107	0.087	0.437	0.282	0.663 **	-	-
Dry weight of seed meat	0.048	0.171	0.109	0.193	0.079	0.288	0.014	0.104	0.08	0.451	0.255	0.661 **	-	-
Dry weight of seed coat	0.04	0.156	0.197	0.326	0.233	0.201	0.049	0.102	0.108	0.229	0.399	0.511 *	-	-
Nut coat and kernel rate	0.076	0.025	0.222	0.238	0.318	0.165	0.2	0.036	0.123	0.062	0.332	0.203	-	-
Nut dry weight	0.353	0.051	0.087	0.151	0.06	0.557 *	0.228	0.06	0.004	0.501	0.221	0.795 **	-	-
Nut kernel rate	0.49	0.193	0.104	0.222	0.061	0.444	0.245	0.074	0.158	0.670 *	0.108	0.830 **	-	-

Note: ** and * represent significant correlations at the 0.01 and 0.05 levels, respectively, and - indicate that there were fewer constants that could not be calculated.

4. Discussion

4.1. Development of SSR Molecular Markers

SSR molecular markers have good genome coverage, high polymorphism, codominance, repeatability, and other characteristics, so they are widely used in the detection of genetic diversity, genotyping, genetic linkage maps, population kinship, and evolutionary analysis [8,15,21,35]. This study provides a basis for the identification and protection of walnut germplasm by developing high-quality SSR markers, which has also been confirmed by previous studies [15,36,37]. Based on the results of genome re-sequencing of Baokexiang, the number of SSR loci with dinucleotide repeats was the highest in this study (Table 4), which was consistent with the research results of Ikhsan et al. [38]. However, SSR loci screened based on transcriptome and chloroplast genome sequencing have mainly been shown to be single nucleotide repeats [24,25,39,40]. Compared to others, SSR markers developed based on transcriptome have greater advantages in locating specific genes. The chloroplast genome has the characteristics of small genome size, low mutation and recombination rates. Therefore, in the future breeding of walnut germplasm, the advantages of various methods can be combined to select the optimal method.

In this study (Table S2), the AA/TT base type was the most abundant, which differed from Itoo et al.'s and Feng et al.'s results. Itoo et al. and Feng et al. pointed out that A/T and AT/AT base types were the most abundant in walnut SSR markers [15,25]. This may be because there were some differences in the screening of SSR markers based on genome and transcriptome sequencing, and it might also be related to the differences between walnut varieties. Among the 60 pairs of primers, 23 pairs obtained specific amplified bands, with 18 pairs (72%) of polymorphic primers, which was higher than the research results of Qi et al. [22] and significantly lower than the research results of Guney et al. [41]. This may be related to the number of experimental samples and the variety.

4.2. Genetic Diversity of Walnut Resources

14 high-quality SSR markers were developed, and 89 alleles were obtained from 47 walnut resources (Tables 5 and 6). Each SSR marker had an average of 4.571 alleles, with a higher number of loci than Shavvon et al. [18], Jin et al. [27], and Wambulwa et al. [6], but lower than the number of SSR marker loci in walnut resources reported by Itoo et al. [15], Cseke et al. [36], and Eser et al. [42]. The main reason was that the distribution range of PIC values between SSR markers was relatively large, with Jr52 only containing two alleles. The He value indicated these 47 walnut resources had rich genetic diversity. The Ho value of this walnut population indicated that the walnut population was under a certain degree of selection pressure. The He value of 14 pairs of SSR markers was higher than the Ho value in 47 walnut resources, which was consistent with the research results of Wang et al. in Shandong walnut resources [21] and Davoodi et al. in Iranian walnut resources [10]. This might be related to genetic variation in walnut germplasm. Meanwhile, the I value

of 47 walnut resources indicated that the walnut population had rich genetic diversity. In addition, there were SSR markers with PIC values ranging from 0.25 to 0.50, indicating moderate polymorphism. The PIC values of SSR markers were greater than 0.50, indicating high polymorphism (Table 6). 12 out of the 14 pairs of SSR markers could accurately evaluate the genetic characteristics of 47 walnut resources, such as genetic relationships and population structure. However, Jr52 and Jr56 are not recommended for subsequent research on genetic diversity analysis and molecular fingerprint construction of walnut resources.

4.3. Cluster Characteristics, Population Structure, and Principal Coordinate Analysis of Walnut Resources

At present, phylogenetic trees, population structure analysis, and principal coordinate analysis are the main research methods used to evaluate the population structure and phylogenetic relationships of plant resources [36,41,43,44]. To evaluate the evolutionary relationship of walnut resources, cluster analysis, population genetic structure, and principal coordinate analysis were used to analyze 47 walnut resources. The results indicated that these three methods had certain differences in the classification results of 47 walnut resources. The results of cluster analysis (Figure 1A) indicated that all of the members of the first sub-branch of branch 1 were ordinary walnuts, but this sub-branch also included two iron walnuts and three hybrid offspring of iron walnuts and ordinary walnuts. In addition, Santai and Yangbipao were separately gathered together, while Yunxin 301, Yunxin 303, and Yunxin 306 were distributed in a dispersed pattern. This indicated that although there was a certain degree of gene exchange between iron walnuts and ordinary walnuts, there was still a certain genetic distance between them. There has been controversy over whether ordinary walnuts and iron walnuts belong to different taxa [39]. The clustering analysis of walnut populations by Yuan et al. showed a significant overlap between ordinary walnuts and iron walnuts [45], which is consistent with the results of this study. In addition, Jinghong 1 and Jinghong 2 were clustered together, Robert Livermore and D2-1 were clustered together, and D2-1 was also a red seed kernel walnut, which further indicated that the genetic distance between these two walnuts was relatively close. The second sub-branch under branch 1 was *J. hopeiensis*, which had a certain genetic distance from ordinary walnuts. Branch 2 only had Liaoyi 1, which was far from ordinary walnuts and *J. hopeiensis*. Liaoyi 1 was the actual offspring of heart-shaped walnuts, while heart-shaped walnut was a variety in the *J. mandshurica* group, which is in line with the current consensus in the academic community [46].

The results of the population genetic structure analysis (Figure 1C,D, Table S3) showed that Group I was divided into ordinary walnuts, and Group II was divided into *J. hopeiensis*. Santai and Yangbipao were divided into the ordinary walnuts group, which was consistent with the results in the phylogenetic tree. This further explained that the genetic distance between iron walnuts and ordinary walnuts was closer, and a certain degree of gene exchange had occurred. Yunxin 301, Yunxin 303, and Yunxin 306 were all divided into the ordinary walnuts group, indicating that the genetic relationship between Yunxin series walnuts and ordinary walnuts was closer, consistent with the clustering results in the phylogenetic tree. In addition, Liaoyi 1 was divided into the J. *hopeiensis* group and belonged to a variety of *J. mandshurica*. *J. mandshurica* is a hybrid of *J. regia* and *J. mandshurica* [39], indicating a closer genetic relationship between Liaoyi 1 and *J. mandshurica*. At the same time, the results of the PCoA analysis (Figure 3) were consistent with those of the cluster analysis (Figure 1A), but there were certain differences between them and the results of the population structure analysis (Figure 1B,C). Santai, Yangbipao, and Yunxin series walnut were divided into the ordinary walnuts group, further confirming the closer evolutionary relationship between iron walnuts and ordinary walnuts, which is consistent with the research results of Yuan et al. [45] and Hu et al. [39]. In addition, PCoA analysis showed that there were differences between the classification of Liaoyi 1 and the results of the population genetic structure analysis. Therefore, the position of Liaoyi 1 in taxonomy and its evolutionary relationship still needs to be studied.

4.4. Identification Ability of Five Core Markers of Walnut Varieties

The evaluation of the discriminative ability of SSR markers using PI and Pisibs values has been applied to chestnuts [17]. After genotyping 47 walnut resources, the identification ability of 14 SSR markers for walnut resources was evaluated by calculating PI and Pisibs values. This research conducted multi-point matching calculations on 14 SSR markers, with PI and Pisibs values of 6.4×10^{-8} and 7.2×10^{-4}, respectively (Table 6). Waits et al. believe that PI values are often over-estimated, and therefore recommend using Pisibs as the upper limit for PI [47]. When the PI value ranged from 1×10^{-4} to 1×10^{-2}, the majority of natural individuals that appeared randomly could be identified. The PI and Pisibs values in this study were significantly lower than the speculated values of Waits et al., indicating that the recognition potential of these 14 SSR markers was enormous. The analysis of 14 SSR markers indicated that the identification abilities of Jr29, Jr40, Jr45, and Jr56 were strong, and they were also suitable for constructing molecular maps of expanded experimental samples.

Based on the PIC, PI, and Pisibs equivalence of SSR markers, at least five core SSR markers were required to fully match the unique fingerprint information of 47 walnut resources (Table S4, Figure 5). The use of molecular markers to construct fingerprint maps is of great significance for more efficient and rapid identification of plant resources [48]. Nie et al. constructed a fingerprint of 146 chestnut resources using 18 SSR markers [17]. Meng et al. constructed a fingerprint of 203 sweet potato resources using four genomic SSR markers and three SSR markers [49]. In the research on the construction of walnut resource fingerprint maps, most studies describe them in numerical form [10,50], and there is still a lack of research on visualizing fingerprint maps. Therefore, this study constructed 47 DNA molecular identification cards for walnut resources using five core SSR marker (Jr45, Jr40, Jr29, Jr35, and Jr11) combinations and visualized the gene site combinations of the five core markers in the form of heat maps. This has a positive impact on the identification of walnut resources and has important guiding significance for the subsequent identification of walnut progeny and new varieties. However, as the number of walnut varieties continues to increase, the above SSR markers may not be able to fully distinguish a large number of walnut varieties. Therefore, it will be necessary to continue developing SSR markers with stronger identification ability and richer genetic diversity in the future.

4.5. Significant Correlation between SSR Markers and Dry Weight of Walnut Nuts

In this study (Table 8), Jr35 was significantly correlated with the dry weight of walnut nuts ($p < 0.05$). At the same time, the dry weight of nuts of AB (124, 136) was the largest, followed by AC (124, 139). The dry weight of nuts of CC (139, 139), AA (124, 124), and BB (136, 136) was lower than that of AB and AC, indicating that heterozygote had significant advantages over homozygote in dry weight of walnut nuts. Although Jr53 was significantly correlated with the dry weight of walnut nuts ($p < 0.01$), its PIC value was too low, with only two genotypes. In addition, BB (175, 175) had only one sample, indicating relatively poor reliability. Previous studies have shown a high correlation between the dry weight of walnut nuts and the diameter and kernel weight of nuts [44,51]. However, there is a certain gap in research on the correlation between genotypes and walnut traits. The kernels of 62 genotypes of Persian walnuts were lighter in color, while 30 genotypes were easily separated from the shell, which was of positive significance for screening high-quality walnuts [52]. However, Buijdoso et al. believed that there was no clear evidence to suggest a clear connection between nut traits and the Hungarian walnut genotypes [53]. We believe that Jr35 can be used as a candidate SSR marker for screening walnut resources with a higher dry weight of walnut nuts. However, the relationship between Jr35 and the dry weight of walnut nuts still needs to be further confirmed in a large number of walnut resources.

In summary, this study enriches the SSR fingerprint of walnut germplasm, which is of great significance for future walnut breeding work and lays a theoretical foundation for the identification, protection, and specific germplasm innovation of excellent walnut germplasm. In the future, with the development of methods such as whole walnut genome

sequencing, microsatellite mapping, transcriptome sequencing, and chloroplast genome sequencing, research on SSR technology in the field of walnut genetic diversity will become more in-depth.

5. Conclusions

Walnut is an important woody oil-bearing tree species in China, with enormous development potential. The characteristics of long childhood and wind pollination pose significant challenges for the identification and screening of excellent germplasm. With the advancement of genome sequencing and other technologies, there has been some progress in the development of genetic markers such as SSR. This study developed 14 highly polymorphic SSR markers from 60 molecular markers and used these markers to fingerprint and analyze the genetic diversity of 47 walnut resources. A total of 64 allele loci were detected. Jr45, Jr40, Jr29, Jr35, and Jr11 were identified as five core SSR markers. Jr35 may be used as a candidate SSR marker for screening walnut resources with higher dry weight of walnut nuts. These SSRs will be helpful for future walnut germplasm innovation and the identification of new varieties. This study has an important impact on the protection of scarce walnut germplasm.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15030405/s1. Table S1. The information of 60 SSR markers; Table S2. Frequency of identified SSR motifs; Table S3. Probabilities (Qi) of 47 walnut cultivars belonging to their subgroup; Table S4. The identification of cultivars through gradually increasing SSR primers; Table S5. Morphological characteristics of walnut germplasm.

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