

# Genetic Diversity of Genus *Morus* Revealed by RAPD Markers

ZHAO WEIGUO AND PAN YILE<sup>1</sup>

Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang City, Jiangsu, China

<sup>1</sup>Corresponding author's e-mail: mbrcaas@pub.zj.jsinfo.net

## ABSTRACT

Forty-five mulberry accessions, which belong to 12 species, 4 varieties, and 1 *B. papyrifera* were examined using RAPD markers in this study. Total 157 bands ranging from 100 to 2500 bp in size, were obtained from 45 mulberry accessions by using 24 random primers (10 bp in length) screened from 182 primers, 113 of these bands showed polymorphism with the ratio of 72.0%. Based on the RAPD data, Nei's genetic similarity coefficient and genetic distance were calculated and analyzed using the UPGMA dendrogram. Additionally, phylogenetic relationship among 46 genotypes was determined. The result from cluster analysis was basically consistent with the morphological classification.

**Key Words:** *Morus* L.; RAPD; Germplasm resources; Genetic

**Abbreviations:** dNTP = Deoxynucleotide triphosphate; CTAB = Cetyltrimethylammonium bromide; PVP = polyvinylpyrrolidone; PCR = Polymerase chain reaction; RAPD = randomly amplified polymorphic DNA; TE = Tris-EDTA buffer; UPGMA = Unweighted pair group method with arithmetical averages

## INTRODUCTION

Mulberry is the most important crop plant in sericulture because it is used for silkworm (*Bombyx mori* L.) rearing for commercial purpose. Mulberries are a group of small trees or shrubs belonging to the family Moraceae, distributed in the temperate and subtropical regions of the northern hemisphere. In China, there are about 3,000 mulberry accessions. According to traditional morphological classification, they are divided into 15 species and 4 varieties (Pan, 2000). Mulberry is the base of sericultural production. The improvement in genetic potential for mulberry yield and quality has been a major contribution to the success of sericultural development in China. The objectives of mulberry breeding are to identify and create genetic variability, to assemble into the productive genotype, and to match the genotype to the appropriate environment. Therefore, evaluating genetic diversity is important for long term improvement in mulberry yield, quality and resistance.

Mulberry is widely distributed geographically, easily adaptable to different climatic conditions, quick in rejuvenation, amenable to various training and pruning methods, able to be propagated by both sexual and asexual ways, easy in hybridization by natural and artificial means, which made the mulberry genetic background rather easily approachable (Dandin, 1998). Many approaches have been reported for the study of genetic diversity in mulberry. Morphology is one of the common methods utilized in mulberry. Karyotypes/cytogenetics, isozyme analysis had been also used to study germplasm and taxonomy, but very little work has been done on characterization of mulberry genetics. So the classification of the genus *Morus* is rather highly debated and arbitrary one.

In recent years DNA-based markers allowing direct comparison of the genetic material of two individual plants have been used quite extensively. The RAPD technique

(Williams *et al.*, 1990) provides a convenient and rapid assessment of the differences in the genetic composition of the related individuals and has been employed in a large number of plants for the determination and assessment of genetic diversity. The genetic identities and relationships of mulberry plants including cultivars, diploids and polyploids have been determined using RAPD technology. Identification of mulberry by DNA analysis has been performed (Xiang *et al.*, 1995; Feng *et al.*, 1996; Zhang *et al.*, 1998; Lou *et al.*, 1998; Esha *et al.*, 2001). Xiang *et al.* (1995) first reported the presence of DNA polymorphisms in genus *Morus* caused by the insertion/deletion of DNA fragments and explored the genetic relationship of the nine species. Feng *et al.* (1996) also used RAPD to investigate the systematics of the genus *Morus*. Though these studies focused on variation among different species of genus *Morus*, very little is known about the intra-species genetic variation existing in mulberry. In this paper we report the results from the application of RAPD profiling, for the determination of differences amongst 45 genotypes of mulberry, which included 12 species and 4 varieties.

## MATERIALS AND METHODS

**Materials.** Forty-five individuals, representing 12 species and 4 varieties of genus *Morus* L. and one individual of *Broussonetia* L., using as outgroup species were sampled in this study (Table I).

**DNA isolation.** Total DNA was extracted from young leaves, following the method described by Doyle and Doyle (1987) with some modification: 1.5 g young leaves were ground in liquid nitrogen to a fine powder and extracted with cetyltrimethylammonium bromide (CTAB) hot extraction buffer [50 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 1.5% polyvinylpyrrolidone (PVP), (w/v) & 1% (v/v)  $\beta$ -mercaptoethanol]. The mixture was incubated at 65°C for 30 min, followed by two extractions with

**Table I. Origin and source of 46 genotypes used in this study**

Sr. No.	Genotypes	Species	2n	Origin
1	Lunjiao 40	<i>M.atropurpurea</i> Roxb.	3	Sunde city, Guangdong province, China
2	Youmaoyansang	<i>M. mongolica</i> Var. <i>diabolica</i> Koidz.	-	Dejiang city, guizhou province, China
3	Baoqing 5	<i>M.cathayana</i> Hemsl.	4	Baoqing city, hunan province, China
4	Yaan 3	<i>M.cathayana</i> Hemsl.	4	Yaan city, sichuan province, China
5	Dejiang 10	<i>M.Laevigata</i> Wall.	6	Dejiang city, guizhou province, China
6	Husang 32	<i>M.multicaulis</i> Perr.	2	Wuxi city, jiangsu province, China
7	Wanbaisang	<i>M.alba</i> Linn.	2	Hefei city, anhui province, China
8	Cuizhisang	<i>M.alba</i> var <i>pendula</i> Dipp.	2	Korea
9	Lijiangshansang	<i>M.bombycis</i> Koidz	-	Lijiang city, yunnan province, China
10	Wenqisang	<i>M.alba</i> var <i>venose</i> Delile.	2	Zhouzhi city, shanxi province, China
11	Jimengsang	<i>M.mongolica</i> Schneid.	2	Jilin province, China
12	Wukelan 1	<i>M.alba</i> Linn.	2	Ozbek
13	Jisang	<i>M.alba</i> Linn.	2	France
14	T11	<i>M. rotundiloba</i> Koidz.	2	Thailand
15	Tezao	<i>M.multicaulis</i> Perr.	2	Algeria
16	T12	<i>M. rotundiloba</i> Koidz.	2	Thailand
17	Ahuhan 1	<i>M.alba</i> Linn.	2	Afghanistan
18	Shuiyuandaye	<i>M.alba</i> Linn.	2	Korea
19	Longchuanqiuyu	<i>M.alba</i> Linn.	2	Korea
20	Sinan 2	<i>M.cathayana</i> Hemsl.	6	Sinan city, guizhou province, China
21	Shenli	<i>M.multicaulis</i> Perr.	2	Ozbek
22	Fu 1	<i>M.multicaulis</i> Perr.	2	Zhenjiang city,jiangsu province, China
23	Suhu 16	<i>M.multicaulis</i> Perr.	2	Zhenjiang city,jiangsu province, China
24	Dalu 1	<i>M.atropurpurea</i> Roxb.	2	Bohai city, yuannan province, China
25	K54	<i>M.atropurpurea</i> Roxb.	2	India
26	Gongxianheiyousang	<i>M.alba</i> var <i>macrophylla</i> Loud.	2	Gongxian city,Sichuan province, China
27	Yu 4	<i>M.atropurpurea</i> Roxb.	2	Viet Nam
28	Fengweiyabian	<i>M.multicaulis</i> Perr.	2	Zhenjiang city,jiangsu province, China
29	Dayesizhimu	<i>M.alba</i> Linn.	2	Japan
30	Jianchi	<i>M.bombycis</i> Koidz.	2	Japan
31	Dajiguan	<i>M.multicaulis</i> Perr.	2	Linju city, shandong province, China
32	Yunnanshuisang	<i>M.Laevigata</i> Wall.	3	Yunnan province, China
33	Xinyizhilan	<i>M.alba</i> Linn.	2	Japan
34	Housang	<i>M.mizuho</i> Hotta.	3	Yuhang city,zhejiang province, China
35	Jinlong	<i>M.mizuho</i> Hotta.	2	Japan
36	Niuersang	<i>M.alba</i> Linn.	2	Yangcheng city, shanxi province, China
37	Chasang	<i>M.Australis</i> Poir.	-	Sichuan province, China
38	Qiner	<i>M.atropurpurea</i> Roxb.	2	Qinzhou city, guangdong province, China
39	Bijie 5	<i>M.wittiorum</i> Hand-Mazz.	6	Biejie city, guizhou province, China
40	Yaosang	<i>M.nigra</i> Linn.	22	Xingjiang autonomous region, China
41	Naxisang	<i>M.multicaulis</i> Perr.	2	Naxi city, sichuan province, China
42	Qinesang 1	<i>M.wittiorum</i> Hand-Mazz.	2	Dejiang city, guizhou province, China
43	Jieque	<i>M.multicaulis</i> Perr.	2	Japan
44	Gui 14	<i>M.wittiorum</i> Hand-Mazz.	4	Biejie city, guizhou province, China
45	Wubingsang	<i>M.alba</i> Linn.	2	Japan
46	Guoshu	<i>B. papyrifera</i> Linn.	-	Nanjiang agricultural university, China

Note: The mulberry materials of No. 1-45 are from Sericulturai Research Intitute, CAAS, China

chloroform/isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids and the pellet obtained was dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0 & 1 mM EDTA, pH 8.0). RNA was removed by digestion with deoxyribonuclease-free ribonuclease and remaining impurities were extracted with chloroform. Total DNA was precipitated using cold ethanol, and the precipitate was washed twice with 75% ethanol and finally dissolved in TE buffer. The purified total DNA was quantified by gel electrophoresis and its quality verified by spectrophotometry. DNA samples were stored at 4°C.

**PCR amplification.** Double-stranded DNA was directly amplified by symmetric PCR in GeneAmp 9700 (Perkin Elmer, Norfolk Connecticut). PCR reaction mixture

consisted of 10xPCR Buffer (100 mM Tris-HCl pH8.3, 500 mM KCl, 0.01% gelatin), 5 pmol of 10-mer primer (Sequence of random primers were published on the brochure of Operon Technology Inc.), dNTP□200 μM of each dATP, dGTP, TTP, TP, 1U Taq DNA polymerase, 2.5 mM MgCl<sub>2</sub>, approximately 25 ng DNA template, and filtered ddH<sub>2</sub>O to a final volume of 20 μL. The 100 μL of mineral oil was overlaid the mixture to prevent evaporation. The procedure was conducted through the following PCR thermal cycles: template pre-denaturation for 2 min at 95°C, then 38 cycles of 30s at 94°C for template denaturation, 30s at 40°C for primer annealing, 90s at 72°C for primer extension, followed by a final extension of 7min at 72°C followed be cooling to 4°C. All amplification products were

visualized on 1.0% agarose gels employing 2 kb ladder (MBI, Shanghai Sangon Biological Engineering & Technology Co. Ltd.) as a molecular weight standard pre-stained with 0.2% ethidium bromide (EB) using 1xTBE and photographed with Polaroid film under UV light.

**RAPDs data analysis.** Each primer generating polymorphic bands was respectively used in the second PCR. All the bands of DNA fragments of 46 genotypes were compared with each other. Only those distinct and polymorphic bands repeated in two-times PCR were determined for sizes and presence (scored as 1) or absence (scored as 0). The data for all the primers were used to estimate the similarity on the basis of the number of bands. The genetic similarities among materials were calculated pairwise by the formula

$I = 2m_{xy} / (m_x + m_y)$  (Nei *et al.*, 1979), in which  $m_x$  and  $m_y$  represent the total amplification bands of each sample, respectively;  $m_{xy}$  is the total number of matches between two materials. When the two genotypes have identical fragment pattern I is equal to 1, or when there is no common band between the two genotypes I is equal to 0. The genetic distance (D) among materials was calculated by the formula  $D = -\ln I$ . The dendrogram based on similarity coefficients was generated using the unweighed pair group methods of arithmetic means (UPGMA) by the Popgene 32 version 3.5 computer package by PHYLIP.

**Table II. Amplification of 24 primers on 45 mulberry genotypes**

Primers	Sequence 5'-3'	G+C Content %	Loci	Polymorphic bands	Percent of polymorphic bands %
O-12	CAGTGCTGTG	60	8	5	62.5
D-20	ACCCGGTCAC	70	9	7	77.8
F-06	GGGAATTCGG	60	11	9	81.8
J-12	GTCCCCTGGT	70	6	5	83.3
O-09	TCCCACGCAA	60	7	5	71.4
D-09	CTCTGGAGAC	60	2	1	50.0
J-15	TGTAGCAGGG	60	6	4	66.7
J-13	—	-	4	3	75.0
F-14	TGCTGCAGGT	60	2	1	50.0
J-16	CTGCTTAGGG	60	8	6	75.0
L-06	GAGGGAAGAG	60	1	1	100.0
G-04	—	-	6	3	50.0
J-01	CCCGGCATAA	60	10	9	90.0
J-05	CTCCATGGGG	70	8	7	87.5
J-20	AAGCGGCCTC	70	5	4	80.0
D-07	TTGGCACGGG	70	7	6	85.7
A-09	GGTAACGCC	70	6	3	50.0
K-01	CATTGAGCC	60	7	5	71.4
J-04	CCGAACACGG	70	8	6	75.0
J-18	TGGTCGCAGA	60	9	7	77.8
D-06	ACCTGAACGG	60	7	5	71.4
D-03	GTCGCCGTCA	70	9	6	66.7
L-07	AGGCGGGAAC	70	8	5	62.5
A-06	GGTCCCTGAC	70	3	1	33.3
<b>Total/Average</b>			<b>157</b>	<b>113</b>	<b>72.0</b>

**Table III. DNA polymorphism of intra-species in mulberry**

Sample	1	2	3	4	5	6	7	8
No. of accessions	9	10	5	2	2	3	3	2
Total bands of intraspecies	123	124	120	118	103	113	112	95
Shared bands of intraspecies	68	69	74	78	72	82	72	94
Polymorphism in intraspecies (%)	44.7	44.4	38.3	33.9	30.1	27.4	35.7	0.99

Note: 1-8 presented the name of species, *M. multicaulis*, *M. alba*, *M. atropurpurea*, *M. mizuho*, *M. Laevigata*, *M. wittiorum*, *M. cathayana* and *M. rotundiloba*, respectively

## RESULTS AND DISCUSSION

### Primer screening and the result of PCR amplification.

All the 46 genotypes screened showed polymorphism with 182 random decamer primers. 24 primers were proved to generate clear and polymorphic bands of various lengths. One hundred fifty seven bands amplified DNA fragments produced from 45 mulberry accessions were approximately between 100 to 2500bp. Of them, 113 bands showed polymorphism, with the ratio of 72.0%. The number of polymorphic bands per primer was 4.7. The number of fragment per primer ranged from 1 to 11 in L-06 and F-06 respectively. The average number of bands per primer was 6.5 (Table I). The results of PCR amplification were given in Fig.1. One hundred and thirteen polymorphic bands were unique and could be used to discriminate the species or varieties. Furthermore, some were species or varieties specific. For instance, two primers F-06 and O-09 generated bands unique to *M. nigra*. In addition, there was rich polymorphism among intra-species, in the range of 27.4%-44.7%. But there was only 1.1% polymorphism in the species of *M. rotundiloba*, probably due to the fact that they were from the same place, Thailand, and had the same characters (Table III). From the data of Table III, we also found that they had higher genetic diversity among intraspecies. Compared with *B. papyrifera*, there were only 11 common bands from total of 157 bands. This demonstrated the existence of significant genetic differentiation.

**Cluster analysis of DNA polymorphism in genus mulberry.** The data of all PCR amplifications was used in

**Table IV. Mean coefficients of genetic similarity (above) and genetic distance (below) among inter-, intra-species of mulberry and *B. papyrifera***

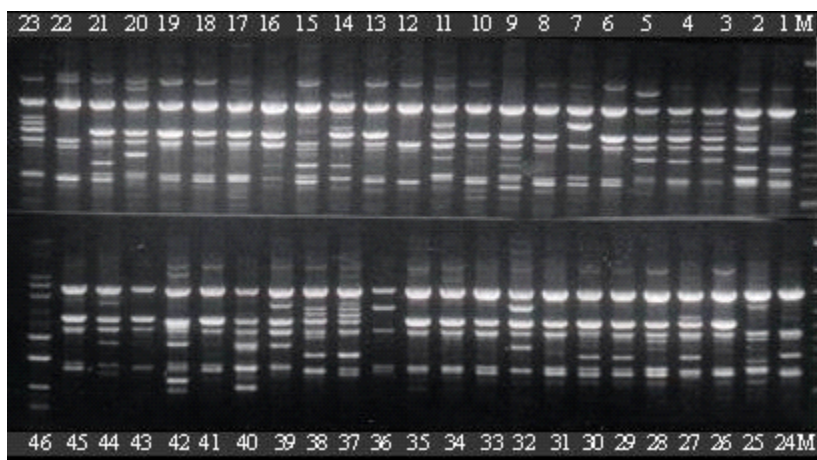
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0.7885	0.9682	0.9194	0.8144	0.9032	0.8855	0.8615	0.8921	0.7785	0.8125	0.7887	0.8099	0.7692	0.6789	0.7356	0.6502	0.6623
2	0.0323	0.7764	0.9376	0.8111	0.9080	0.8856	0.8600	0.9015	0.7839	0.8242	0.7877	0.8107	0.7560	0.6759	0.7349	0.6509	0.6500
3	0.0841	0.0644	0.7894	0.7692	0.8822	0.8689	0.8570	0.8540	0.7451	0.7516	0.7307	0.7917	0.7763	0.6241	0.7219	0.6068	0.6213
4	0.2053	0.2094	0.2624	0.6549	0.7514	0.7148	0.7531	0.7881	0.7761	0.8260	0.8036	0.7661	0.7603	0.7253	0.7092	0.6654	0.6209
5	0.1018	0.0965	0.1253	0.2858	****	0.8673	0.8319	0.8570	0.6917	0.7352	0.7051	0.7207	0.7522	0.6814	0.6372	0.6195	0.6283
6	0.1216	0.1215	0.1405	0.3357	0.1424	****	0.8584	0.8030	0.6737	0.7126	0.7031	0.7473	0.6726	0.6372	0.6460	0.5575	0.5664
7	0.1491	0.1509	0.1543	0.2836	0.1841	0.1527	****	0.8433	0.6972	0.7203	0.7200	0.7473	0.6903	0.6549	0.6460	0.5752	0.5664
8	0.1142	0.1037	0.1578	0.2381	0.1543	0.2194	0.1705	0.7876	0.7369	0.8181	0.7754	0.7825	0.7305	0.6835	0.7027	0.6100	0.5822
9	0.2504	0.2435	0.2943	0.2535	0.3686	0.3950	0.3607	0.3054	0.7404	0.8183	0.8480	0.7341	0.7187	0.6313	0.6688	0.7277	0.5632
10	0.2076	0.1933	0.2856	0.1912	0.3076	0.3389	0.3280	0.2007	0.2005	0.7080	0.8597	0.7580	0.7142	0.7087	0.6587	0.6829	0.5886
11	0.2373	0.2386	0.3138	0.2187	0.3495	0.3523	0.3285	0.2544	0.1649	0.1512	0.8112	0.7412	0.7175	0.7255	0.6869	0.6660	0.6067
12	0.2108	0.2099	0.2336	0.2664	0.3275	0.2913	0.2913	0.2452	0.3091	0.2770	0.2995	0.9912	0.7865	0.6321	0.7599	0.5523	0.5914
13	0.2624	0.2797	0.2533	0.2740	0.2847	0.3967	0.3707	0.3141	0.3303	0.3366	0.3320	0.2402	****	0.6991	0.7080	0.5841	0.6460
14	0.3873	0.3917	0.4715	0.3211	0.3836	0.4507	0.4233	0.3806	0.4601	0.3443	0.3210	0.4588	0.3579	****	0.5664	0.6549	0.5575
15	0.3071	0.3080	0.3258	0.3436	0.4507	0.4369	0.4369	0.3529	0.4023	0.4175	0.3755	0.2746	0.3454	0.5685	****	0.5221	0.6018
16	0.4304	0.4294	0.4995	0.4074	0.4789	0.5843	0.5530	0.4943	0.3179	0.3814	0.4064	0.5937	0.5377	0.4233	0.6499	****	0.5133
17	0.4121	0.4308	0.4760	0.4766	0.4647	0.5685	0.5685	0.5409	0.5741	0.5300	0.4998	0.5252	0.4369	0.5843	0.5079	0.6669	****

Note: the number of 1-17 represent *M. multicaulis*, *M. alba*, *M. atropurpurea*, *M. bombycis*, *M. alba var pendula*, *M. alba var venose*, *M. alba var macrophylla*, *M. mizuho*, *M. cathayana*, *M. laevigata*, *M. wittiorum*, *M. rotundiloba*, *M. mongolica vardiabolica*, *M. mongolica*, *M. australis*, *M. nigra*, and *B. papyrifera*, respectively

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 coefficient ranged from 0.4690 to 0.9912. The wide variation in genetic similarity coefficient among the different genotypes revealed by RAPD techniques reflected a high level of polymorphism at the DNA level. Earlier studies by Xiang *et al.* (1995), Feng *et al.* (1996), and Esha *et al.* (2001) using the same technique also observed a large genetic variation among different *Morus* genotypes. Such a high level of polymorphism reflects the outcrossing nature of the species. The highest similarity (0.9912) was found between T11 and T12. The result indicated that they almost have the same genetic constituents. The reason was probably due to that both T11 and T12 belong to *M. rotundiloba* and originated

from the same place. The least similarity between *B. papyrifera* and Yaan 3 was ascribed to their genome difference, because *B. papyrifera* and Yaan 3 were from different genus. For further revealing the genetic evolution relationship between different mulberry species and *B. papyrifera*, the mean genetic similarity coefficient of interspecies was analyzed (Table IV). It was observed from Table IV that mean genetic similarity coefficient among *M. multicaulis*, *M. alba* and the varieties of *M. alba* was above 0.8000, indicating their closer relationship. The wild species and cultivated species of mulberry had a rather remote relationship since the genetic similarity of any pairwise comparisons went below 0.7000. Compared with cultivated species of mulberry, *M. nigra* had the highest remote relationship, followed by *M. mongolica* and *M. cathayana*.

**Fig.1. The RAPD agarose gel electrophoresis profiles of 46 genotypes.** The profiles were obtained using primers D-20. Numbers refers to the samples listed in Table 1. M: DNA marker

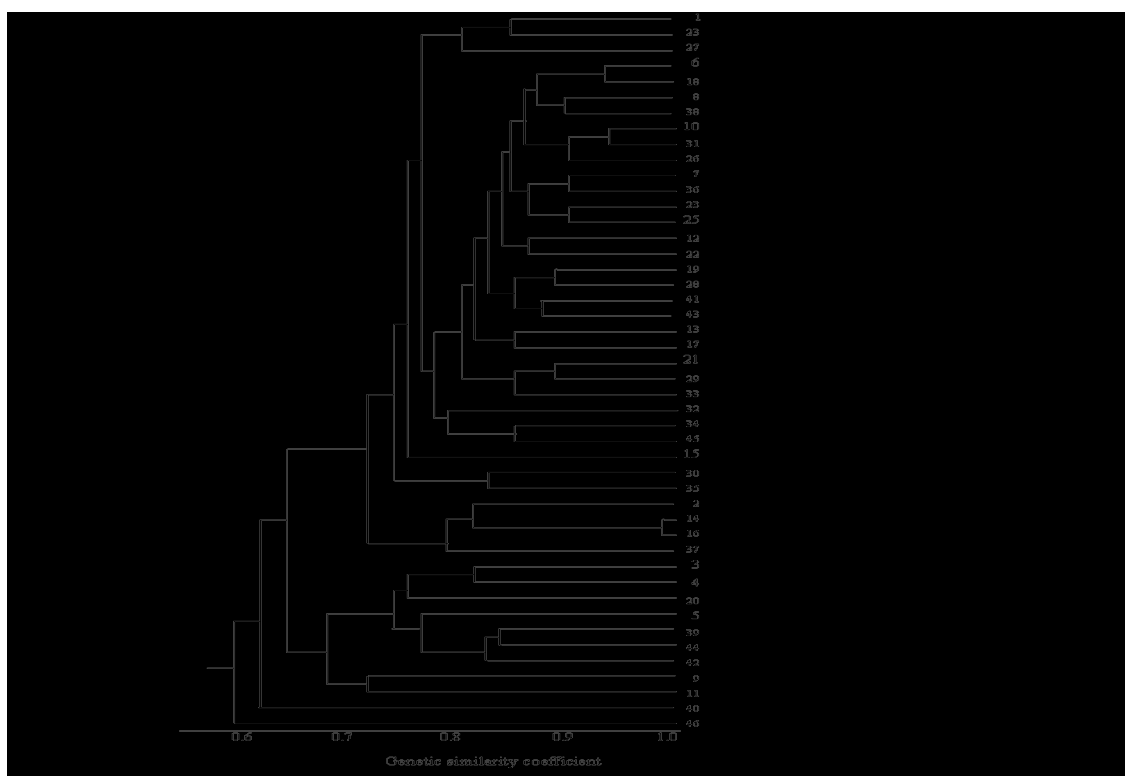


Cluster analysis was carried out on the matrix of similarity coefficient with UPGMA method and the phylogeny tree was constructed (Fig. 2). From clustering analysis, it was found that *M. multicaulis*, *M. alba* and the varieties of *M. alba*, which belong to macromorus in the classification of the genus *Morus* (Koidzumi, 1917), clustered together, had closer relationship and should be clustered into a single species. Three *M. cathayana* accessions, including Baoqing 5, Yaan 3 and Sinan 2, clustered together and formed a subgroup. Three *M. wittiorum* accessions, including Bijie 5, Qinesang 1 and Gui 14, cultured together. Two *M. rotundiloba* koidz. Accessions, including T11 and T12, clustered together with *M. australis* and *M. mongolica* var. *diabolica*, which belong

to *dolichostylae* in the classification of the genus *Morus*. *M. nigra* with highest chromosome number among angiosperms ( $2n=308$ ), having the name of the king of mulberry, formed a cluster alone. Compared with other mulberry species, it had a more remote relationship. This explained the reason why it was difficult to hybridize it with other mulberry species. The results from cluster analysis were in general agreement with our morphologic classification.

The result from current study indicated that RAPDs offer a reliable and an efficient method of assessing genetic diversity and thus provide an avenue for help in the selection of parents for breeding purpose.

Fig. 2. Dendrogram of 46 genotypes by UPGMA analysis. 1-46 represent the number of genotypes shown in Table 1



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