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Full Length Research Paper

Analysis of genetic relationships of mulberry (*Morus* L.) germplasm using sequence-related amplified polymorphism (SRAP) markers

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Sequence-related amplified polymorphism (SRAP) is a novel molecular marker technique designed to amplify open reading frames (ORFs). Here, for the first time, SRAP was used to identify mulberry germplasm. Twenty-three mulberry accessions were screened using SRAP techniques, with 12 primer combinations selected for their reproducibility and polymorphism. Out of 83 PCR fragments scored, 59 (71.1%) were polymorphic, with an average of 4.9 polymorphic bands and 6.9 bands per primer combination. Mean gene diversity and PIC were 0.1611 and 0.1353, respectively. The genetic similarity coefficient ranged from 0.6905 to 0.9524, with an average of 0.8330. The phylogenetic tree was obtained using the UPGMA method using the total number of amplified SRAP fragments. The results from cluster analysis were in general agreement with our morphologic classification.

Key words: Mulberry, molecular marker, genetic diversity, SRAP.

INTRODUCTION

Mulberry (genus *Morus*), a perennial tree or shrub, is an economically important plant used for sericulture and is the sole food plant for the domesticated silkworm, *Bombyx mori*. Mulberry is widely distributed in Asia, Europe, North and South America, and Africa, and it is cultivated extensively in East, Central, and South Asia for silk production. Because it adapts easily to different ecological conditions, and is easily hybridized, both naturally and artificially, abundant mulberry germplasm resources are available, making its genetic background rather complicated and highly heterozygous (Dandin, 1998). There are about 3,000 mulberry germplasm resources in China comprising 15 species and four varieties (Pan, 2000).

Previous studies on genetic diversity in the genus *Morus* have been developed using morphological characters and molecular techniques. Molecular markers are useful complements to morphological and phenological characters because they are plentiful, are independent of tissue or environmental effects, and allow accession identification in the early stages of development. Such techniques reveal polymorphisms at the DNA level and are a very powerful tool for characterization and genetic diversity estimation. Many molecular marker techniques have been successfully used in identification and genetic diversity analysis in mulberry, such as RAPD (Xiang et al., 1995; Feng et al., 1996; Zhao and Pan, 2004), AFLP (Sharma and Sharma, 2000; Wang and Yu, 2001), SSR (Aggarwal et al., 2004; Zhao et al., 2005), and ISSR (Vijayan and Chatterjee, 2003; Awasthi et al., 2004; Vijayan, 2004; Vijayan et al., 2004a, b; Zhao et al., 2006, 2007a, b). RAPD is simple, convenient, and inexpensive, but poor consistency and low reproducibility limit its utilization (Roodt et al., 2002). AFLP technology is now

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Table 1. Names and origins of tested mulberry accessions.

No.	Accession	Species	Origin
1	Yu 2	*M. multicaulis* Perr.	Zhenjiang city, Jiangsu province, China
2	Yu 711	*M. multicaulis* Perr.	Zhenjiang city, Jiangsu province, China
3	Sanglian	*M. alba* Linn.	Fujian province, China
4	Gongxianheiyousang	*M. alba* var. *macrophylla* Loud.	Gongxian city, Sichuan province, China
5	Wenqisang	*M. alba* var. *venose* Delile.	Zhouzhi city, Shanxi province, China
6	Chuizhisang	*M. alba* var. *pendula* Dipp.	Korea
7	Lunjiao 40	*M. atropurpurea* Roxb.	Shunde city, Guangdong province, China
8	Changnongshansang	*M. bombycis* Koidz	Shandong province, China
9	T12	*M. rotundiloba* Koidz.	Thailand
10	Bijie 5	*M. wittiorum* Hand-Mazz.	Biejie city, Guizhou province, China
11	Jimengsang	*M. mongolica* Schneid.	Jilin province, China
12	Yaosang	*M. nigra* Linn.	Xingjiang autonomous region, China
13	Yaan 3	*M. cathayana* Hemsl.	Yaan city, Sichuan province, China
14	Dejiang 10	*M. laevigata* Wall.	Dejiang city, Guizhou province, China
15	Yunnanshuisang	*M. laevigata* Wall.	Yunnan province, China
16	Qianesang 1	*M. wittiorum* Hand-Mazz.	Dejiang city, Guizhou province, China
17	Yu 54	*M. multicaulis* Perr.	Zhenjiang city, Jiangsu province, China
18	Yu 82	*M. multicaulis* Perr.	Zhenjiang city, Jiangsu province, China
19	T11	*M. rotundiloba* Koidz.	Thailand
20	Jianchi	*M. bombycis* Koidz	Japan
21	Baojing 5	*M. cathayana* Hemsl.	Baoqing city, Hunan province, China
22	Lijiangshansang	*M. bombycis* Koidz	Lijiang city, Yunnan province, China
23	Husang 39	*M. multicaulis* Perr.	Zhenjiang city, Jiangsu province, China

now widely used for genomic fingerprinting (Zhang et al., 1999; Karaca et al., 2002) due to its high polymorphism (Vos et al., 1995). However, AFLP is complex, requires multiple steps, and shows pseudo-polymorphism when methylation-sensitive restriction enzymes are used. SSRs are stable, abundant, highly polymorphic and reproducible, but they require the development of working primers for each species, which makes the method laborious and costly (Xiao et al., 2008a, b). Sequence-related amplified polymorphism (SRAP) is a novel molecular marker technique based on two-primer amplification that preferentially amplifies open reading frames [(ORFs) Li and Quiros, 2001]. The forward primers preferentially amplify exonic regions, and the reverse primers preferentially amplify intronic regions and regions with promoters. The observed polymorphism originates in the variation in the length of these extrons, introns, promoters, and spacers, both among individuals and among species (Li and Quiros, 2001). With this unique primer design, SRAP markers are more reproducible, more stable, and less complex than other molecular marker techniques. SRAP markers are more powerful for revealing genetic diversity among closely related cultivars than SSR, ISSR, or RAPD markers on buffalo grass (Budak et al., 2004). The SRAP marker system has been used to investigate genetic diversity in plant species, including *Brassica* (Li and Quiros, 2001), *Cucurbita* (Ferriol et al., 2003), buffalo grass (Budak et al., 2004), cotton (Lin et al., al., 2004), and other plant species (Qiao, 2007). But it has not previously been reported in mulberry. Here, we established the SRAP analytic system in mulberry and analyzed the genetic diversity, which will be valuable for germplasm identification and conservation, use of the mulberry germplasm, construction of core collections and mulberry genetic breeding.

MATERIALS AND METHODS

Plant materials

Table 1 lists the accessions of mulberry used in the current study and their origins. Twenty-three mulberry materials, including 10 species and three varieties, were obtained from the National Mulberry Gene Bank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu Province, China.

DNA extraction

Total DNA was extracted from young leaves of mulberry following the CTAB method described by Zhao and Pan (2004). Purified total DNA was quantified using gel electrophoresis, and DNA quality was analyzed using UV absorption spectrophotometry. DNA samples were stored at -20ºC until use.

Table 2. Sequences of SRAP forward and reverse primers used in the study.

Forward primer F		Reverse primer R	
Name	**Sequence (5′–3′)**	**Name**	**Sequence (5′–3′)**
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGTAA	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTCC	Em7	GACTGCGTACGAATTGAG
Me8	TGAGTCCAAACCGGTGC	Em8	GACTGCGTACGAATTGCC
		Em9	GACTGCGTACGAATTTCA

Table 3. Total and polymorphic fragment numbers per SRAP primer combination.

Code	Primer combination	NAB	NPB	PPB%	GD	PIC
1	Me5/Em8	10	8	80	0.1512	0.1304
2	Me4/Em3	6	2	33.3	0.1210	0.1092
3	Me7/Em3	11	7	63.6	0.0440	0.0411
4	Me2/Em9	3	3	100	0.1084	0.1019
5	Me7/Em9	3	2	66.7	0.2394	0.1906
6	Me6/Em9	7	6	85.7	0.1836	0.1525
7	Me7/Em1	9	7	77.8	0.2058	0.1701
8	Me5/Em1	4	3	75	0.1645	0.1437
9	Me7/Em5	3	2	66.7	0.2420	0.1919
10	Me4/Em1	16	12	75	0.1989	0.1630
11	Me1/Em9	6	3	50	0.1928	0.1525
12	Me1/Em2	5	4	80	0.0817	0.0771
Total		83	59			
Average		6.9	4.9	71.1	0.1611	0.1353

NAB = Number of amplified bands; NPB = number of polymorphic bands; PPB = percent of polymorphic bands; GD, gene diversity; PIC, polymorphic information content.

SRAP analysis

Primers were designed according to the principles of SRAP primers (Li and Quiros, 2001) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Ltd. Company, China. A total of 72 SRAP primer pairs, all combinations of eight forward primers and nine reverse primers, were assayed on the two accessions (Table 2). Twelve primers were chosen for SRAP analysis of genetic diversity based on reproducible production of bands (Table 3).

PCR reactions were carried out in a volume of 15 µl containing 10 ng total DNA, 10 × PCR buffer (200 mM Tris-HCl pH 8.4, 2.5 mM $MgCl_2$, 500 mM KCl), 0.25 mM of each dNTP, 6 pM of each primer, and 1 unit of Taq DNA polymerase. PCR cycling conditions for all accessions were as follows: 5 min initial denaturation step (94ºC), then five cycles of 1 min of denaturing at 94ºC, 1 min of annealing at 35ºC, and 1 min of elongation at 72ºC. In the following 35 cycles, the annealing temperature was increased to 50ºC, ending with a final extension step of 7 min at 72ºC. DNA amplification fragments were separated in a 2.5% agarose gel at 90 W for 4 h in 1 × TBE buffer (100 mM Tris-borate, pH 8.0, 2 mM EDTA) and stained with ethidium bromide.

Data analysis

Only distinct, reproducible, well-resolved fragments were scored as present (1) or absent (0) for each of the SRAP markers. The observed number of alleles, Nei's genetic similarity and genetic distance estimated by Nei's coefficient between pairs were analyzed using Popgene software, version 3.5 (www.ualberta.ca/~fyeh/popgene.pdf). The gene diversity, polymorphism information content (PIC) per locus were calculated with the program Power Maker 3.25 (Liu and Mouse, 2005). The UPGMA algorithm was used to construct a phylogram from a distance matrix using MEGA4 software (Tamura et al., 2007).

RESULTS

Levels of polymorphism revealed by SRAP markers

From the prescreening assays with two mulberry accessions, a total of 72 primer combinations were assayed. Primer banding patterns that were difficult to score and

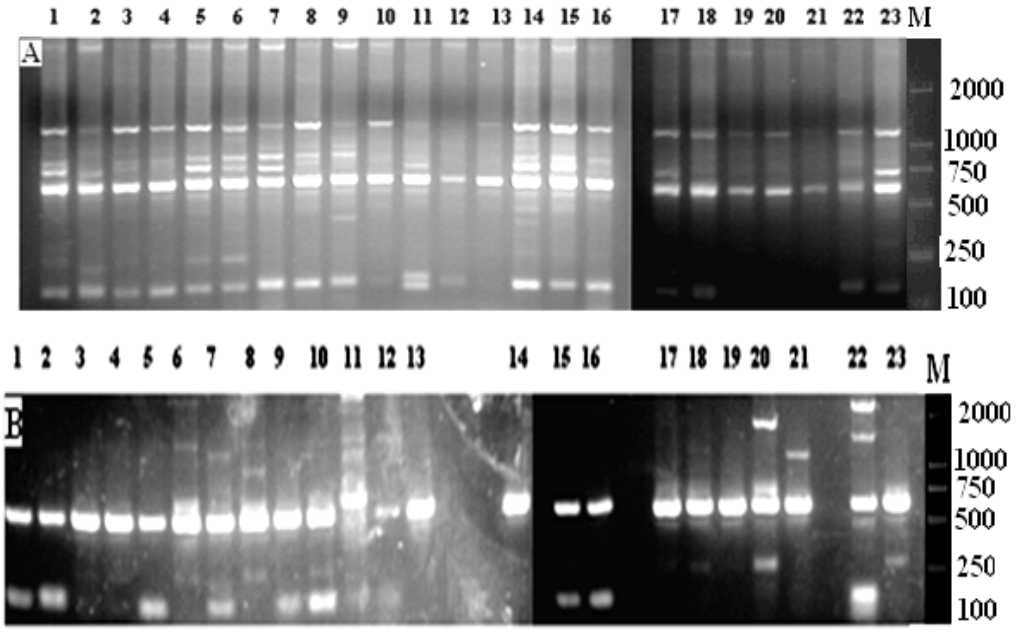

Figure 1. SRAP fingerprint of 23 mulberry accessions generated by primer combination Me7/Em1 (A) and Me6/Em9 (B). The accession codes are the same as those shown in Table 1. M is the DL 2000 marker.

those that failed to amplify consistently in all genotypes were excluded. Consequently, only 12 combinations were selected (Table 3). A total of 83 amplicons were produced, of which 59 were polymorphic (71.1%). The number of amplicons produced by each primer set ranged from 3 (Me2/Em9, Me7/Em9, and Me7/Em5) to 16 (Me4/Em1), with an average of 6.9 amplicons per primer set, whereas the number of polymorphic amplicons ranged from 2 (Me4/Em3, Me7/Em9, Me7/Em5) to 12 (Me4/Em1), with an average of 4.9 amplicons per primer set. The percentage of polymorphic markers produced by each primer pair ranged from 33.3% (Me4/Em3) to 100% (Me2/Em9). The observed average number of alleles was 1.7024. The gene diversity varied from 0.0440 (Me7/Em3) to 0.2420 (Me7/Em5), with an average value of 0.1611. PIC values ranged from a low of 0.0411 (Me7/Em3) to a high of 0.1919 (Me7/Em5), with an average of 0.1353 (Table 2). Figure 1 illustrated the electrophoresis pattern obtained with SRAP markers in different accessions of genus *Morus*.

Genetic variation and cluster analysis based on SRAP markers

The data from all PCR amplification bands were used in the similarity evaluation. The genetic similarity matrix among all materials used in the present work was obtained after multivariate analysis using Nei's coefficient (data not shown). From the coefficient matrix, similarity coefficients ranged from 0.6905 to 0.9524, with an average of 0.8330, revealing high levels of genetic variation among the mulberry accessions studied. The highest similarity (0.9524) was between Dejiang 10 and Yunnanshuisang, both belonging to *Morus laevigata* Wall., indicating that they are closely related. The lowest genetic similarity coefficient (0.6905) was between Yaosang and T11, indicating that they are relatively remote, possibly because of differences in their genomes, as they are from different mulberry species.

An UPGMA cluster of the 23 mulberry accessions by using the total number of amplified SRAP fragments, and three major clusters were formed (Figure 2). Yaosang and Lijiangshansang were generally the most distant in comparison with the other accessions, forming independent cluster I and II groups; the other mulberry accessions grouped into cluster III.

Cluster III was divided into four subgroups: A, B, C, and D. From cluster analysis among mulberry accessions, we found that T11 and T12 belong to *Morus. rotundiloba* with dolichostylae in the morphological classification of the genus *Morus* (Koidzumi, 1917) and clustered together to form the A subgroup. In the B subgroup, Changnongshansang and Jianchi belonging to *M. bombycis* clustered together, then to Jimengsang, all of which belong to the dolichostylae in the morphological classification of the genus *Morus*, indicating that they are closely related. Yu 711 and Yu 82 belong to *M. multicaulis*; Lunjiao 40 belongs to *Morus atropurpurea* and was integrated into cluster C, all of whose accessions contained the guangdongsang pedigree. In the D subgroup, two *M. laevigata* accessions, Dejiang 10 and

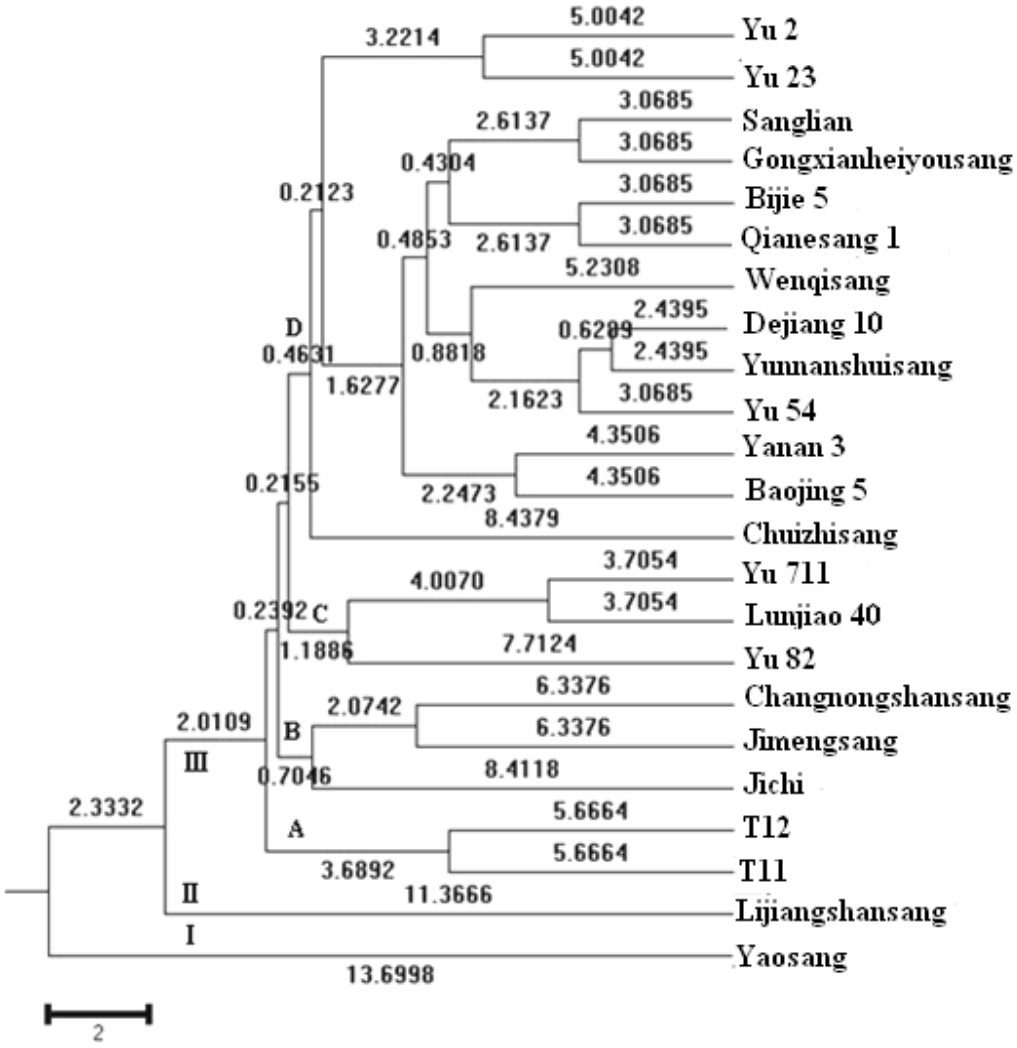

Figure 2. Dendrogram obtained using UPGMA for 23 mulberry accessions based on SRAP markers. The numbers correspond to those listed in Table 1. The numbers above the branches are branch lengths.

Yunnanshuisang, clustered together; two *Morus wittiorum* accessions, Bijie 5 and Qinesang 1, clustered together; and two *Morus cathayana* accessions, Yaan 3 and Baoqing 5, clustered together (Figure 2).

DISCUSSION

We successfully used SRAP, for the first time, to analyze the genetic diversity of 23 mulberry accessions. Primer combinations that produced only a few or many fragments were rejected, and those with poor reproducibility in repetitive experiments were not scored. Only the 83 fragments amplified by 12 primer combinations selected from the screened 72 primer combinations after repetitive experiments were scored. The amplified fragments could be classified into strong, intermediate, or weak categories on the basis of their intensity; only the strong and intermediate fragments were scored, and the weak categories were rejected. This variation in amplification preference could be a result of the copy number of the fragment in the genome and/or the degree of complementarity of the end sequences to those of the primers (Hu and Vick, 2003). Out of 83 fragments scored, 59 (71.1%) were polymorphic and were sufficient to distinguish 23 mulberry accessions, indicating the very high discriminating ability of the SRAP technique. The SRAP primers amplified dominant markers that were scored as two alleles per locus. An average of 1.70 observed alleles per locus was amplified for primer tested in this study. Mean gene diversity and PIC were 0.1611 and 0.1353, respectively (Table 2). The genetic diversity revealed by SRAP was higher than that of mulberry local varieties revealed by ISSR markers (Zhao et al., 2007b). SRAP analysis did not show any correlation among the number of amplified fragments, the degree of polymorphism and the mulberry

ploidy level. This study also found a smaller number of amplification bands (6.9 bands/per primer combination) compared with previous reports (10-20; Li and Quiros, 2001; Budak et al., 2004; Ferriol et al., 2004). It is plausible that the types of specimen analyzed and the different detection methods employed caused this difference, as more amplification products are produced by 6% PAGE electrophoresis and visualized with simple silver staining.

The SRAP molecular marker systems revealed considerable genetic diversity in the 23 mulberry accessions of diverse origin, with similarity coefficients ranging from 0.6905 to 0.9524. Hirano (1982) examined isozymes and several sap proteins and found no significant difference among mulberry species. However, the high level of genetic diversity observed in this study for this marker system reflects the outcrossing nature of the species and is consistent with results from previous studies carried out on mulberry genotypes using different molecular markers (Xiang et al., 1995; Feng et al., 1996; Zhao and Pan, 2004; Sharma and Sharma, 2000; Awasthi et al., 2004), thereby confirming the great diversity in mulberry genotypes.

The dendrogram obtained using the UPGMA method consisted of three major clusters (Figure 2). UPGMA cluster analysis showed clear genetic relationships among the 23 mulberry accessions. Cluster I and II, including Yaosang and Lijiangshansang, respectively; cluster III, including other mulberry accessions. *Morus nigra*, with the highest chromosome number among angiosperms (2n = 308), formed a single cluster. Compared with the other mulberry species, it has a more remote relationship. This is why it is difficult to hybridize this species with other mulberry species. A similar phenomenon was observed using RAPD markers (Zhao and Pan, 2004). The results from cluster analysis were in general agreement with our morphological classification, but there were also some differences. For instance, many accessions of *M. multicaulis*, *Morus alba*, and varieties of *M. alba* did not cluster together and were mixed with other species; however, according to the classification of the genus *Morus*, most of these belong to macromorus (Koidzumi, 1917).

In conclusion, these results obtained by SRAP analysis of mulberry accessions were in general agreement with our morphological classification, suggesting that SRAP is a simple and effective molecular marker technique and could be successfully applied to the study of genetic relationships, to the conservation and identification of mulberry collections, and to plant breeding. Our results also suggest that both morphological and molecular tools should be used for the classification of the genus *Morus*.

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