Molecular Characterization of Mulberry Accessions in Turkey by AFLP Markers

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Abstract. Mulberries (Morus L.) show a great deal of genetic variability and adaptability to various environments. There are more than 24 species of mulberries in cultivated and wild forms. In Turkey, three Morus species, M. alba L., M. nigra L., and M. rubra L., are grown. In this study, we attempted to characterize 43 Morus accessions originating from distinct regions of Turkey using fluorescent dye amplified fragment length polymorphism (AFLP) markers and capillary electrophoresis. The accessions belonged to M. alba, M. nigra, and M. rubra; M. alba consisted of white- and purple-fruit samples. Eight primer combinations generated a total of 416 bands, 337 of which were polymorphic (80.5%). Resolving powers of the AFLP primers ranged from 0.410 to 0.942 making a total of 5.015, whereas the polymorphic information content ranged from 0.662 to 0.898 with an average of 0.812. Unweighted pair-group method of arithmetic mean (UPGMA) clustering of the accessions showed three major groups representing M. nigra, M. rubra, and M. alba accessions. The M. alba group had two subgroups that were not correlated with fruit color. The UPGMA dendrogram of average taxonomic differences confirmed these results. The principle coordinate analysis demonstrated that M. nigra accessions had limited genetic variation. In conclusion, our study indicated that M. nigra and M. rubra are molecularly distinct from M. alba. Our results also suggest that M. nigra accessions having a low level of morphological variation are molecularly similar.

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Molecular markers have been commonly used to characterize mulberry cultivars and wild accessions. For example, RAPD and intersimple sequence repeat (ISSR) markers were used to study the genetic relationships of Japanese and Indian cultivars to assess molecular variability (Vijayan, 2004; Vijayan et al., 2004a). ISSRs were also combined with simple sequence repeat (SSR) to reveal genetic variation among wild and cultivated mulberry species (Zhao et al., 2007a). Indeed, ISSRs have been the most common marker system used in mulberries (Kar et al., 2008; Vijayan and Chatterjee, 2003; Vijayan et al., 2004a, 2004b, 2004c, 2006; Zhao et al., 2006, 2007b).
Single primer amplification reactions were also used to characterize 27 mulberry cultivars (Bhattacharya et al., 2005).

Among polymerase chain reaction (PCR)-based molecular markers, amplified fragment length polymorphisms (AFLPs) are highly reproducible multilocus marker system developed by Vos et al. (1995). This method has several advantages over previously introduced methods such as RAPDs and ISSRs and has been extensively used for a wide range of species. High levels of polymorphism and high degrees of discriminative capacity are the main advantages of AFLPs for closely related accessions. Standard AFLP methods based on two cutting enzymes require labeling of selective primers, which necessitates the use of isotopes or fluorescent dyes.

Although the AFLP method has been used to identify genetic variability of many different plant species, the use of this powerful and reliable method in mulberries has been very limited. Molecular studies conducted by AFLPs have been limited to assessing genetic diversity in a mulberry germplasm collection (Sharma et al., 2000) identifying the origin of introduced mulberry accessions in Italy (Botton et al., 2005) and analyzing artificial triploid mulberries (Wang and Yu, 2001). The objectives of this study were to characterize 43 accessions of M. alba, M. nigra, and M. rubra from Turkey using AFLP markers to determine whether AFLP markers are appropriate for this subgroup of Morus germplasm and to have a better understanding about the taxonomical relationships among these three species.

Materials and Methods

**PLANT MATERIALS.** Cultivated mulberry genotypes of M. nigra [black mulberry (33 accessions)], M. rubra [red mulberry (5 accessions)], and M. alba [white- and purple-fruited mulberry (5 accessions)] were sampled across Turkey. Distribution of origin and location of trees for these genotypes are displayed in Table 1. Four of seven ecogeographical regions of Turkey were represented: Black Sea (3 accessions), Central Anatolia (15 accessions), Eastern Anatolia (10 accessions), and Mediterranean (17 accessions).

**DNA EXTRACTION AND AFLP ANALYSIS.** Genomic DNA was extracted from leaf tissue by the CTAB method of Doyle and Doyle (1987) with minor modifications (Kafkas et al., 2005). Concentration of extracted DNA was estimated by comparing band intensity with λ DNA of known concentrations after 0.8% agarose gel electrophoresis and ethidium bromide staining. DNA was diluted to 50 ng·μL⁻¹ for AFLP reactions.

Details of AFLP assay, adaptor and primer sequences, PCR conditions for preselective and selective amplifications, and selective primer designation were according to Vos et al. (1995). Genomic DNA was restricted with EcoRI/MseI enzyme combination and double-stranded adaptors specific to each site were ligated. Preselective amplification was carried out with primers complementary to the adaptors with an extra selective base on each primer (EcoRI-A/MseI-C). Selective amplification was performed with eight primer combinations involving three MseI (M) and three EcoRI (E) primers (E_AAG/M_CAC, E_AAG/M_CCA, E_AAG/M_CCG, E_ACC/M_CAC, E_ACC/M_CCT, E_AGG/M_CGA, E_AGG/M_CCG, E_AGG/M_CCC). Fragments were resolved using capillary electrophoresis on an ABI 3130 Genetic Analyzer [Applied Biosystems (ABI), Foster City, CA] with the data collection software 3.0 (ABI). AFLP fragment analysis was performed with GeneScan Analysis Software 4.0 (ABI) and the data were assembled in binary format.

**DATA ANALYSIS.** The ability of the most informative primer pairs to differentiate between the genotypes was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (1999) using the formula Rp = \( \sum Ib \), where \( Ib = 1 - (2 \times 0.5 - p) \), and \( p \) is the proportion of the 69 accessions containing the I band. The polymorphism information content (PIC) of each marker was calculated using PIC = 1 – \( \sum p_i \) where \( p_i \) is the band frequency of the \( i \)th allele (Smith et al., 1997). Jaccard’s similarity coefficients (Sneath and Sokal, 1973).
were calculated for all pairwise comparisons among the 43 *Morus* genotypes. A dendrogram was generated using NTSYSpc version 2.11V (Exeter Software, Setauket, NY) (Rohlf, 2004) based on unweighted pair-group method of arithmetic mean (UPGMA) cluster analysis. For this dendrogram, the bootstrap values for the clusters were calculated by making 1000 replicates using PAUP program (Swofford, 1998). In addition to the dendrogram of the 43 genotypes, AFLP band frequencies were calculated for each of the four classes [*M. nigra*, *M. rubra*, *M. alba* (purple-fruited), and *M. alba* (white-fruited)] based on the average taxonomic distance parameter. Cluster analysis using the UPGMA method was performed to construct a dendrogram from the distance matrix. The representativeness of both dendrograms was evaluated by estimating cophenetic correlation for the dendrograms and comparing it with the similarity matrix and similarity interval matrix using Mantel's matrix correspondence test (Mantel, 1967). The result of this test is a cophenetic correlation coefficient, *r*, indicating how well the dendrogram represents similarity data. The similarity matrix data were also subjected to principal coordinate (PCoA) analysis using the NTSYSpc (version 2.11V; Exeter Software, Setauket, NY). The genotypes were plotted on first three dimensions using the G3D procedure of SAS (version 6; SAS Institute, Cary, NC).

**Results and Discussion**

**Level of polymorphism and discriminating capacity of the AFLP primer pairs.** The eight primer combinations used to study the 43 *Morus* accessions generated a total of 416 bands (Table 2). The number of bands produced by each primer combination ranged from 35 (*E*_{AAG}/*M*_{CCA}) to 90 (*E*_{ACC}/*M*_{CGA}) with an average of 52 bands. Of 416 bands, 337 were polymorphic (80.5% polymorphism). The percentage of polymorphic bands varied considerably among the primer combinations. For example, all of the 40 bands generated by *E*_{ACC}/*M*_{CTT} were polymorphic, whereas the *E*_{AAG}/*M*_{CCA} combination yielded 71.4% polymorphic bands.

When we attempted to characterize Turkish mulberry accessions representing *M. alba*, *M. nigra*, and *M. rubra*, the AFLP analysis successfully differentiated the genotypes and it was confirmed that AFLP is a powerful marker system. AFLP markers have been previously used in the analysis of mulberry populations. Sharma et al. (2000) studied 43 mulberry accessions from ecogeographic regions of Japan and other parts of the world. In their collections, 21 species and 2×, 3×, 4×, 6×, and 22× ploidy levels were presented. Each of five primer combinations they studied generated an average of 110 amplification products and their percentage of polymorphic bands ranged from 69.7 to 82.3. Botton et al. (2005) characterized

<table>
<thead>
<tr>
<th>AFLP primer combinations</th>
<th>Total bands (no.)</th>
<th>Polymorphic bands (no.)</th>
<th>Polymorphism (%)</th>
<th>Resolving power</th>
<th>Polymorphism information content</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E</em><em>{AAG}/<em>M</em></em>{CAC}</td>
<td>40</td>
<td>31</td>
<td>77.5</td>
<td>0.445</td>
<td>0.891</td>
</tr>
<tr>
<td><em>E</em><em>{AAG}/<em>M</em></em>{CCA}</td>
<td>35</td>
<td>25</td>
<td>71.4</td>
<td>0.488</td>
<td>0.898</td>
</tr>
<tr>
<td><em>E</em><em>{AAG}/<em>M</em></em>{CCG}</td>
<td>57</td>
<td>45</td>
<td>78.9</td>
<td>0.410</td>
<td>0.896</td>
</tr>
<tr>
<td><em>E</em><em>{ACC}/<em>M</em></em>{CAC}</td>
<td>54</td>
<td>44</td>
<td>81.5</td>
<td>0.622</td>
<td>0.808</td>
</tr>
<tr>
<td><em>E</em><em>{ACC}/<em>M</em></em>{CGA}</td>
<td>40</td>
<td>40</td>
<td>100.0</td>
<td>0.942</td>
<td>0.662</td>
</tr>
<tr>
<td><em>E</em><em>{ACC}/<em>M</em></em>{CTT}</td>
<td>90</td>
<td>78</td>
<td>86.7</td>
<td>0.683</td>
<td>0.789</td>
</tr>
<tr>
<td><em>E</em><em>{AAG}/<em>M</em></em>{CCC}</td>
<td>50</td>
<td>36</td>
<td>72.0</td>
<td>0.742</td>
<td>0.753</td>
</tr>
<tr>
<td><em>E</em><em>{AAG}/<em>M</em></em>{MCC}</td>
<td>50</td>
<td>38</td>
<td>76.0</td>
<td>0.683</td>
<td>0.800</td>
</tr>
<tr>
<td>Total</td>
<td>416</td>
<td>337</td>
<td>—</td>
<td>5.015</td>
<td>—</td>
</tr>
<tr>
<td>Mean</td>
<td>52</td>
<td>42</td>
<td>80.5</td>
<td>—</td>
<td>0.812</td>
</tr>
</tbody>
</table>

AFLP = amplified fragment length polymorphism.
48 accessions from three species, *M. alba*, *M. latifolia* Poir., and *M. bombycis* Koidz. Their accessions originated from diverse regions of the world such as Italy, China, Japan, India, the Middle East, Philippines, and Brazil. In their study, they used six primer combinations and scored an average of 110 bands per combination, 72.2% of which were polymorphic. Although our accessions represent only three diploid species, all originating from Anatolia, their level of polymorphism was comparable to those of previous studies. This is an indication of the high degree of polymorphism among the accessions tested.

Rp values ranged from 0.410 (E<sub>AAG</sub>/M<sub>CCG</sub>) to 0.942 (E<sub>ACC</sub>/M<sub>CCT</sub>) with a total of 5.015. The PIC values of the primer combinations ranged from 0.662 to 0.898 with an average of 0.812. The PIC values we recovered in this study were much higher than those of Zhao et al. (2007a) in which they studied 27 mulberry accessions from several *Morus* species by 15 SSR primers generating 138 reliable bands, 91.3% of which were polymorphic.

**Genetic Relatedness Among the Morus Species and Accessions.** A dendrogram was obtained by the UPGMA method using the total number of AFLP bands (Fig. 1). There were three groups in the dendrogram. Group I consisted of *M. nigra* accessions. Indeed, all *M. nigra* accessions were grouped in this cluster and the node separating these accessions from the others were highly supported by the bootstrap value (100%). This indicates that *M. nigra* is distinct from the other species studied. Another dendrogram was generated using frequency data within the entries (Fig. 2). This dendrogram, exhibiting average taxonomic difference, also confirmed the distinctness of *M. nigra*. The same conclusion can be made from Figure 3 showing the results of PCoA. The bootstrap values supported some of the nodes within Group I (Fig. 1); based on these values, five subgroups were identified. However, PCoA failed to clearly separate *M. nigra* accessions, indicating that they had limited levels of molecular variation. Indeed, although *M. nigra* accessions were sampled from various ecological conditions, they displayed only limited morphological variation for many horticultural traits (data not presented).

Three *M. rubra* accessions (R1, R2, and R7) formed Group II, although two other *M. rubra* accessions (R6 and R8) were grouped among the *M. alba* accessions (Fig. 1). The bootstrap values further separated Group II into two subgroups. PCoA revealed the same results (Fig. 3). The dendrogram constructed from average taxonomic differences supported the separation of *M. rubra* from *M. alba* (Fig. 2).

The third group in the UPGMA dendrogram consisted of all of the *M. alba* accessions along with two *M. rubra* accessions (Fig. 1). The bootstrap values separated the accessions of Group II into two subgroups. However, this separation was not highly supported (52%) and not based on fruit color (white- versus purple-fruit). The dendrogram exhibiting average taxonomic differences of entries (Fig. 2) and the PCoA (Fig. 3) analyses were also not successful in differentiating white- and purple-fruit *M. alba* accessions.
The UPGMA dendrogram of AFLP bands even separated the closely related *M. nigra* accessions. Thus, our results were in general agreement with those of Sharma et al. (2000) and Botton et al. (2005). In our analyses, the accessions were most tightly clustered by their species and there were no apparent interrelationships based on the origin of the accessions. Regarding *Morus* taxonomy, our analysis shed light into relationships of *M. alba*, *M. nigra*, and *M. rubra*. Our results indicated that *M. nigra* is distinct from the other species. Previous studies on the relationships of the *Morus* species conducted by AFLP came to the same conclusion. Sharma et al. (2000) and Botton et al. (2005) used a representative of *M. nigra* that was found distinct from *M. alba* and *M. nigra* accessions. In fact, the *M. nigra* accession of Botton et al. (2005) was different from all other entries in their study. In the study of Zhao et al. (2007b), a representative of *M. nigra* was also separated from all the cultivated accessions studied.

The results of the present study may benefit breeders in selecting the most diverse genotypes with similar fruit characteristics to begin crossing and selection programs. This may result in increased mulberry growing for fruit production rather than just sericulture.

**Literature Cited**


