Research Article

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Molecular characterization of mulberry (*Morus* spp.) genotypes via RAPD and ISSR

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Abstract

BACKGROUND: In recent years, DNA-based markers have been used quite extensively because of their many advantages over the traditional morphological and biochemical markers. Many studies have shown that molecular markers are useful in delineating the genetic relationships among closely related mulberry genotypes and cultivars. Thus, in the present study, polymer chain reaction based DNA fingerprinting techniques were used to investigate the genetic relationships among mulberry genotypes growing in different agro-climatic regions of Turkey.

RESULTS: 20 RAPD primers generated a total of 173 bands, of which 157 (90.75%) were polymorphic. As for 11 ISSR primers, 124 bands (96.55%) were polymorphic in a total of 128. The similarity index for RAPD technique ranged between 0.24–0.98; 25İs203 with 25İs112 were found to be the closest genotypes, while 24Ke10 and 25İs123 were the most distant ones. According to the ISSR result, the genetic similarity index changed between 0.21–095; 25İs203 with 25İs112 genotypes were the closest, while 25İs08 and 01KaD2 were the most distant ones.

CONCLUSION: The RAPD and ISSR markers were found to be promising for assessing genetic diversity in mulberry genotypes.

Keywords: RAPD; ISSR; mulberry; genetic similarity

INTRODUCTION

Mulberries belong to the genus *Morus* in the Moraceae family. About 12 mulberry species are cultivated worldwide for their foliage, fruit, timber or for ornamental use. Among them, *Morus alba, M. rubra* and *M. nigra* have been used for their fruits in southwest China in the Far East (*M. alba*), in North America (*M. rubra*) and in Central Asia, Anatolia, Southern Caucasus and Iran (*M. nigra*), which are genetic diversity centers.[1] In Turkey, *M. alba, M. nigra* and *M. rubra* varieties are grown. Mulberry is an important crop in Turkey and has been cultivated for centuries. About 2.3 million mulberry trees are present in Turkey and approximately 65 000 tons of mulberry fruit are produced annually. Although mulberry grows in every region of Turkey, the highest production is obtained from eastern Anatolia and the Mediterranean regions.[2]

Mulberry is a very important plant in the sericulture economics of the country since its foliage is used for rearing silkworms.[3] Therefore, studies on mulberries are mostly related to leaf properties. Eliminating fruit was considered to be one of the most important characters for mulberry breeding, because the mulberry fruit is not necessary for sericulture. However, the mulberry fruit is grown in many European and Middle Eastern countries. In Japan, it has been evaluated as a product that could stimulate upland farming.[4] Anatolia is one of the genetic diversity centers of mulberry species. Consequently, Turkey has a rich genetic potential. Numerous genotypes possessing fruit characteristics have been destroyed through timber use. This situation has caused a large decrease in valuable germplasm and exhaustion of mulberry genotypes. Although Turkey has very rich mulberry populations, selection studies related to desirable characters have been poorly conducted. If high-yielding and quality fruit phenotypes were selected and then propagated, mulberry production could be increased.

In recent years, DNA-based markers have been used quite extensively[5] because of their advantages over traditional morpho-biochemical markers.[6,7] Morphological markers are few and might have epistatic effects. Isozyme markers are also few in number and may be affected by the environment. Therefore, DNA markers which are not affected by the environment have been suggested for the determination of genetic similarity among the genotypes.[8] DNA markers such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), sequence-characterized amplified regions (SCAR) and amplified fragment length polymorphism (AFLP) have advantages in producing large amounts of markers and can be used for various purposes such as fingerprinting, marker-assisted selection, genetic mapping, etc.[9] Two of them – RAPD and ISSR markers – were used to study the molecular characterization of mulberry cultivars and genotypes.[10,11] ISSR has been the most common marker system used in mulberries.[12–19] Furthermore, in mulberry ISSR has been

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Table 1. Fruit properties of 21 mulberry genotypes

Genotypes	Region	TC	AFW (g)	FW (cm)	FL (cm)	SSC (%)	TTA (%)
23 Mrk 09	Elazığ	Table	3.95	1.52	2.68	22.4	0.14
25 Uz 05	Erzurum	Table	4.18	1.45	3.15	19.1	0.10
25 Uz 08	Erzurum	Table	3.70	1.52	2.52	20.02	0.10
25 İs 204	Erzurum	Table	3.90	1.58	3.06	21.6	0.49
24 Ke 09	Erzincan	Dried	2.25	1.28	1.82	25.73	0.17
24 Ke 10	Erzincan	Dried	2.42	1.37	2.26	24.9	0.14
25 İs 146	Erzurum	Dried	2.28	1.34	2.16	25.2	0.37
25 İs 147	Erzurum	Dried	3.19	1.39	2.53	23.9	0.34
25 Uz 01	Erzurum	Pekmez	3.69	1.47	2.43	24.3	0.10
25 Uz 10	Erzurum	Pekmez	3.69	1.59	2.30	20.4	0.18
25 Uz 18	Erzurum	Pekmez	4.14	1.56	2.57	22.6	0.12
25 İs 110	Erzurum	Pekmez	3.88	1.53	2.56	21.8	0.46
25 İs 112	Erzurum	Pekmez	2.95	1.33	2.70	21.4	0.30
25 İs 123	Erzurum	Pekmez	3.57	1.37	2.60	23.7	0.35
25 İs 203	Erzurum	Pekmez	3.68	1.41	2.32	19.5	0.39
25 İs 205	Erzurum	Pekmez	3.79	1.52	2.48	21.5	0.48
25 İs 206	Erzurum	Pekmez	4.28	1.70	2.78	21.9	0.35
25 To 04	Erzurum	Pekmez	3.68	1.62	2.91	23.8	0.11
25 İs 08	Erzurum	Juice	3.02	1.26	2.16	15.5	0.16
01 Ka D1	Adana	Şıra	3.80	1.71	2.41	19.3	1.31
01 Ka D2	Adana	Şıra	4.14	1.66	2.40	15.3	1.27

TC, type of consumption; AFW, average fruit weight; FW, fruit width; FL, fruit length; SSC, soluble solids content; TTA, total titratable acidity.

successfully used to study the genetic divergence among selected genotypes and cultivars.[3,20]

These studies proved that molecular markers are useful in delineating the genetic relationships among closely related mulberry genotypes and cultivars. Thus in the present study PCR-based DNA fingerprinting techniques were used to investigate the genetic relationships among mulberry genotypes growing in different agro-climatic regions of Turkey.

MATERIALS AND METHODS

Plant material

Twenty-one mulberry genotypes were originally collected from four geographic regions (Adana, Elazığ, Erzincan and Erzurum). Three of them were *M. nigra* L. (01KaD1, 01KaD2 and 25İs08) and the others are *M. alba* L. These genotypes were classified and described according to consumption type (Table, Dried, Pekmez, Fruit Juice and Şıra) (Table 1).

DNA extraction

Total genomic DNA was extracted from fully expanded leaves of actively growing shoots, with the 'NucleoMagPlant_KFmL' plant DNA extraction kit (Thermo Fisher Scientific, Inc., Vantaa, Finland). The required dilution of DNA was done by quantification of the DNA on 0.8% agarose gel, followed by staining with ethidium bromide, using λ DNA as a standard.

RAPD and ISSR fingerprinting

Polymerase chain reaction (PCR) amplifications of genomic DNA with RAPD primers were conducted according to Kafkas *et al.*,[21] modified from Williams *et al.*[22] Twenty decamer oligonucleotide

Table 2. Primers and polymorphism of RAPD and ISSR

Primer	AT	TBN	PBN	PR (%)	PIC	RP – $\overline{RP}$
RAPD						
OP-B01	36	12	10	83.33	0.566	4.190–0.349
OP-B02	36	4	4	100.00	0.914	2.095–0.523
OP-B03	36	8	8	100.00	0.611	2.476–0.309
OP-B08	36	12	10	83.33	0.450	3.428–0.285
OP-G02	36	8	7	87.50	0.871	2.380–0.297
OP-G03	36	9	9	100.00	0.952	2.952–0.328
OP-G04	36	8	7	87.50	0.576	4.285–0.535
OP-G05	36	11	10	90.90	0.525	4.190–0.380
OP-G08	36	6	5	83.33	0.479	2.571–0.428
OP-G10	36	8	6	75.00	0.670	1.904–0.238
OP-G11	36	8	8	100.00	0.804	3.714–0.464
OP-G12	36	6	6	100.00	0.539	2.000–0.333
OP-G13	36	9	7	77.77	0.459	1.714–0.190
OP-G14	36	8	8	100.00	0.381	1.523–0.190
OPA-01	36	9	8	88.88	0.495	3.333–0.370
OPA-02	36	9	9	100.00	0.549	3.047–0.338
OPA-04	36	13	13	100.00	0.574	4.476–0.344
OPA-06	36	5	5	100.00	0.883	3.333–0.666
OPA-12	36	8	8	100.00	0.644	2.761–0.345
OPA-13	36	12	9	75.00	0.419	2.666–0.222
Total		173	157	–	–	–
Means		8.65	7.85	91.63	0.618	2.951–0.357
ISSR						
UBC-807	50	16	15	93.75	0.79	5,619–0,351
UBC-808	52	10	10	100.00	0.56	3,809–0,380
UBC-809	52	11	10	90.91	0.68	5,428–0,493
UBC-810	50	12	11	91.67	0.46	4,476–0,373
UBC-811	52	7	7	100.00	0.58	1,904–0,272
UBC-812	50	7	7	100.00	0.68	3,047–0,435
UBC-826	52	15	15	100.00	0.39	5,238–0,349
UBC-827	52	11	11	100.00	0.55	3,809–0,346
UBC-835	54	11	11	100.00	0.68	5,904–0,536
UBC-861	54	7	6	85.71	0.66	2,857–0,408
UBC-881	60	21	21	100.00	0.83	12,857–0,612
Total		128	124	–	–	–
Means		11.64	11.27	96.55	0.62	4.995–0.414

AT, annealing temperature; TBN, total bands number; PBN, polymorphic band number; PR, polymorphism rate; RP, resolving power; $\overline{RP}$, means of resolving power.

primers were used to investigate mulberry genotypes. PCR amplification utilized 20 μL of reaction mixture containing 75 mmol L^{-1} Tris–HCl, 20 mmol L^{-1} $(NH_4)_2SO_4$, 0.1% Tween 20, 2.0 mmol L^{-1} $MgCl_2$; 100 μmol L^{-1} each of dGTP, dATP, dCTP, and dTTP; 100 pmol L^{-1} primer, 1 U Taq polymerase and 10 ng genomic DNA. The PCR schedule followed was 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 36 °C for 60 s, 72 °C for 2 min and a final incubation at 72 °C for 5 min. The PCR product was resolved on a 1.8% agarose gel in TBE buffer (89 mmol L^{-1} Tris–HCl, 89 mmol L^{-1} borate, 2 mmol L^{-1} ethylenediaminetetraacetic acid) at a constant current of 90 V for approximately 4 h, and visualized with ethidium bromide under UV light.

Eleven ISSR primers were selected for this study based on the results of earlier studies.[3,19] PCR amplifications of genomic DNA were conducted according to Zietkiewicz *et al.*[23] The

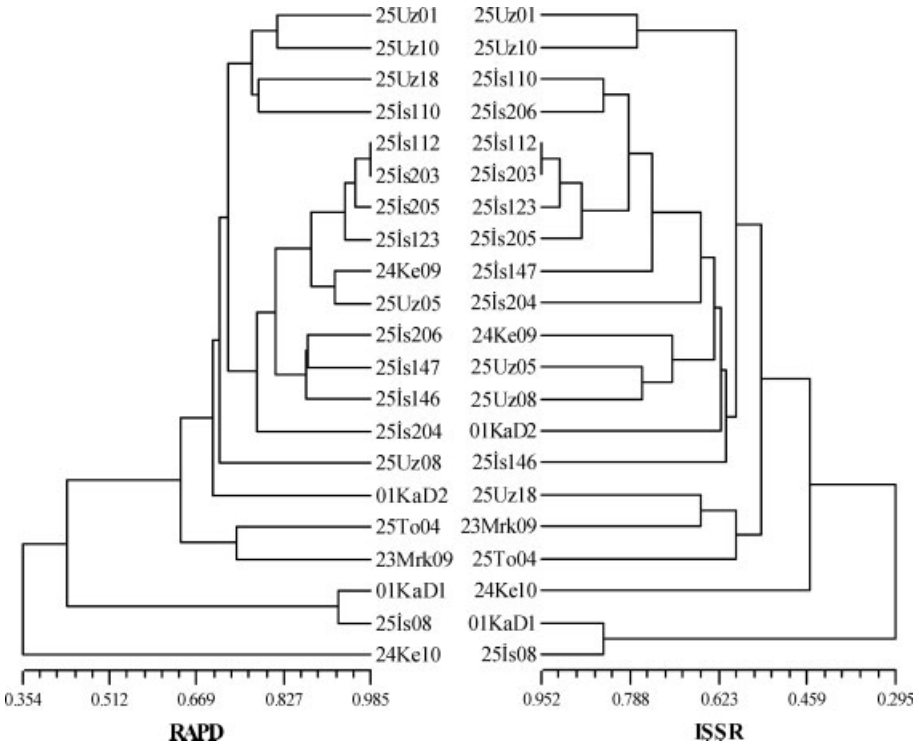

Figure 1. RAPD and ISSR dendrograms derived from UPGMA cluster analysis.

PCR amplification utilized 20 µL of reaction mixture containing 75 mmol L^{-1} Tris–HCl, 20 mmol L^{-1} $(NH_4)_2SO_4$, 0.1% Tween 20, 2.0 mmol L^{-1} $MgCl_2$; 100 µmol L^{-1} each of dGTP, dATP, dCTP, and dTTP; 100 pmol L^{-1} primer, 1 U Taq polymerase and 10 ng genomic DNA. The PCR schedule followed was 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 40–60 °C for 1 min, 72 °C for 2 min and a final incubation at 72 °C for 7 min. The PCR products were separated on a 1.5% agarose gel.

Data analysis

Only distinct, reproducible, well-resolved fragments were scored as present (1) or absent (0) for each of the RAPD and ISSR markers. Genetic similarity among the genotypes was estimated on the basis of the RAPD and ISSR band profiles generated by each primer with the genomic DNA of these genotypes, following the similarity coefficient of Jaccard,[24] in which the polymorphism information content (PIC) of each marker was calculated using a modified form of the original formula

$$\mathrm{PIC} = 1 - \sum P_i^2$$

where P_i is the band frequency of the ith allele.[25] The resolving power of primers was calculated from the formula

$$\mathrm{RP} = \sum I_b$$
$$I_b = 1 - (2 \times |0.5 - p|)$$

where p is the rate of I band in all genotypes.[26] Genetic distance estimated by Jaccard coefficient between pairs and dendrograms based upon the unweighted pair group method with arithmetical averages (UPGMA) were analyzed using NTSYSpc 2.2.[27] The correlation coefficient among the matrices and cophenetic correlation were tested by Mantel's Z-statistics.[28]

RESULTS AND DISCUSSION

DNA polymorphism among the mulberry genotypes

All the primers selected for this study generated DNA polymorphism among 21 mulberry genotypes. A total of 173 RAPD markers were generated by the 20 primers; 157 of these markers were polymorphic, indicating 91.63% DNA polymorphism among the genotypes (Table 2). While these results were found to be higher than some other reports,[5,10,11,14] they were lower than others.[29–31] The size of the amplified products ranged from 400 to 2200 bp, with 4–13 bands per primer.

Of the total 128 bands produced by the 11 ISSR markers, 124 bands were polymorphic, generating 96.55% polymorphism (Table 2). The amplified product sizes ranged from 520 to 2200 bp, with 6–21 bands per primer. The results clearly showed that ISSR and RAPD primers had close values in revealing DNA polymorphism among the mulberry genotypes.

Genetic similarity among the mulberry genotypes

Genetic similarity was estimated from the RAPD and ISSR markers by following the methods of Jaccard.[24] The maximum genetic similarity for RAPDs (0.985) was recorded between 25İs203 and 25Is112, while the minimum (0.241) was found between 25İs08 and 25Ke10. Further, the average genetic similarity was calculated as 0.652. On the other hand, the maximum genetic similarity (0.952) was recorded again between 25İs112 and 25İs203, while the minimum (0.212) was found between 01KaD2 and 25İs08 in the ISSR analysis. In addition, the average genetic distance was calculated as 0.551 (Table 3). Finally, the average genetic distances obtained from RAPD and ISSR analysis indicate considerable genetic diversity among the selected mulberry genotypes.

The similarity matrix based on RAPD and ISSR datasets is graphically represented as a dendrogram using the UPGMA method shown in Fig. 1. The dendrograms of RAPD and ISSR

Table 3. Genetic similarity among the 21 genotypes of mulberry realized from RAPD and ISSR (upper half) markers

	25Uz01	25Uz10	25Uz18	25Is110	25Is112	25Is123	25Is203	25Is205	25Is206	25To04	24Ke09	24Ke10	25Is146	25Is147	23Mr09	25Uz05	25Uz08	25Is204	01KaD1	01KaD2	25Is08
25Uz01		0.773	0.649	0.741	0.674	0.626	0.678	0.688	0.694	0.458	0.561	0.420	0.600	0.652	0.525	0.573	0.575	0.578	0.312	0.500	0.315
25Uz10	0.815		0.648	0.659	0.565	0.538	0.570	0.583	0.616	0.526	0.526	0.528	0.580	0.560	0.539	0.521	0.571	0.505	0.360	0.464	0.366
25Uz18	0.748	0.806		0.667	0.583	0.571	0.607	0.567	0.620	0.591	0.522	0.547	0.542	0.598	0.656	0.517	0.658	0.537	0.277	0.489	0.295
25Is110	0.752	0.774	0.782		0.788	0.736	0.770	0.739	0.838	0.570	0.608	0.457	0.564	0.724	0.563	0.571	0.647	0.611	0.289	0.596	0.277
25Is112	0.819	0.684	0.637	0.784		0.916	0.952	0.867	0.867	0.553	0.624	0.448	0.615	0.756	0.511	0.620	0.663	0.700	0.304	0.663	0.280
25Is123	0.774	0.646	0.601	0.739	0.943		0.917	0.856	0.791	0.523	0.646	0.453	0.621	0.725	0.500	0.594	0.615	0.670	0.282	0.635	0.270
25Is203	0.820	0.686	0.628	0.772	0.985	0.943		0.910	0.826	0.523	0.644	0.438	0.653	0.739	0.484	0.608	0.648	0.703	0.298	0.649	0.287
25Is205	0.807	0.674	0.628	0.772	0.956	0.930	0.956		0.791	0.489	0.638	0.411	0.698	0.747	0.469	0.589	0.608	0.660	0.318	0.627	0.295
25Is206	0.803	0.711	0.716	0.808	0.820	0.786	0.807	0.807		0.568	0.656	0.458	0.579	0.779	0.542	0.602	0.702	0.644	0.306	0.628	0.295
25To04	0.610	0.660	0.680	0.691	0.575	0.553	0.566	0.578	0.643		0.581	0.508	0.460	0.548	0.591	0.540	0.667	0.453	0.313	0.494	0.282
24Ke09	0.780	0.684	0.637	0.770	0.886	0.876	0.887	0.900	0.820	0.563		0.429	0.590	0.670	0.539	0.729	0.689	0.602	0.305	0.604	0.307
24Ke10	0.358	0.424	0.478	0.385	0.313	0.296	0.309	0.309	0.375	0.500	0.313		0.424	0.459	0.500	0.422	0.520	0.417	0.338	0.363	0.362
25Is146	0.791	0.717	0.680	0.781	0.833	0.813	0.835	0.861	0.863	0.587	0.847	0.331		0.681	0.506	0.602	0.607	0.574	0.267	0.545	0.255
25Is147	0.795	0.731	0.692	0.785	0.799	0.766	0.800	0.813	0.872	0.621	0.826	0.330	0.870		0.578	0.616	0.678	0.606	0.297	0.608	0.299
23Mr09	0.699	0.764	0.721	0.759	0.644	0.620	0.635	0.635	0.724	0.742	0.644	0.457	0.702	0.701		0.534	0.592	0.448	0.233	0.472	0.247
25Uz05	0.783	0.684	0.649	0.786	0.863	0.854	0.864	0.878	0.795	0.585	0.919	0.336	0.810	0.802	0.682		0.765	0.649	0.286	0.600	0.287
25Uz08	0.703	0.675	0.664	0.731	0.674	0.671	0.676	0.701	0.756	0.575	0.724	0.430	0.760	0.748	0.718	0.780		0.640	0.315	0.604	0.318
25Is204	0.763	0.662	0.600	0.712	0.793	0.774	0.794	0.794	0.722	0.535	0.793	0.325	0.765	0.727	0.646	0.850	0.703		0.313	0.589	0.315
01KaD1	0.432	0.396	0.421	0.449	0.423	0.447	0.436	0.436	0.418	0.385	0.442	0.252	0.465	0.432	0.429	0.456	0.443	0.403		0.236	0.836
01KaD2	0.738	0.686	0.620	0.672	0.683	0.658	0.685	0.697	0.709	0.550	0.732	0.313	0.754	0.742	0.642	0.708	0.661	0.738	0.349		0.212
25Is08	0.456	0.401	0.438	0.474	0.446	0.469	0.459	0.459	0.444	0.381	0.465	0.241	0.490	0.457	0.434	0.480	0.470	0.427	0.927	0.374	

grouped the 21 mulberry genotypes into four and five major clusters, respectively, showing great genetic diversity among them.

The genetic similarity coefficients revealed a substantial amount of genetic similarity among the mulberry genotypes, though the genotypes were collected from different cities of varied climatic conditions, such as subtropical (Adana) and cold temperature (Elazığ, Erzincan, Erzurum). Genotypes from the same geographic regions show closer genetic similarity than those from geographically distant regions, but we found closer relatedness between 01KaD1 and 25Is08 in RAPD and ISSR results. This situation may have occurred from grafting buds or cuttings being transported from one region to another. *Morus alba* genotypes grouped in one cluster (main cluster) except for the 24Ke10 genotype, whereas *M. nigra* genotypes were outside the main group, except for the 01KaD2 genotype in the both ISSR and RAPD dendrograms. 01KaD2 and 24Ke10 could be a hybrid between *M. alba* and *M. nigra* or vice versa. Genetic similarity values were low between genotypes from different species. For example, 01KaD1 and 25Is08 genotypes which belong to *M. nigra* species were separated from the main cluster that contained *M. alba* genotypes.

A good correlation was found between the two matrices (0.846). Similar results were also found in cophenetic correlation between matrices and dendrograms: 0.959 for RAPD and as 0.958 for ISSR As a result, the dendrograms reflected matrices at a high level.

Our results suggest that RAPD and ISSR markers are useful for mulberry genetic diversity analysis and germplasm characterization, and that putative species-specific markers may be obtained which can be converted to SCAR after further studies. These results may also benefit for breeders in selecting the most diverse genotypes having similar fruit characteristics in breeding programs.

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