DETERMINATION OF BIO-VARIATION AMONG DIFFERENT MULBERRY SPECIES GROWN IN TOKAT REGION OF TURKEY BY MOLECULAR MARKERS

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Abstract. The aim of this research is to determinate diversity of mulberries grown in North Anatolia between or within species by ISSR markers. In the study, 38 different mulberry genotypes collected from Tokat region in Turkey were used. ISSR-PCR analyses were carried out on the DNA of genotypes isolated using mini-CTAB extraction method. The levels of polymorphism between genotypes were determined using the UBC-ISSR primers. A total of 96 bands were obtained from 15 UBC ISSR primers. Out of 96 bands, 80 bands were polymorphic. The number of bands obtained per primer ranged between 4 and 11, the average number of bands were determined as 6.4. The average number of polymorphic bands per primer was 5.33. Similarities and differences between genotypes have been studied at the molecular level. The data used for statistical analysis were obtained by the evaluation of ISSR bands. Similarity coefficient and UPGMA dendrogram were built using the Basic Coordinates Analysis. According to the dendrogram, the genotypes have been divided in two main groups, one small and one large group. While the small group was only comprised of black mulberry genotypes, the large group included white mulberry, weeping mulberry and wild white mulberry genotypes. Different mulberry species have been divided into different subgroups within the large group. The polimorfism level within the species was lowest in black mulberry genotypes, and this was followed by weeping mulberry, white mulberry and wild white mulberry genotypes, respectively.
Keywords: *similarity, diversity, dendrogram, North Anatolia, mulberry*

Introduction

Mulberry is a perennial fruit species belonging to the genus *Morus* of the family Moraceae (Datta, 2002; Anonymous, 2006). *Morus*, a genus of flowering plants in the family Moraceae, deciduous trees commonly known as mulberries, grow wild and under cultivation in many temperate world regions. The origin of mulberry is Asia (Awasthi et al., 2004). Over 150 species names have been published, and although differing sources may cite different selections of accepted names, only 10–16 are generally cited as being accepted by the vast majority of botanical authorities. *Morus* classification is even further complicated by widespread hybridisation, wherein the hybrids are fertile. Mulberry trees are either dioecious or monoecious and sometimes will transform from one sex to another. The flowers are held on short, green, pendulous, nondescript catkins that emerge in the axils of the current season's growth and on spurs on older wood. They are wind pollinated and some cultivars will set fruit without any pollination (Anonymous, 2009).

Black, red, and white mulberry are widespread in southern Europe, the Middle East, northern Africa and Indian subcontinent, where the tree and the fruit have names under regional dialects. The most important mulberry species are *Morus alba* (white

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mulberry), *Morus australis* (Chinese mulberry), *Morus indica* (Indian mulberry), *Morus microphylla* (Texas mulberry), *Morus nigra* (Black mulberry), *Morus rubra* (Red-purple mulberry) and *Morus serrata* (Himalian mulberry) (Tutin, 1996; Vijayan et al., 2004). It is sometimes difficult to distinguish mulberry species morphologically and pomologically from each other, especially the fruits of *Morus nigra*, *Morus rubra* and *Morus pendula* species, those with black fruits. In fact, cheaper black fruits are sometimes sold in markets instead of *Morus nigra* fruits in high prices (Günes and Cekic, 2004; Erdogan and Pirlak, 2005). Therefore, determining the difference in the species and between species at the molecular level is important in terms of consumers as well as in terms of scientific literature.

The mulberry plants are deciduous and are produced for their fruit and leaves in all parts of Turkey (Anonymous, 2009). The species *M. alba*, *M. nigra* and *M. rubra* are common in Turkey (Ercisli, 2004; Ercisli and Orhan, 2007; Özgen et al., 2009). The main mulberry production areas of Turkey are Black Sea Region and Eastern and Central Anatolian Regions. Turkey has very old mulberry cultivation, and mulberries are one of the main fruits grown by Turkish farmers. The four mulberry species (*Morus alba*, *Morus nigra*, *Morus rubra*, *Morus laevigata*) can be seen in different agro-climatic regions in Turkey. There is no registered mulberry cultivar in Turkey but each region has its own local genotypes which are propagated by budding or grafting over many years (Ercisli and Orhan, 2007).

Tokat Province of Turkey, at the mid Black Sea region of Anatolia, has a hot-summer Mediterranean climate with considerable maritime and continental influences. Most parts of province are suitable for mulberry production, and some species of mulberry spread over the province. Although there is no intensive mulberry production by producers, one or two mulberry trees can be seen almost in every orchard, especially near by homes.

The aim of this research is to determinate diversity of mulberries grown in North Anatolia (Tokat Province) between or within species by ISSR markers.

Material and methods

Plant material

For research purposes, a total of 38 different mulberry genotypes from Tokat region in Turkey were used (*Table 1; Fig. 1*). Out of total number 15 belongs to black (*Morus nigra*), 13 to white (*Morus alba*), 5 to pendulous (*Morus pendula*), 2 to everbearing mulberry (*Morus alba*) and 3 to wild mulberry (*Morus alba*).

Figure 1. *The localization of Tokat province in Turkey, where the samples were collected*

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Table 1. *The latitudes, longitudes and elevations of the samples taken*

	Sample code	Elevation (m)	Latitude	Longitude
1	K1	608 m	40° 20.014N	36° 31.065E
2	K2	730 m	40° 18.863N	36° 32.191E
3	K3	679 m	40° 18.964N	36° 32.925E
4	K4	700 m	40° 18.307N	36° 33.092E
5	K5	671 m	40° 17.390N	36° 33.017E
6	K6	689 m	40° 17.860N	36° 33.454E
7	K7	677 m	40° 18.229N	36° 33.682E
8	K8	589 m	40° 20.551N	36° 31.812E
9	K9	667 m	40° 21.387N	36° 31.565E
10	K10	658 m	40° 21.336N	36° 31.582E
11	K11	613 m	40° 20.356N	36° 33.272E
12	K12	615 m	40° 20.317N	36° 33.321E
13	K13	649 m	40° 20.497N	36° 34.120E
14	K14	2211 m	40° 17.269N	36° 33.065E
15	K15	2214 m	40° 17.266N	36° 33.064E
16	B1	730 m	40° 18.804N	36° 32.169E
17	B2	676 m	40° 18.506N	36° 32.925E
18	B3	659 m	40° 18.024N	36° 33.086E
19	B4	740 m	40° 17.423N	36° 33.136E
20	B5	589 m	40° 20.551N	36° 31.812E
21	B6	617 m	40° 20.442N	36° 32.796E
22	B7	615 m	40° 20.391N	36° 33.175E
23	B8	611 m	40° 20.225N	36° 33.341E
24	B9	618 m	40° 20.219N	36° 34.123E
25	B10	2214 m	40° 17.266N	36° 33.063E
26	B11	2185 m	40° 17.293N	36° 33.016E
27	B12	2211 m	40° 17.269N	36° 33.065E
28	B13	2270 m	40° 17.504N	36° 33.539E
29	S1	2270 m	40° 17.504N	36° 33.539E
30	S2	589 m	40° 18.804N	36° 32.169E
31	S3	658 m	40° 18.506N	36° 32.925E
32	S4	619 m	40° 18.024N	36° 33.086E
33	S5	626 m	40° 17.423N	36° 33.136E
34	Y1	679 m	40° 18.964N	36° 32.925E
35	Y2	667 m	40° 21.387N	36° 31.565E
36	YB1	659 m	40° 18.024N	36° 33.086E
37	YB2	619 m	40° 20.456N	36° 32.363E
38	YB3	618 m	40° 18.024N	36° 33.086E

DNA extraction

Total genomic DNA was extracted according to the CTAB method (Doyle and Doyle, 1987). DNA sample concentration was determined using a fluorometer employing a Hoechst dye (Hoefer Inc., San Francisco, CA, USA), and the DNA

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samples were diluted to a final concentration of 10 ng/µl with 1 × TE buffer and stored at −20 °C prior to polymerase chain reaction (PCR) amplification.

ISSR amplification

15 primers that produced clear and reproducible fragments were selected out of a hundred UBC primers, which were previously tested for further analyses (*Table 2*). ISSR amplification was performed in a 20 µl volume containing 20 ng genome DNA, 1 × Taq buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 µM primer, 0.5 units of Taq DNA polymerase. The amplification reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of 45 s at 94 °C, annealing at 50–56 °C for 45 s, extension at 72 °C for 90 s, and ended with extension at 72 °C for 7 min. The amplified products were resolved electrophoretically on 2.0% agarose gels run at 100 V in 1.0 × TBE buffer, visualized by staining with ethidium bromide (0.5 µg/ml), and photographed under ultraviolet light (Charters et al., 1996; Rafalski et al., 1996; Cekic et al., 2001) The amplifications were repeated twice and only clear repetitive bands were used in data analysis, and molecular weights were estimated using a 100 bp DNA marker (Vivantis).

Table 2. *Band number, polymorphic band number and polymorphic band ratio (%) in mulberry genotypes*

	ISSR primer code	Primer sequence and anchors	Band number	Polymorphic band number	Polymorphic band ratio (%)
1	807	AGAGAGAGAGAGAGAG**T**	11	9	81.81
2	808	AGAGAGAGAGAGAGAG**C**	5	5	100.00
3	810	GAGAGAGAGAGAGAGA**T**	8	6	75.00
4	811	GAGAGAGAGAGAGAGA**C**	7	6	85.71
5	826	ACACACACACACACAC**C**	6	6	100.00
6	835	AGAGAGAGAGAGAGAG**YC**	7	6	85.71
7	841	GAGAGAGAGAGAGAGA**YC**	6	4	66.67
8	842	GAGAGAGAGAGAGAGA**YG**	10	9	90.00
9	844	CTCTCTCTCTCTCTCT**RC**	4	3	75.00
10	856	ACACACACACACACAC**YA**	4	3	75.00
11	881	GGGTGGGTGGGTGGGT	5	4	80.00
12	888	**BDB**CACACACACACACA	7	6	85.71
13	889	**DBD**ACACACACACACAC	4	3	75.00
14	890	**VHV**GTGTGTGTGTGTGT	7	6	85.71
15	891	**HVH**TGTGTGTGTGTGTG	5	4	80.00
Average			6.4	5.33	82.76
Total			**96**	**80**	

Data analysis

The amplified fragments were scored for band presence (1) or absence (0) with all the accessions studied. Similarities and differences between genotypes have been studied at the molecular level. The data used for statistical analysis were obtained by the

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evaluation of ISSR bands. Similarity coefficient and UPGMA dendrogram were built using the Basic Coordinates Analysis (Rolf, 1992).

Results and discussion

On the basis of the number, intensity and reproducibility of ISSR bands 15 primers were selected out of a hundred UBC primers, which were previously tested (Cekic et al., 2001). Bands with the same mobility were treated as identical fragments. Weak bands with negligible intensity and smear bands were both excluded from final analysis. Each primer was evaluated for total band number (TBN), polymorphic band number (PBN) and the ratio of polymorphism (PR= PBN/TBN X 100).

The number of scored bands varied from four to eleven with an average of 6.4 bands per primer and an average of 5.33 polymorphic bands per primer. In total, 80 bands out of 96 derived from 15 primers were polymorphic (*Table 2; Fig. 2*).

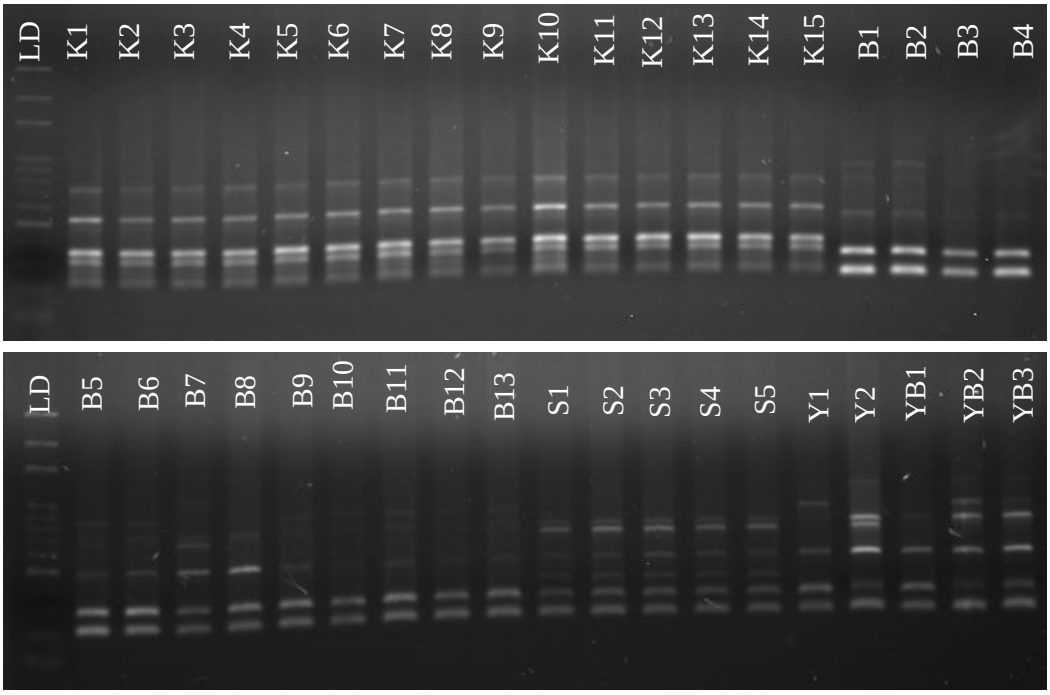

Figure 2. *Agarose gel of polymorphic DNA amplification profiles of different mulberry genotypes (K: Black mulberry, B: White mulberry, S: Pendulous mulberry, Y: Everbearing mulberry (long season harvesting), YB: Wild mulberry) obtained with UBC ISSR primer 811. (LD: 100 bp LADDER -Vivantis)*

Cluster analysis

Genetic similarity among varieties was estimated using dissimilarity coefficient matrix based on ISSR bands scored. Pairwise values of dissimilarity coefficients ranged from 0.27 for genotypes with the same scored bands to 1.00 for the most similar genotypes. The dendrogram was constructed based on the similarity matrix, using UPGMA method (Rohlf, 1992). The 38 mulberry genotypes were divided into two main clusters (*Fig. 3*), in which black mulberry genotypes in one group and the rest of the genotypes falling under the other group. While the first major group contained only

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black mulberries, the second major cluster was further separated into subgroups. The first subgroup contained black mulberry genotypes, respectively which showed very close ISSR profile exhibited the highest genetic similarity ranges (0.95-1.00). On the other hand, the similarity of the second varied 0.75 to 0.96, in which different mulberry species (White mulberry, Pendulous mulberry, Everbearing mulberry, Wild mulberry) separated in different clusters at various stages (*Fig. 2*).

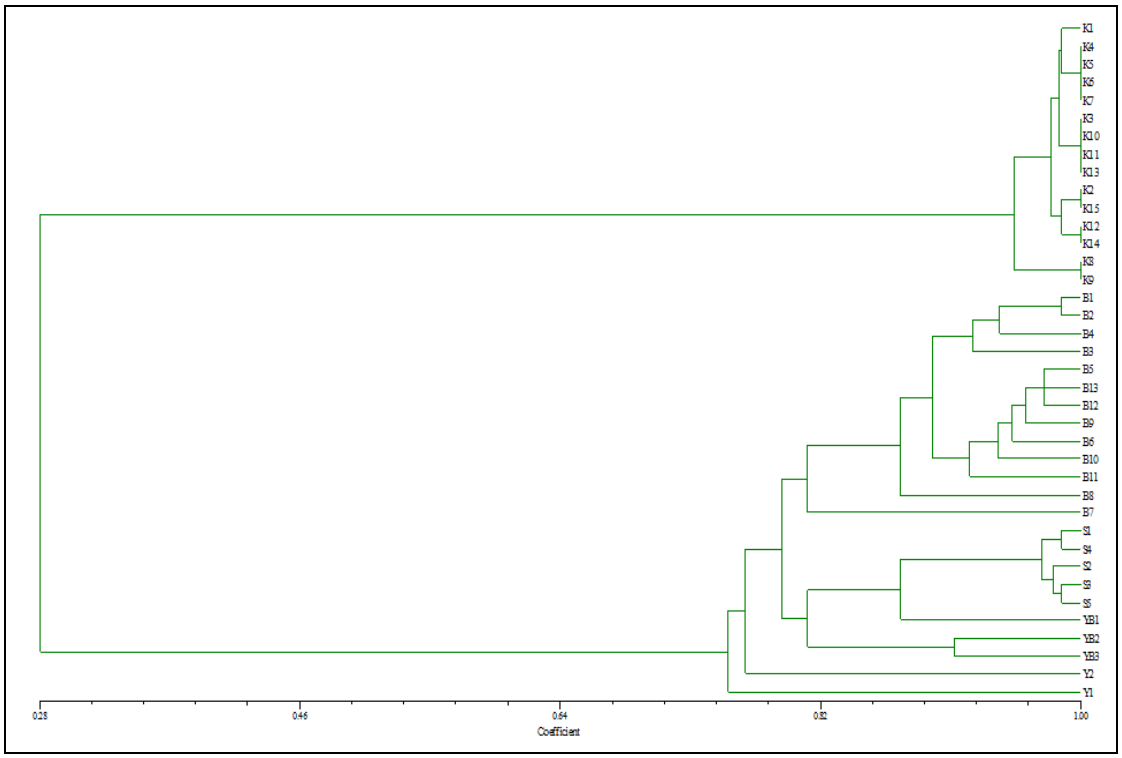

Figure 3. *Dendrogram showing genetic relationships among value of ISSR markers for mulberry genotypes (K: Black mulberry, B: White mulberry, S: Weeping (Pendulous) mulberry, Y: Everbearing mulberry (long season harvesting), YB: Wild mulberry)*

While the small main group in the dendrogram only consists of black mulberry genotypes, the major groups include white mulberry, weeping mulberry, everbearing and wild white mulberry genotypes. However, in the major group, mulberry genotypes belonging to different species constituted their subgroups. The level of polymorphism between black mulberry genotypes was very low. While no polymorphism was observed in the great majority of 15 genotypes of black mulberry genotypes, the lowest similarity level in this species is 94% in dendrogram. The lowest level of similarity within the other species were 96% in the weeping mulberry, 80% in the white and wild white mulberry and 75% in the everbearing mulberry genotypes.

The wild and everbearing white mulberry genotypes were most likely grown from seed. Therefore, these mulberry genotypes showed the least similarities and the greatest polymorphism was obtained from these genotypes. Although 100% similarities were not obtained within the group, polymorphism was low in weeping mulberry genotypes. Significant differences were determined within the group in white mulberry genotypes, and 100% similarities were not observed. The majority of black mulberry genotypes were similar to each other, most of which can be caused by vegetative propagation from

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a single source by grafting, cutting or dipping. As a matter of fact, previous studies indicate that seeds of black mulberry genotypes hardly germinate without any pre-treatment (Güneş and Çekiç, 2004). In addition, this example is true because the sample area is narrow and there is only one or two black mulberry trees in producer gardens. The results of this study will then shed light on the work to be done for vegetative propagation in this area. Also, the principle component analysis generally put the genotypes into different clusters as parallel to morphological differences of the mulberry species (*Fig. 4*).

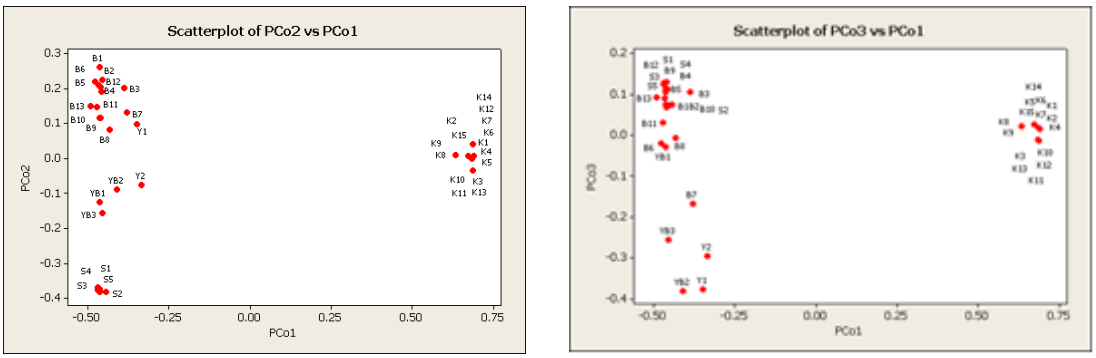

Figure 4. *The principle component analysis of mulberry genotypes according to 15 ISSR primers*

Conclusion

The results of the study mean that molecular tools are more reliable than the phenotypic observations as the well-known fact. Screening of the 38 genotypes of different mulberry species revealed that banding profiles obtained with fifteen ISSR primers were enough to distinguish the diversity between species or within the species. The results indicated that the ISSR technique is effective to develop genotype-specific banding patterns valuable for genotype identification. Since ISSR-PCR technique does not require previous DNA sequence information and uses very small quantity of DNA, it is considered as one of the most widely used techniques for genotype identification and genetic diversity studies. These results mean that molecular tools are more reliable than the phenotypic observations for evaluating variations and monitoring genetic stability.

The wide variation in genetic distance among the different mulberry species revealed by ISSR techniques reflected a high level of polymorphism at the DNA level. The genetic similarity of different mulberry species is low as indicated ISSR analyses. The variability in black mulberry genotypes within the species has been characterized as quite low. We can conclude that the saplings of black mulberry were mostly obtained by budding or cutting from local sources. There are some flows in other mulberry species from outside the province as we can see wide variation within the species.

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