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Genetic Diversity and Relationship Analysis of 21 Walnut Varieties Based on SSR Markers

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Abstract In order to grasp the germplasm resources of walnut in Yunnan province, the genetic diversity and relationship of 21 walnuts varieties commonly planted in Yunnan province were analyzed using SSR fluorescent labeling technology. The results showed that the average number of allelic loci of 12 pairs of primers used in 21 walnut species samples was 7.916 7. The mean number of alleles, Shannon Wiener index (I) and polymorphic information content (PIC) per locus were 4.3516, 1.643 6 and 0.705 5. Twelve pairs of polymorphism information content (PIC) were higher than 0.25, indicating that the 12 pairs of primers had good polymorphism in the samples. In the phylogenetic analysis, the 21 walnut varieties were divided into 7 subgroups, indicating that the 21 walnut varieties had rich genetic diversity, extensive gene exchange and complex genetic background in the breeding process.

Keywords Walnut; SSR; Genetic analysis; Clustering analysis

Walnut is a perennial woody nut and oil-bearing species of the walnut genus Juglandaceae (Xi et al., 1996). Walnut is a tree with important economic value, widely distributed more than 40 countries or regions in Asia, Europe, America, Africa; Walnut planting in China has a long history (Yang, 1984), and is generally considered as the origin and distribution center of the genus Walnut (Xi, 1987). Among the more than ten species of walnut genera in the world with cultivation value, 5 species (*J. regia* L., *J. sigillata* Dode., *Mandshuria Maxim*., *J. Hopenensis* Hu. and *J. Nigra* L.) were originally produced in China and widely cultivated (Wu et al., 2009). Meanwhile, walnut germplasm resources for selection and breeding in China are extremely rich. There are two kinds of walnut, which are widely cultivated as fruit trees in China. The genus Walnut is distributed in 25 provinces (regions) of China. Chinese walnut production amounted to 2019 1 586 357 t, accounted for 43.31% of world output (https://kns.cnki.net/kcms/detail/11.2864.TS.20200904.1717.006.html). Yunnan province is a major producing area of walnuts in China, with its output and output ranking among the top in the world. In 2018, the walnut output of Yunnan province reached 1.16×10^6 T, with a comprehensive output of 31.8 billion yuan, ranking first in the country in terms of area, output and comprehensive output (Li et al., 2020). ' Yangbi ' walnut and ' Santai ' walnut were the main cultivated in Yunnan province, which had the advantages of high yield, high quality, aesthetic appearance, sweet and delicious taste, easy processing and strong resistance (Geng et al., 2019). The cultivation of walnut varieties in Yunnan province played an important role in the development of walnut industry. Walnut planting area in Yunnan province is extensive and variety resources are abundant, which provides sufficient materials for further breeding walnut varieties.

In a narrow sense, genetic diversity refers to the sum of genetic variation of different individuals within a species or within a species population (Shen, 2001). Genetic diversity is the result of organisms' long-term adaptation to environmental. Genetic variation provides an important basis for species' sustainable survival and development in the face of habitat changes. The evolutionary potential, economic and ecological value of a species also depend on its

genetic diversity (Wang and Ho, 1996). The study of genetic diversity of species can help us to better understand the genetic relationship and genealogical relationship of various varieties of this species, and is also an important basis for exploring the excellent traits of this species (Shi Liming, 1990, Life Sciences, 4: 158-164). DNA molecular marker technology based on DNA polymorphism is one of the important means to measure genetic diversity and can visualize the degree of genetic variation at the DNA level (Zhou et al., 2018). Among them, simple Sequence repeat (SSR) is a widely used technique.

SSR technology has the advantages of simple operation, high polymorphism, wide coverage, codominance and high repeatability, as well as high universality among related species, which can well detect the genetic diversity among similar species or different populations of the same species (Gupta and Varshney, 2000; Yuan et al., 2000). There have been studies on the genetic analysis of walnut varieties through SSR technology. Freeman et al. (2004) used SSR technology to analyze the differentiation relationship of walnut varieties in different regions and the genetic pedigree of the population. Jia et al. (2019) successfully designed 5547 pairs of SSR primers with good polymorphism and multiple duplications through genetic studies and transcriptional data set analysis of leptopecan. Xiao et al. (2014) established the optimal PCR reaction system by selecting 12 pairs of primers from 27 pairs of primers in the experiment of 18 walnut varieties and amplifying 9.8 exit points per primer. Chen et al. (2019) studied the genetic diversity and distribution characteristics of 153 yunnan walnut populations at 3 elevations in Jinsha River basin using SSR technology. Christopoulas et al. (2010) carried out genetic similarity analysis on 56 walnut varieties all over the world, while Chen et al. (2014) carried out DNA typing analysis on 35 major walnut varieties in northern China. Wang et al. (2005) analyzed the genetic diversity of 8 natural populations of Walnut in China. Zhou et al. (2018) detected a total of 59 alleles in 11 sichuan walnut varieties with 11 pairs of fluorescent primers, with an average effective allele of 3.604 per pair of primers. Bao et al. (2018) used 25 pairs of SSR primers to conduct experiments on 21 natural populations of Tibet Walnut. The average observed allele and the average effective allele of each pair of primers were 8.6 and 3.6 respectively. On the basis of previous studies, this study intends to conduct genetic analysis and genetic relationship research on 21 new walnut varieties that have not been studied by SSR technology, in order to further understand the germplasm resources and clustering relationship of walnut, and provide basic data for further research on walnut biodiversity and walnut breeding.

1 Results and Analysis

1.1 PCR amplification

The amplified PCR products were subjected to agarose gel electrophoresis (2 uL sample +6 uL bromophenol blue) for 12 min at a voltage of 300 V to obtain the identification gel map (Figure 1). Electrophoresis results showed that the bands were clear and bright, indicating that the amplified DNA was of good quality.

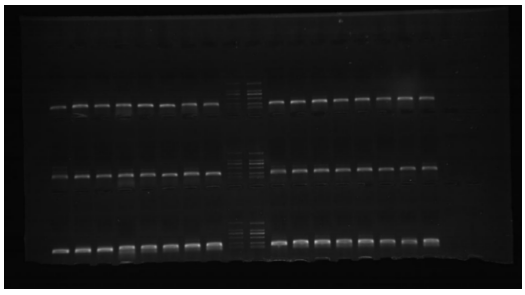

Figure 1 The PCR products were detected by agarose gel electrophoresis

1.2 Primer polymorphism

A total of 95 alleles were detected by 12 pairs of primers in 21 walnut cultivars (Table 1), and the number of alleles of WJR265 primers was the lowest, with 6. The number of alleles of WJR202 primers was the highest, with 11. The average number of alleles per primer pair was 7.916 7, and the total number of effective alleles was 52.219 2. The values varied from 2.018 3 to 7.350 0, with an average of 4.356. The results showed that the average number of

observed alleles in the 12 pairs of primers was significantly higher than the average number of effective alleles, indicating that the detected alleles were unevenly distributed at the sites. Shannon Wiener index ranged from 1.169 1 to 2.107 4, with an average of 1.643 6. The numerical value range of polymorphic information content (PIC) was 0.486 4~0.848 6, with an average value of 0.705 5, and the PIC of 12 pairs of primers was all greater than 0.25, indicating that these 12 pairs of primers were highly polymorphic in the tested varieties. The shannon index and polymorphic information content of the 12 primers indicated that the genetic diversity of the 12 primer pairs was relatively average. The variation range of heterozygosity (Ho) was 0~0.882 4 and the mean value was 0.523 4. The expected heterozygosity (He) ranged from 0.516 8 to 0.871 1, and the mean value was 0.750 4. Among them, the average expected heterozygosity was higher than the average observed heterozygosity, indicating that there was a certain inbreeding rate among the varieties, and that the 12 pairs of primers could be used to distinguish the 21 walnut varieties, and the tested walnut varieties had a high genetic diversity.

Table 1 The genetic diversity of 12 selected SSR loci

Locus	*Na*	*Ne*	*I*	*PIC*	*Ho*	*He*
WJR007	9	7.350 0	2.075 3	0.848 6	0.857 1	0.885 0
WJR031	7	4.793 5	1.703 8	0.761 5	0.714 3	0.810 7
WJR069	9	4.955 1	1.805 5	0.770 2	0.619 0	0.817 7
WJR073	7	2.831 4	1.385 6	0.616 1	0.263 2	0.664 3
WJR100	7	2.910 9	1.426 9	0.628 9	0.285 7	0.672 5
WJR115	7	4.281 5	1.648 3	0.735 7	0.882 4	0.789 7
WJR142	7	3.340 9	1.535 1	0.674 2	0.095 2	0.717 8
WJR202	11	6.681 8	2.107 4	0.834 4	0.714 3	0.871 1
WJR265	6	4.938 3	1.654 5	0.766 3	0.850 0	0.817 9
WJR279	7	2.390 2	1.195 0	0.535 4	0.571 4	0.595 8
WJR281	8	2.018 3	1.169 1	0.486 4	0.428 6	0.516 8
WJR061	10	5.727 3	2.016 0	0.808 1	0	0.845 5
Mean	7.916 7	4.351 6	1.643 6	0.705 5	0.523 4	0.750 4

1.3 Clustering analysis

The cluster analysis results of 21 samples showed that (Figure 2), 21 samples were divided into 7 groups. Among them: groups VI b including ' Boniao', 'Hui5', 'Hui6', 'Yaoxing', 'Jingzhongwanshu' and 'Wei2'; Groups VI a includes: 'Ningxiang', 'Santai', 'Yangpao', 'Zigui' and 'Zijin'; Groups V a includes: 'Muyou', 'Yangzaoxiang' and 'zhaoyang1 '; Groups IV a includes: 'Huixiaomi' and 'li53'. Groups III a includes: 'Heiyangjing' and 'Longjia'; Groups II a includes: 'Shenshuang' and 'shenyong'; 'Wanlong' separate into groupsIa.

2 Discussion

In this study, the distribution of effective alleles of 12 pairs of primers used in 21 walnut species samples was 2.018 3 (WJR281)~7.350 0 (WJR007), the average number of alleles observed at each locus was 7.916 7, and the effective number of alleles was 4.356. The average effective alleles of the primers tested in this study were compared with other studies (Chen, 2014; Bao et al., 2018) were basically consistent, which indicated that the primers used in this experiment could basically meet the requirements of genetic diversity of the test target species population, and the SSR markers used could be used to study the genetic structure and phylogenetic analysis of walnut varieties.

Genetic drift, breeding system, gene flow, gene mutation and other internal factors as well as external factors such as habitat fragmentation and population isolation caused by environmental changes and human interference can all affect the level and distribution pattern of genetic diversity of species (Wen et al., 2010). The comparison of UPGMA cluster analysis results and geographical origin of test varieties showed that there was a certain correlation between the two. Collected from Yongsheng county, Lijiang city, Yunnan province 'Shengshuang' and 'shengyong' were gathered in groupsIIa, collected from Huize county, Qujing city, Yunnan province 'Hui5', 'Hui6' and 'Jingzhongwanshu' were gathered in VIb. Collected from the west of Yunnan 'NingXiang' (Changning county,

baoshan city), 'Heiyangjing' (Yunlong County, Dali city), 'ziguiv' (Dayao county, Chuxiong city) were gathered in groups VI a. But also appeared different situations: collected from Honghe city, Yunnan province (in southern Yunnan) the 'zijing' is clustering as groups VI a, collected from Jin Chengjiang county, Hechi city, Guangxi province (in west Guangxi) the 'Muyou', collected from the Yangbi County, Dali city (in northwest Yunnan) 'Yangzaoxiang', collected from Zhaoyang county, Zhaotong city (in northeast Yunnan) 'Zhaoyang 1' is clustering as groups V a, it shows that there are complex bubble walnut varieties lineage relationships. 'Heiyangjing' collected from the big chestnut village, baofeng township, Yunlong county, Dali city is the local solid wild walnut, being able to source the Dali varieties 'Longjia' Yunlong county and distinguish the 'Wanlong', prove the experiment using SSR primer combination has a certain reliability, another experiment found that 12 pairs of primers do not distinguish well 'Hui5', 'Hui6', therefore, if you want to learn more about the genetic diversity and genetic relationship of bubble walnut in southwest China, the need to develop more suitable SSR primers or increase the other SSR primers.

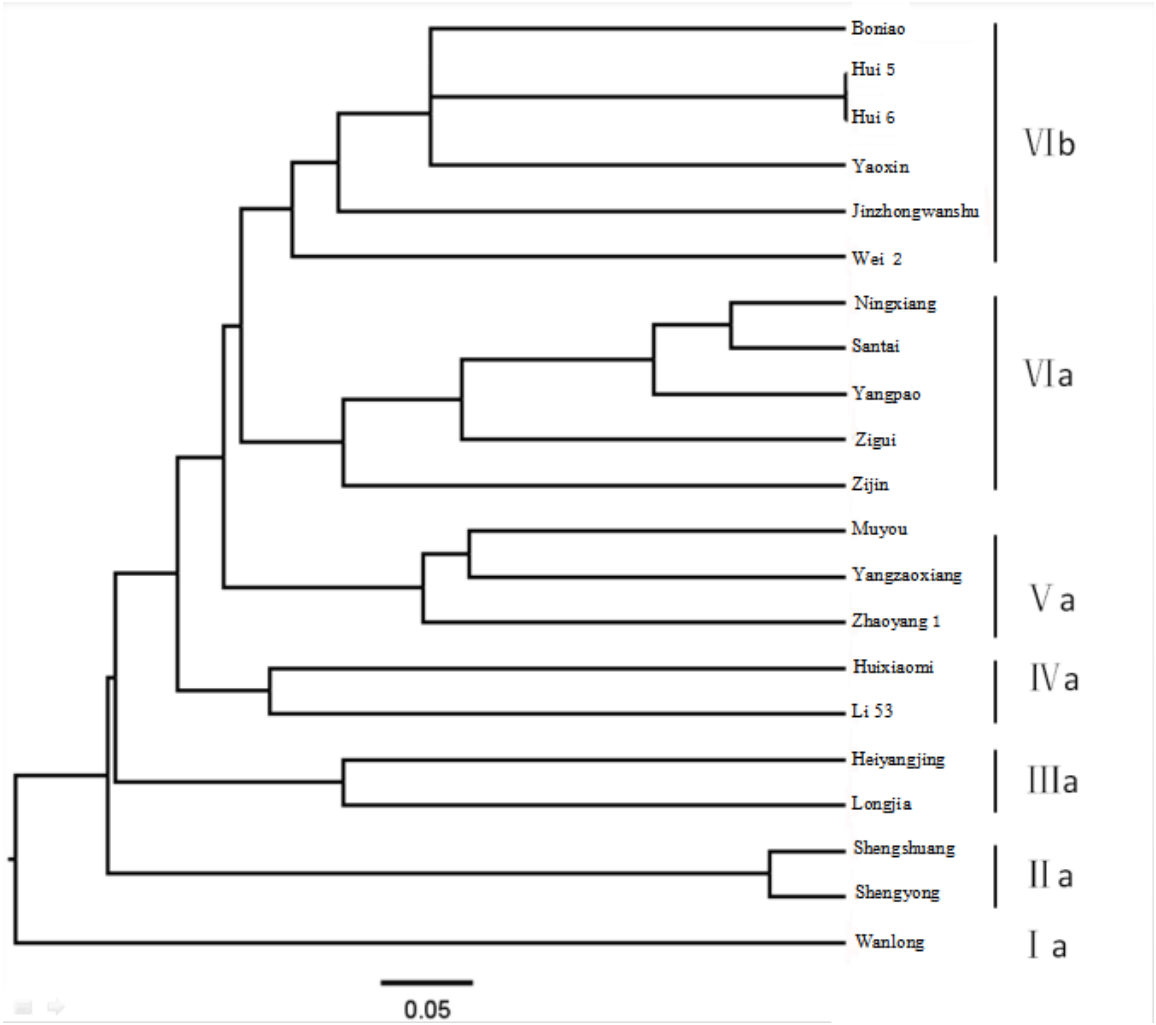

Figure 2 Clustering analysis (UPGMA) of 21 ruglans regia l varieries based on SSR markers

3 Materials and Methods

3.1 Material source

Through the bubble walnut planting and the investigation of germplasm resources in Yunnan province, this study chose the 21 unused SSR technology research of walnut varieties, Fumin county in Kunming, Yunnan province from 2018 township Luo Mian bubble walnut germplasm collection base collected the 21 varieties of walnut samples (Table 2), the samples taken from tender leaf is disinfected.

Table 2 The walnut name and breeding sources

Number	Varieties	Code	Source
1	Boniao	BN01	Nandan County, Guangxi
2	Hui 5	H5	Huize County, Yunnan
3	Hui 6	H6	Huize County, Yunnan
4	Huixiaomi	HXM	Huize County, Yunnan
5	Heiyangjing	HYJ	Yunlong County, Yunnan
6	Jingzhongwanshu	JZWS	Huize County, Yunnan
7	Li 53	L53	Yulong County, Yunnan
8	Longjia	LJ	Yunlong County, Yunnan
9	Muyou	MY	Jingchengjiang County, Guangxi
10	Ningxiang	NX	Changning County, Yunnan
11	Shengshuang	SS	Yongsheng County, Yunnan
12	Santai	ST	Dayao County, Yunnan
13	Shengyong	SY	Yongsheng County, Yunnan
14	Wanlong	WL	Yunlong county, Yunnan
15	Wei 2	W2	Weixi County, Yunnan
16	Yangpao	HYJ	Biyang County, Yunnan
17	Yaoxin	YX	Dayao County, Yunan
18	Yangzaoxiang	YZX	Biyang County, Yunann
19	Zigui	ZG	Dayao County, Yunnan
20	Zijin	ZJ	Shiping County, Yunnan
21	Zhaoyang 1	ZY1	Zhaoyang County, Yunnan

3.2 Instruments and equipment

L550 desktop low-speed large-capacity centrifuge (Hunan Xiangyi Centrifuge Instrument Co., LTD.), H1650-W high-speed small-capacity centrifuge (Hunan Xiangyi Centrifuge Instrument Co., LTD.), C300 Chemiluminescence imaging system (Azure Biosystems), C1000 Touch™ PCR instrument (Bio-Rad Bioler), Ultraviolet Fluoroscope (Bio-Rad Bioler), Sub-Cell Electrophoresis Apparatus (Bio-Rad Bioler), ABI 3730XL Genetic Analyzer (ABI American Applied Biology Co., LTD.), Boge HB-T2-D metal bath heater (Hangzhou Boge Technology Co., LTD.), Pipette (Semmer Feier Technology Co., LTD.), 96-hole reaction plate (Shanghai Yisheng Biotechnology Co., LTD.), etc.

3.3 Genomic DNA extraction

The experiment referred to the study of Li et al. (2013) and used the improved CTAB method to extract DNA. The quality was detected by 1% agar-gel electrophoresis, the extracted DNA concentration was determined by nanodrop-2000, and the DNA integrity was detected by agar-gel. All samples were diluted to 20 ng/L and stored at -20℃ for later use.

3.4 Polymorphism primer screening

Primers were synthesized according to literature (Chen et al., 2014) or transcriptomic data, and 12 pairs of SSR primers were selected as candidate primers for polymorphic primer screening in this study (Table 3).

3.5 PCR amplification and product detection

The PCR system was as follows: enzyme Mixture 20 μL, forward primer 1 μL (concentration 10 pmol/L), reverse primer 1 μL (concentration 10 pmol/L), DNA template 1 μL, Mixture 10 μL, and finally added with ddH_2O to 20 μL. The PCR reaction procedure was as follows: 2 min at 98℃, 10 s at 98℃, annealing for 10 s (the temperature was determined by the primer used) at 72℃ for 10 s, cycling for 30 times, and then storing at 72℃ for 5 min and 4℃. The PCR products were sampled and subjected to agarose gel electrophoresis (2 μL sample +6 μL bromophenol blue) for 12 min at a voltage of 300 V. Agarose gel electrophoresis images were obtained.

Table 3 Information of 12 SSR markers

Primer	Forward primer (5'-3')	Reverse primer (5'-3')	Type of SSR
WJR007	AATGCATGACATGGTGGTCA	GTATGAAACAATCATTTTCACTCA	(AT)n
WJR031	AGCTTCCCCCATTCTCCTAA	GGACCTCCACAACCAAAAGA	(AT)n
WJR061	CAAGACCACAGCACAGCATAA	GGGAGTGCTGGAATCGAATA	(AT)n
WJR069	CAGTACCCTTGGTTGAAGGA	GTGCATTAGTGCCCAAACCT	(AT)n
WJR073	CATGCATGCAGGCTTTAAAAT	CGCATCCGGAGTAGTTCTTT	(AT)n
WJR100	CGACGATTCGGTGAAGAAAT	GAAAACCCAGTTTCTGTCGG	(AT)n
WJR115	CTCACCCTTGTAGAGCGAGG	TGCAAACTCAGTGCTAAAATCAA	(GA)n
WJR142	AGACCTCAAAAGACGAAAAC	TGTGGCTGTCCATAAAGTCTTG	(AT)n
WJR202	GTTTCTACACCAGCAGCACG	CTTCATCCGGATATTGTGGC	(AT)n
WJR265	TGGCTATTGCAAAATCAGGTC	CAAAAGCATGTAGGTCGGGT	(AAT)n
WJR279	TTCATTACGTGGGGAAAAGC	TCTTGGCTCCCATTATCTGC	(GA)n
WJR281	TTCCATGGCTCTCTACCACA	ATGGAGCTGGTTCCTGACAC	(TC)n

3.6 The data processing

Popgen32 software was used to calculate various genetic diversity indicators for each SSR locus and population, including observed alleles (Na), effective alleles (Ne), Shannon index (I), polymorphism information index (PIC), observed heterozygosity (Ho), and expected heterozygosity (He).

After the same electrophoresis migration of all SSR primers, strip statistics were performed on agarose gel electrophoresis images, that is, at the same location, strip marked as "1" and strip marked as "0". The standard genetic distance of Nei between individuals was calculated in Popgen32 software (Ma and Lu, 1992), and the cluster tree of individuals was established by using the unweighted group average (UPGMA) method based on the standard genetic distance. The specific method is as follows: The UPGMA tree is constructed in the software sucking sound -1_2_30, and the landscaping and editing of the cluster tree are carried out in the software FigTree Version 1.4.2.

Authors' contributions

Wang Yi is the experimental designer and executor of this research. Xu Lingwen completed the data analysis and the writing of the first draft of the paper; Yuan Xiaolong, Hao Jiabo, Zhang Yu, Ma Jun and Wang Fei participated in the experimental design and analysis of the experimental results. Wang Yi and Lu Bin are the architects and leaders of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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