INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0472/2019/22–3–427–434
DOI: 10.17957/IJAB/15.1081
http://www.fspublishers.org

Full Length Article

Genetic Diversity of Mulberry Cultivars using ISSR and SRAP Molecular Markers

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Abstract

Mulberry, a kind of Morus (Moraceae), is an important perennial economic plant, with leaves used to feed silkworm. It is important to reveal the relationship between the genetic variation and evolution of mulberry and to reveal the phylogenetic of its germplasm. In the present study, the genetic diversity of the gene structure characteristics of 23 main mulberry in Sichuan Province (China) based on inter-simple sequence related (ISSR), sequence-related amplified polymorphism (SRAP) markers and sequencing analysis was reported. 12 ISSRs and 5 SRAPs were exploited, chosen to evaluate the polymorphism level and the discriminating capacity from 100 ISSR primers and 50 SRAP primer. The 12 ISSRs generated 116 repeatable amplified bands, of which 93 (80.24%) were polymorphic. The amplified fragment size was between 0.1–1.5 KB. The 5 SRAP primers produced 31 repeatable amplified bands, of which 24 (77.42%) were polymorphic. The amplified fragment size was also between 0.1–1.5 KB. The study indicated that, ISSR and SRAP markers were reliable and effective to assess the genetic relationship and genetic diversity of 23 main mulberry varieties of Sichuan Province (China). The results provided the scientific basis for the establishment and identification of DNA fingerprint of mulberry varieties germplasm resources.

Keywords: Mulberry; ISSR; SRAP; Genetic diversity; Cluster analysis

Introduction

Mulberry, a multipurpose agro-forestry plant belongs to the family of Moraceae, is widely used in agriculture (Andallu *et al*., 2012). New varieties of mulberry with high quality and yield are an important material foundation of the silk industry. The leaf quality is the key factor affecting the yield and quality of cocoon and silk. China has a long history as the origin center of the world's sericulture production (Satarupa, 2016). Mulberry breeding in China started varieties selection from 1930 s by conventional mutation and polyploidy breeding to get more than 100 mulberry varieties approved by national or provincial approval committee (Boonsri *et al*., 2012; Akkarachaneeyakorn and Tinrat, 2015; Chen *et al*., 2015a). The misname phenomenon of mulberry is very common recently because of the frequent exchanges between different countries and regions. In the past, the mulberry identification was done by morphological method that is affected by environmental conditions and the subjective factors.

It is important to explore the genetic relationship between different varieties of mulberry and to collect mulberry seed resources with high quality genetic diversity. At present, the ISSR markers which are the developed simple sequence repeat (SSR) markers are kinds of widely used DNA molecular markers (Banu *et al*., 2015; Perera *et al*., 2015; Shafiei-Astani *et al*., 2015). ISSR molecular marker technology has the characteristics of simple, fast and high efficiency compared with RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) molecular markers (Sen and Alikamanoglu, 2012; Jing *et al*., 2013; Sharma *et al*., 2014; Venkat *et al*., 2014; Bhattacharyya *et al*., 2015; Sousa *et al*., 2015). ISSR molecular marker is widely used in the analysis of varieties relationship, genetic diversity, variety identification and genetic linkage mapping because of these advantages.

Sequence-related amplified polymorphism (SRAP) is a new marker system based on PCR (Zong *et al*., 2015). The purpose of SRAP is to analyze the polymorphic sequence of the open reading frame with the upstream promoter or intron detection in the genome sequence without any

To cite this paper: Zhang, N., M. Wang, Y. Hou, Y. Zeng, G. Liu, X. Ding and G. Huang, 2019. Genetic diversity of mulberry cultivars using ISSR and SRAP molecular markers. *Intl. J. Agric. Biol*., 22: 427–434

additional conditions. The target fragment can be directly amplified through polymerase chain reaction (Ji *et al*., 2014). The SRAP molecular marker has the characteristics of high efficiency, high yield, good repeatability, easy to sequence and to clone the target fragment (Chen *et al*., 2011; Huang *et al*., 2012, 2014; Fan *et al*., 2015). It has been successfully applied to genetic diversity analysis, genetic map construction and gene cloning of crop (Jiang and Liu, 2011).

This study used ISSR molecular marker and SRAP molecular marker technology to study the relationship among Sichuan province 23 mulberry varieties and make genetic diversity evaluation at the molecular level, which will provide scientific basis for selection of planting mulberry varieties in Sichuan province of China in the future.

Materials and Methods

Plant Materials

The experimental material was collected from the Sericulture Research Institute, Sichuan Academy of Agricultural Sciences (Table 1). Sampling time was from late March to early July 2015. The 10 g samples (tender leaf) with no pests were put into 50 mL centrifuge tube and stored under -80ºC in ultra-low temperature freezer (Chen *et al*., 2015b).

Genomic DNA Extraction

The mulberry leaf DNA was extracted using the plant genomic DNA Purification Kit (Beijing Tiangen Biotechnology Limited Company) by using the improved CTAB method (chloroform instead of 1:4 chloroform and isoamyl alcohol) (Chen *et al*., 2014a; Chen *et al*., 2016).

ISSR Amplification

The ISSR primer is synthesized by BGI Shenzhen Technology Services Limited. A total of 100 ISSR primers were used for PCR amplification from the DNA templates (Jehan *et al*., 2014). ISSR-PCR reaction system was 25 μL, including 40 ng template DNA, 1 μL primer (10 ng/μL) (the primers were dissolved in TE), 1 μL Taq DNA polymerase, 2.5 μL PCR buffer and 0.5 μL dNTPs (10 mmol/L) and then, ddH_2O was added to 25 μL final volume (Shen *et al*., 2006).

ISSR-PCR amplification was performed in BIO-RAD T100 Thermal Cycler PCR machine. The PCR reaction conditions are as follows: initial denaturation at 94℃ for 2 min, denaturation at 94℃ for 40 s, annealing at 35℃ for 45 s (the Tm was set by the specific annealing temperature of each primer), extension at 72℃ for 90 s, 36 cycles, extension at 72℃ for 10 min, hold at 4℃.

Agarose (2%) gel electrophoresis was used to separate the ISSR products. The electrophoresis results were photographed by Quantity One gel imaging system. Each primer PCR reactions was repeated three times to ensure the ISSR markers' reproducibility and reliability.

SRAP Amplification

The SRAP primer is synthetized by BGI Shenzhen Technology Services Limited. A total of 50 SRAP primers were used for PCR amplification from the DNA templates (Chen *et al*., 2014b). SRAP-PCR reaction system is 25 μL, including 40 ng template DNA, 1 μL primer (10 ng/μL) (the primers were dissolved in TE), 1 μL Taq DNA polymerase, 2.5 μL PCR buffer and 0.5 μL dNTPs (10 mmol/L) and then, ddH_2O was added to 25 μL final volume (Shen *et al*., 2009; Xue *et al*., 2010; Polat *et al*., 2012; Liu *et al*., 2014).

SRAP-PCR amplification was performed in BIO-RAD T100 Thermal Cycler PCR machine (BIO-RAD T100 Thermal Cycler, United States). The PCR reaction conditions were as followed: initial denaturation at 94℃ for 5 min, denaturation at 94℃ for 1 min, annealing at 35℃ for 1 min, extension at 72℃ for 1 min, 5 cycles, denaturation at 94℃ for 1 min, annealing at 50℃ for 1 min, extension at 72℃ for 1 min, 35 cycles, extension at 72℃ for 10 min, hold at 4℃ (Nie *et al*., 2014; Costa *et al*., 2014; Robarts and Wolfe, 2014).

Agarose (2%) gel electrophoresis was used to separate the ISSR products. The electrophoresis results were photographed by Quantity One gel imaging system. Each primer PCR reactions was repeated three times to ensure the ISSR markers' reproducibility and reliability.

Data Analysis

The clear, stable and repeated amplified bands were analyzed. NT Software system was used to analyze the genetic dendrogram among the varieties. Both ISSR and SRAP amplified bands were analyzed using the score of 1 means presence of amplified bands and a score of 0 means absence of amplified bands. The similarity matrix of the examined 23 samples was calculated based on Nei's genetic distance using the NTSYS-pc software. The genetic distance among the landraces was expressed as a distance tree using un-weighted pair-group method with arithmetic averages (UPGMA) and simple matching coefficient (Peng *et al*., 2014). The products amplified by SRAP-PCR were sequenced and DNAMAN software was used for multiple sequence alignment to get the genetic similarity among the 23 varieties and to compare the relationship among them (Jena *et al*., 2015). The genetic distances of the 23 mulberry varieties were calculated based on the SRAP-PCR and ISSR-PCR amplified bands from total DNA using NT sys 2.10e software (Li *et al*., 2010; Liu *et al*., 2011, 2015).

Results

Amplification of ISSR

A total of 12 primers selected from 100 ISSR primers were used to analyze the genetic diversity of 23 mulberry varieties. The polymorphic fingerprints are shown in Fig. 1, 2 and 3.

Table 1: The experimental materials

Order	Material name	Order	Material name	Order	Material name
1	Xiang7920	9	Chuan7637	17	Chuan799-1
2	Chuan826	10	Shushen1	18	Santai1
3	Husang32	11	Chuansang48-3	19	Tai90 Tong
4	Yousang	12	Chuansang98-1	20	Wuhedashi
5	Chuan7431	13	Taiwanguosang 72C002	21	Jialing30
6	Xinzhiyilai	14	Shigu11-6	22	Hongguo1
7	Jialing20	15	Chuansang83-5	23	Hongguo2
8	Nongsang14	16	Chuansang83-6		

Table 2: List of ISSR primers, amplification conditions and the experiment results

Primer name	Sequence (3'-5')	Annealing temperature℃	Number of Amplified bands	Number of polymorphic bands	Polymorphic percentage%
ISSR04	(GTC)6	60	7	5	71.43
ISSR08	(AG)6TC	42	5	4	80
ISSR11	(GAG)4GC	48	12	11	91.67
ISSR14	(CT)8TG	54	12	9	75
ISSR15	(GA)GG	56	9	8	88.89
ISSR19	(CAG)4GC	48	7	7	100
ISSR20	(CT)8TC	54	10	7	70
ISSR29	(AG)8YC	55	11	8	72.23
ISSR30	(GT)6CC	44	8	6	75
ISSR39	(CA)6GT	48	13	11	84.62
ISSR40	(CAC)4GC	48	8	6	75
ISSR54	(GA)8YT	53	14	11	78.57
Total			116	93	80.24
Mean			9.6	7.8	

The list of ISSR primers, amplification conditions and the experiment results were shown in Table 2. The results showed that 116 clear and repeatable bands were amplified, including 93 polymorphic bands, which accounted for 80.24% of the total bands. The average amplification using one pair of ISSR primers was 9.7 amplified bands including 7.8 polymorphic amplified bands. The number of amplified bands ranged from 5 to 14. Among them, the number of bands amplified using ISSR08 was the least (only 5), while the number of bands amplified using ISSR54 was the maximum (14) with the size of amplified bands between 0.1 and 1.5 kb.

Analysis of Genetic Diversity Based on ISSR Markers

The genetic relationships of 23 mulberry varieties were also analyzed (Fig. 4 and Table 3). The cluster analysis indicated that the mulberry varieties can be divided into 2 categories: Xiang7920, Chuan826, Husang32, Yousang, Xinzhiyilai, Chuan7431, Jialing20, Nongsang14, Chuan7637, Shushen1, Chuansang48-3 and Chuansang98-1 are divided into the first category (I) and Taiwanguosang72C002, Shigu11-6, Chuansang83-6, Hongguo2, Chuan799-1, Wuhedashi, Jialing30, Santai-1, Tai90Tong and Hongguo1 were divided into the second category (II). The genetic similarity coefficient of variation of the 23 mulberry varieties were 0.51–0.89. The genetic similarity coefficient between

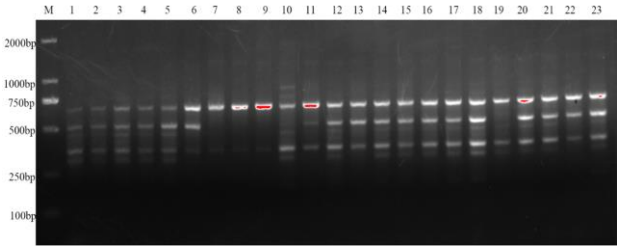

Fig. 1: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using ISSR04 as the primer

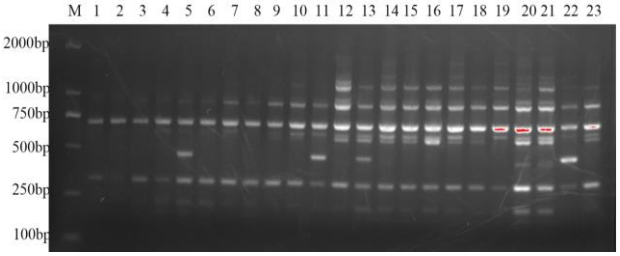

Fig. 2: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using ISSR11 as the primer

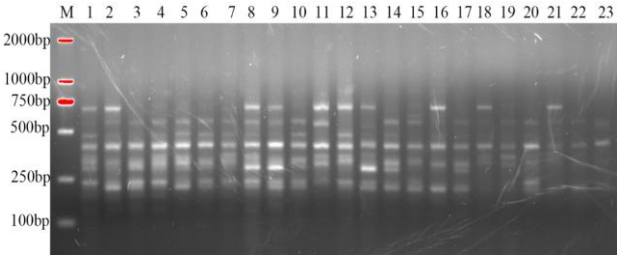

Fig. 3: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using ISSR15 as the primer

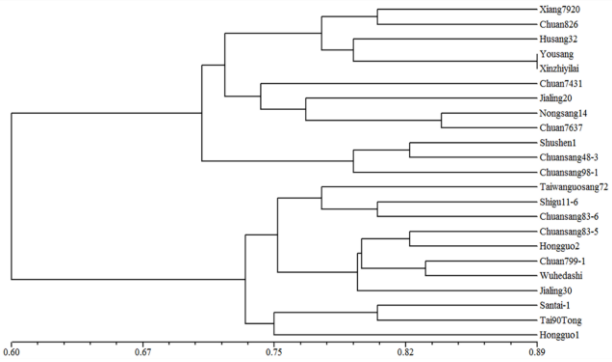

Fig. 4: Clustering analysis of 23 mulberry varieties based on ISSR markers

Wuhedashi and Nongsang 14, Chuan799-1 and Nongsang14, Chuansang98-1 and Hongguo1, Husang32 and Shigu11-6 was the smallest (only 0.51), while the genetic similar coefficient between Yousang and Xinzhiyilai was the largest (0.89).

Table 3: Genetic consistency between 23 mulberry trees obtained by ISSR analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
2	0.8017																					
3	0.7845	0.7586																				
4	0.7845	0.8103	0.7931																			
5	0.7069	0.6983	0.6810	0.7500																		
6	0.7414	0.7500	0.7845	0.8879	0.7414																	
7	0.6552	0.6466	0.6638	0.7155	0.7586	0.7241																
8	0.7155	0.7586	0.6724	0.7931	0.6983	0.7672	0.7155															
9	0.7586	0.7845	0.6638	0.7500	0.7586	0.7414	0.8103	0.8362														
10	0.6466	0.7241	0.6379	0.7759	0.7328	0.7328	0.6638	0.7759	0.7672													
11	0.6379	0.7155	0.6466	0.8190	0.6724	0.7759	0.6897	0.7845	0.7414	0.8190												
12	0.6466	0.6897	0.6724	0.7414	0.6810	0.6983	0.6293	0.6897	0.6983	0.7931	0.7845											
13	0.4914	0.5862	0.5172	0.6207	0.4914	0.6121	0.5086	0.5345	0.5603	0.5690	0.6121	0.5690										
14	0.6034	0.5603	0.5086	0.6293	0.6034	0.6034	0.5690	0.5431	0.6724	0.6466	0.6034	0.6810	0.7500									
15	0.6034	0.6638	0.6466	0.6466	0.5517	0.6552	0.5690	0.5431	0.6034	0.6466	0.6207	0.6293	0.7672	0.7586								
16	0.5776	0.6724	0.5690	0.6724	0.6121	0.6466	0.5603	0.5517	0.6121	0.6552	0.6293	0.6897	0.7931	0.8017	0.7672							
17	0.5345	0.5948	0.6293	0.6466	0.5862	0.6379	0.5690	0.5086	0.5862	0.5948	0.5690	0.5948	0.7672	0.7759	0.8103	0.8017						
18	0.5776	0.6552	0.6207	0.6379	0.6121	0.6121	0.6293	0.5862	0.6466	0.5862	0.5776	0.6379	0.7069	0.6810	0.7328	0.7759	0.7672					
19	0.6207	0.6638	0.6293	0.6638	0.6207	0.6552	0.6207	0.6121	0.6897	0.6293	0.5862	0.5948	0.6466	0.7241	0.7241	0.7672	0.7759	0.8017				
20	0.5345	0.5603	0.6121	0.6121	0.5690	0.6379	0.5172	0.5086	0.5517	0.5948	0.5517	0.6466	0.7155	0.7241	0.7931	0.7500	0.8276	0.6983	0.6897			
21	0.6034	0.6638	0.6810	0.6293	0.5517	0.6379	0.5690	0.5431	0.5862	0.5776	0.5690	0.6638	0.6983	0.7069	0.8103	0.7500	0.7931	0.7672	0.7586	0.7931		
22	0.5862	0.6466	0.6466	0.6121	0.5690	0.6552	0.5690	0.5259	0.5862	0.5431	0.6034	0.5086	0.6983	0.6379	0.7586	0.7328	0.7241	0.7328	0.7586	0.7069	0.6897	
23	0.5948	0.6724	0.6379	0.6897	0.6293	0.6983	0.6121	0.5690	0.6293	0.6724	0.6638	0.6897	0.7069	0.7328	0.8190	0.7931	0.7845	0.7931	0.7672	0.7845	0.7672	0.8017

Table 4: List of SRAP primers, amplification conditions and the experiment results

Primer name	Forward primer (5'-3')	Reverse primer (5'-3')	Number of Amplified bands	Number of polymorphic bands	Polymorphic percentage %
SRAP01	TGAGTCCAAACCGGATA	GACTGCGTACGAATTAAT	5	3	60
SRAP02	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGC	7	6	85.71
SRAP03	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTGAC	6	4	66.67
SRAP10	TGAGTCCAAACCGGAAA	GACTGCGTACGAATTCAT	9	8	88.89
SRAP11	TGAGTCCAAACCGGAAC	GACTGCGTACGAATTCTA	4	3	75
Total			31	24	77.42
Mean			6.2	4.8	

Amplification of SRAP

The results showed that 31 clear and repeatable bands were amplified, including 24 polymorphic bands, which accounted for 77.42% of the total bands (Table 4). The average amplification using one pair of SRAP primers was 6.2 amplified bands including 4.8 polymorphic amplified bands. The number of amplified bands ranged from 4 to 9. Among them, the number of bands amplified by SRAP11 was the least (only 4), while the number of bands amplified by SRAP10 was the highest (9). The size of amplified bands using these SRAP primers were between 0.1 and 1.5 kb (Fig. 5–9).

Analysis of Genetic Diversity Based on SRAP Markers

The genetic distances of the 23 mulberry varieties were calculated and the genetic relationships of 23 mulberry varieties were also analyzed (Fig. 10 and Table 5). The cluster analysis indicated that the mulberry varieties can be divided into 4 categories: Xiang7920, Chuan7637, Chuan826, Chuansang98-1, Jialing20, Chuansang83-5 and Chuansang83-6 belong to the first category (I); Husang32, Shushen1, Xinzhiyilai, Chuan7431, Jialing30, Hongguo1, Shigu11-6,

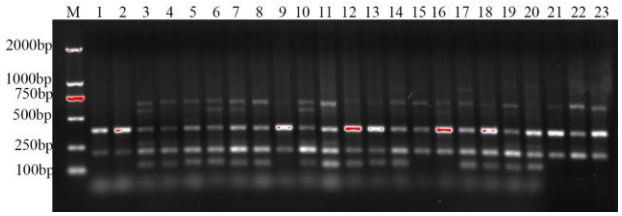

Fig. 5: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using SRAP01 as the primer

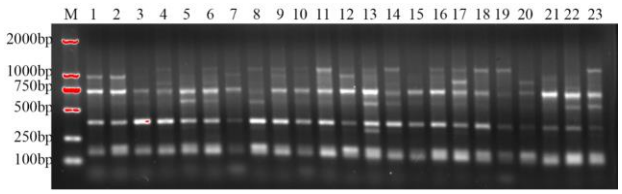

Fig. 6: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using SRAP02 as the primer

Taiwanguosang72C002, Yousang, Chuansang48-3, Chuan799-1, Santai-1 and Tai90Tong belong to the second major categories (II); Wuhedashi belong to the third categories (III); and Nongsang14 and Hongguo2 belong to the fourth categories (IV).

Table 5: Genetic consistency between 23 mulberry trees obtained by SRAP analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
2	0.8065																					
3	0.6452	0.7097																				
4	0.7742	0.7097	0.7419																			
5	0.7742	0.6129	0.8387	0.7742																		
6	0.5806	0.6452	0.8065	0.6774	0.7742																	
7	0.7742	0.8387	0.8710	0.8065	0.7097	0.7419																
8	0.6452	0.6452	0.6129	0.7419	0.6452	0.7419	0.6774															
9	0.8710	0.8065	0.7097	0.7742	0.7419	0.7097	0.7742	0.5806														
10	0.6129	0.6129	0.9032	0.7097	0.8065	0.9032	0.7742	0.6452	0.7419													
11	0.7742	0.6452	0.7419	0.8710	0.8387	0.7419	0.7419	0.7419	0.7742	0.8387												
12	0.8710	0.8710	0.7097	0.7742	0.6774	0.6452	0.8387	0.7097	0.8065	0.6129	0.7097											
13	0.6452	0.6452	0.7419	0.6774	0.8387	0.7419	0.6129	0.6774	0.6452	0.7097	0.7419	0.7097										
14	0.6452	0.7097	0.8065	0.8065	0.8387	0.7419	0.7419	0.7419	0.7097	0.7742	0.8065	0.6452	0.7419									
15	0.6774	0.8065	0.8387	0.7097	0.7419	0.7097	0.7742	0.6452	0.7419	0.7419	0.7097	0.7419	0.7097	0.7742								
16	0.7419	0.8710	0.7742	0.8387	0.6774	0.7742	0.8387	0.7742	0.8065	0.7419	0.7742	0.8065	0.6452	0.7742	0.8710							
17	0.6774	0.6129	0.7097	0.8387	0.7419	0.7742	0.7097	0.6452	0.8065	0.8065	0.8387	0.6774	0.6452	0.7097	0.7419	0.7419						
18	0.7097	0.7097	0.6774	0.7419	0.7097	0.7742	0.6774	0.6129	0.8387	0.7742	0.8065	0.7097	0.6774	0.7419	0.7097	0.7097	0.9032					
19	0.6452	0.6452	0.6774	0.7419	0.7097	0.6774	0.6774	0.6774	0.7097	0.7742	0.8710	0.6452	0.6129	0.7419	0.7097	0.7097	0.8387	0.8710				
20	0.7097	0.7097	0.7419	0.8065	0.7097	0.5484	0.7419	0.6774	0.6452	0.6452	0.7419	0.7097	0.6774	0.7419	0.7097	0.7097	0.6452	0.6129	0.6129			
21	0.7742	0.7742	0.8065	0.7419	0.8387	0.6774	0.7419	0.5484	0.8387	0.7742	0.8065	0.7097	0.6774	0.8065	0.8387	0.7742	0.7742	0.8065	0.8065	0.6774		
22	0.6129	0.6774	0.7742	0.6452	0.8065	0.6452	0.6452	0.5161	0.6774	0.6774	0.6452	0.6129	0.7097	0.7742	0.7419	0.6774	0.6129	0.6452	0.6452	0.5806	0.8387	
23	0.6129	0.6774	0.6452	0.7097	0.6774	0.7097	0.6452	0.7742	0.6774	0.6129	0.6452	0.6129	0.6452	0.7742	0.7419	0.8065	0.6129	0.5806	0.5161	0.6452	0.6452	0.6774

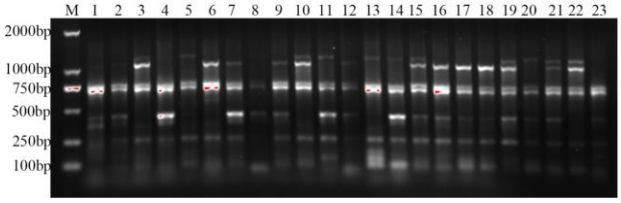

Fig. 7: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using SRAP03 as the primer

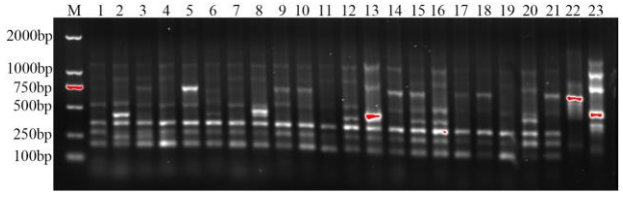

Fig. 8: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using SRAP10 as the primer

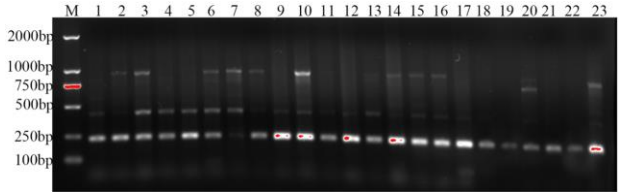

Fig. 9: Electrophoresis of PCR amplified products of the total DNA of 23 mulberry varieties leaves using SRAP11 as the primer

The genetic similarity coefficients of variation of 23 mulberry varieties were 0.52–0.90. The genetic similarity coefficient between Tai90 Tong and Hongguo2, Nongsang14 and Hongguo1 was the smallest (only 0.52), while the genetic similar coefficient between Husang32 and Shushen1, Xinzhiyilai and Shushen1, Chuan799-1 and Santai1 was the largest (0.90).

Sequencing Analysis

The SRAP11-PCR amplicons of the 23 mulberry varieties was recovered using the universal DNA purification kit and sent to BGI Tech for sequencing. DNAMAN software was used for multiple sequence alignment to get the genetic similarity coefficient and genetic distance map (Fig. 11). The genetic similarity coefficient of the 23 mulberry varieties was more than 91.28%. The 23 mulberry varieties were divided into 2 categories using the sequencing analysis: Tai90 Tong, Yousang, Chuan799-1, Hongguo2, Shushen1, Hongguo1, Xiang7920, Chuansang98-1, Jialing20 and Jialing30 belong to the first major categories (I). Chuan7431, Nongsang14, Xinzhiyilai, Wuhedashi, Shigu11-6, Chuansang83-5, Santai1, Taiwanguosang72C002, Chuan7637, Chuan826, Chuansang83-6, Chuansang48-3 and Husang32 belong to the second categories (II).

Discussion

The origin and domestication of mulberry have not been elucidated until now. SRAP marker was reported to be used for analysis on the genetic relationships of 24 mulberry (*Morus* L.) accessions in the lower regions of the Yellow River. The results showed that both species of *M. alba* and *M. multicaulis* presented the abundant variations and some accessions proved to be inter-specific hybrid progenies by SRAP genetic information (Dechang *et al*., 2015). ISSR markers were also used for molecular characterization and identification associated with yield traits in mulberry. The results indicated that the greater resolving power of the ISSR markers was evident from the dendrograms (Vijayan *et al*., 2010). Using step-wise multiple regression analysis, a number of markers associated with total shoot length, leaf weight, number of branches, internodal distance, protein, leaf moisture percentage, and leaf chlorophyll could be

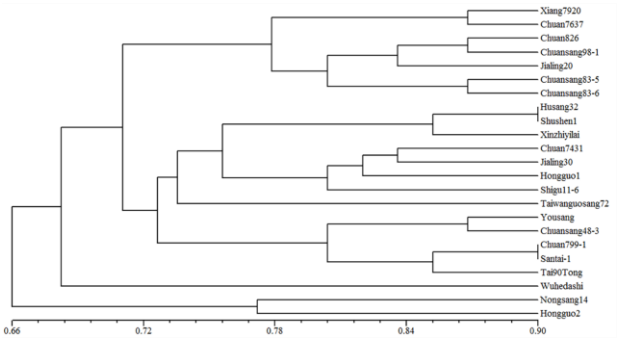

Fig. 10: Clustering analysis of 23 mulberry varieties based on SRAP markers

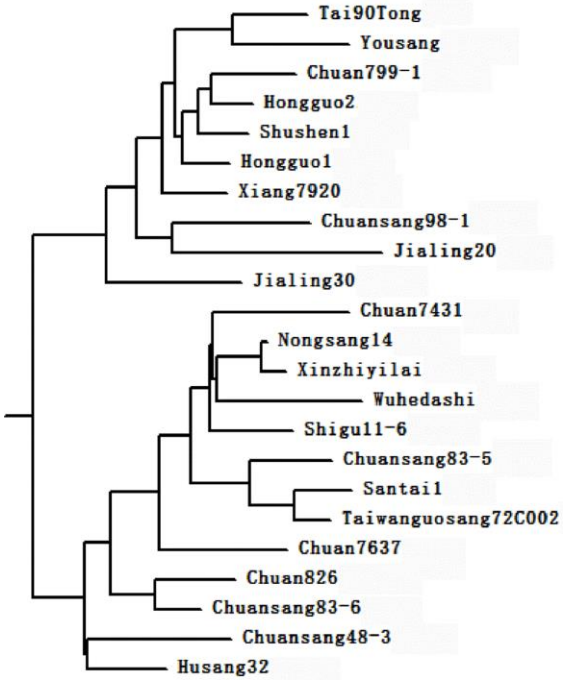

Fig. 11: Clustering analysis of 23 mulberry varieties based on sequencing analysis of SRAP marker

identified. These markers could be of much use in marker assisted selection (MAS) breeding programmes in mulberry, especially when no genetic information in terms of linkage maps and Quantitative Trait Locis (QTLs) is available to plant with high heterozygosity and a long juvenile period (Prasanta *et al*., 2008).

Among the markers, ISSR, SRAP and sequence analysis methods can divided Xiang7920, Jialing20 and Chuansang98-1 into the same category, Chuansang83-5 and Chuangsang83-6 into the same category, Jialing30 and Hongguo1 into the same category, Chuan799-1and Tai90Tong into the same category and Taiwanguosang72C002, Shigu11-6 and Santai1 into the same category, which indicated that all the three methods are feasible. Wuhedashi was divided into a single category using SRAP maker method, but was divided into another category with Taiwanguosang72C002, Shigu11-6 and Santai1 using ISSR maker method and sequence analysis, which indicated that SRAP marker is more suitable than ISSR marker and sequence analysis for Wuhedashi. Nongsang14 and Hongguo2 were classified as a category using SRAP maker method, but were separated into the totally different categories using both ISSR maker method and sequence analysis, which indicated that SRAP and ISSR maker methods or sequence analysis can be combined for identification of these two varieties. It worth noting that Husang32, Xinzhiyilai, Chuan7431 and Chuansang48-3 were classified in the same category using SRAP maker method and sequence analysis, but divided into the other category using ISSR maker method, which showed that ISSR and SRAP maker methods or sequence analysis can be combined for these four varieties. A similar situation appeared in these two species of Yousang and Chuan826. These results indicated that the molecular marker technique is useful and powerful for the detection of genetic variation among the 23 mulberry species. These three methods have their own advantages and disadvantages. And need to be chosen the appropriate identification methods according to the variety that needs to be selected.

The cultivation of the 23 mulberry varieties is very wide and representative (Aramwit *et al*., 2011; Ann *et al*., 2015). At present, the main cultivars of mulberry are used for its fruits, tea, fodder and high resistant mulberry varieties. New mulberry varieties will be introduced in the future, which will make the mulberry hybrid population more complex. Therefore, more perfect mulberry germplasm resources fingerprint, rapid detection technology and more stable molecular marker technology need to be established in the future for the production and breeding. In addition, the clear construction of the parent's genetic map of mulberry germplasm resources, the study of offspring traits segregation law, the QTL mapping for important traits and the assisted breeding molecular marker of mulberry is of great significance.

Conclusion

In conclusion, the present study showed that a great genetic diversity occurred among 23 main mulberry varieties of Sichuan Province (China) due to their morphological differentiation. ISSR markers, SRAP markers and Sequence analysis not only have certain applicability in the evaluation of genetic diversity and genetic relationships of mulberry varieties, but also contribute to the identification of 23 mulberry germplasm resources.

Acknowledgments

This project was supported by the Science and Technology Support Project of Sichuan Province (2018JY0087 and 2018NZ0055), the Cultivate Major

Projects of Sichuan Province (16CZ0018), Nanchong science and Technology Bureau of Sichuan Province (16YFZJ0043), Talent Program of China West Normal University (17YC328,17YC136,17YC329), National Training Project of China West Normal University (17c039), Innovative Team Project of China West Normal University (CXTD2017-3), the project for Promoting the Innovation Ability of Sichuan Province (2016ZYPZ-030) and the 13th Five Years Plan " Corps Breeding Research Projects of Sichuan Province" (NO.2016yzgg-13-01 and 2016yzgg-13-02).

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[Received 27 Mar 2018; Accepted 25 Jan 2019; Published (online) 12 Jul 2019]