Molecular characterization of mulberry genotypes and species in Turkey

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Abstract

Mulberries are one of the most important traditional fruit in Turkey and either consumed fresh or processed into several traditional products. Mulberry trees (genotypes) belong to *Morus alba* L., *Morus nigra* L., and *Morus rubra* L. shows high phenotypic diversity. In this study, 26 genotypes of *Morus nigra*, 21 genotypes of *Morus rubra* and 26 genotypes and landraces of *Morus alba* sampled from different parts of eastern Anatolia region in Turkey were fingerprinted using 16 simple sequence repeat (SSR) markers. Among SSR markers, the number of alleles per locus ranged from 2 (SS01, SS05) to 11 (MulSTR3) with an average of 5.19. A total of 83 alleles were obtained for the 16 SSR markers. The polymorphic information content ranged from 0.43 to 0.84 with an average of 0.61 per locus. In this study, MulSTR3, MulSTR5, MulSTR6, SS04 and SS20 were found to be the most useful SSR markers to detect genetic differences between mulberry species, genotypes and landraces. Genetic similarity ratio ranged from 0.24 to 0.94 with a mean similarity value of 0.41 indicating a high level of polymorphism among the mulberry species, genotypes and landraces. Clustering based on Jaccard's similarity coefficient and an unweighted pair group method with arithmetic mean (UPGMA) revealed 3 main clusters that corresponded with species. The results of the present study indicate that white mulberry landraces and genotypes were more diverse than the red and black mulberry genotypes. Therefore, white mulberry genotypes can be a good source for the mulberry-breeding programme due to its better genetic diversity as well as its high economical and yield properties.

Keywords: landraces; molecular characterization; mulberry species; seed propagation; SSR

Introduction

The fruiting mulberry placed in the *Morus* genus, Moraceae family. The widely distributed and used mulberries in the world come from the three main species: white mulberry (*Morus alba*), red mulberry (*Morus rubra*), and the black mulberry (*Morus nigra*) (Gungor and Sengul, 2008). The three major mulberry species originated from different areas of the world. The red mulberry (the American mulberry) is native to eastern

United States. The white mulberry, bred for silkworm production and fruit cultivation, is native to China. The black mulberry is native to Asia (Ercisli and Orhan, 2007; Kafkas *et al*., 2008; Vijayan *et al*., 2008).

Mulberries can be found in many parts of the world; yet they originated in the Far East along the Silk Road from China to Turkey. As the silkworm's food of choice, mulberry trees flanked the famous route that traded goods and culture between China, Persia, Europe, the Horn of Africa and the Arabian Peninsula. Mulberry trees belong to different *Morus* species today spread throughout all regions from the tropics to the sub-arctic and from sea level to altitudes as high as 4000 m (Darias-Martin *et al*., 2003; Arabshahi-Delouee and Urooj, 2007; Ercisli and Orhan, 2007).

Mulberry fruits contain considerable amounts of biologically active ingredients that might be associated with some potential pharmacological activities. Thus, they are beneficial for health and are categorized as super foods. In most mulberry-growing countries, mulberry fruit is commonly eaten fresh, dried, or processed into wine, fruit juice, and jam for its delicious taste, pleasing colour, low calorie content, and high nutrient content (Gungor and Sengul, 2008; Yuan and Zhao, 2017; Zhang *et al*., 2018).

Genetic variations are the raw materials of plant breeding and have been increasingly narrowed due to various reasons. Landraces and unnamed genotypes are repository of gene pool that enrich biodiversity and maintain and stabilize ecosystem in a sustainable way to make it functional. They are also genetically diverse and locally adapted and associated with a set of farmers' practices of seed selection and field management as well as with a farmers' knowledge base (Camacho-Villa *et al*., 2005). Determination of the level of variation within the landraces and unnamed genotypes is very important for their preservation. For landraces and unnamed genotypes to be employed in plant breeding programs their levels of genetic variation should first be determined (Dwivedi *et al*., 2016).

Continuous seed propagation for thousands of years in Turkey has given rise to a great number of seedling mulberry trees, which represent valuable mulberry genetic resources. Although many valuable mulberry trees were cut for their precious timber, there are still important mulberry populations of great genetic variation having resistance to pests and diseases, yield and fruit quality in Anatolia which has led to many researches of selection (Aslan, 1998; Ercisli and Orhan, 2008; Orhan, 2009).

Mulberry genotypes vary greatly in phenotypically (Thangavelu *et al*., 1997; Cam and Turkoglu, 2004; Orhan *et al*., 2007), and tools for distinguishing among them are lacking. Therefore, a method to easily and accurately identify mulberry genotypes is greatly needed; such a method would also benefit breeders and planters. Mulberry genotypes are usually distinguished based on morphology (Aslan, 1998; Cam and Turkoglu, 2004; Orhan, 2009). However, morphological studies are preliminary breeding approaches, and morphological traits are usually quantitative, meaning that they are easily controlled by the environment and thus exhibit high variability (Andersen and Lubberstedt, 2003). In addition, similar characters cannot be distinguished by morphology (Orhan, 2009). To overcome these problems, molecular methods such as randomly amplified polymorphic DNA (RAPD) (Orhan *et al*., 2007), inter-simple sequence repeat (ISSR) (Vijayan *et al*., 2005) and amplified fragment-length polymorphism (AFLP) (Kafkas *et al*., 2008) has been used in mulberry classification. All above methods approved insufficient for the identification of mulberry genotypes; thus, there is a great need to establish an accurate and easy way to distinguish mulberry genotypes, landraces, cultivars and to determine relationships between species as well. Among available molecular markers, microsatellite markers or SSR (Simple Sequence Repeat) are widely used in genetic analyses of plants including mulberry, since they show considerable advantages such as co-dominance, multiallelism, high frequency and random distribution in the genome, and high level of polymorphism (Ercisli *et al*., 2011; Guney *et al*., 2018; Garcia-Gomez *et al*., 2019). SSR markers are characterized by sequences, also called motifs, from one to six nucleotides, which are repeated in tandem, considering that the polymorphism produced consists of the difference in the number of replicates of motifs in each allele (Eyduran *et al*. 2016).

To our knowledge, a detailed compilation of research regarding the SSR based molecular characterization of mulberry species, genotypes and landraces is lacking. Knowledge of genetic diversity and

relationships among the mulberry germplasm will play a significant role in local and regional breeding programmes. Therefore, the objective of the present study was to determine the genetic relationship among 73 mulberry genotypes/landraces using SSR markers to identify genetically divergent genotypes for breeding.

Materials and Methods

Plant material

In total 73 mulberry genotypes (26 genotypes of *Morus nigra*, 21 genotypes of *Morus rubra* and 26 genotypes of *Morus alba*) sampled from Eastern Anatolia region in Turkey in 2016 were used in SSR analysis.

DNA extraction

Genomic DNA was extracted from young leaf tissues using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the instructions provided by the manufacturer. Subsequently, an RNase treatment was performed the eluted DNA samples. Purity and concentration of the DNA were checked both on 1% (w/v) agarose gels and by NanoDrop® ND-1000 Spectrophotometer.

SSR analysis

Sixteen nuclear SSR markers of primers flanking SSR sequences previously characterized and developed for *Morus* were used. These are MulSTR1, MulSTR2, MulSTR3, MulSTR4, MUlSTR5, MulSTR6 (Aggarwal *et al*., 2004) and SS01, SS02, SS04, SS05, SS06, SS09, SS17, SS18, SS19 and SS20 (Zhao *et al*., 2005) (Table 1).

Polymerase Chain Reaction

PCR was conducted in a volume of 10 µL and contained 15 ng genomic DNA, 5 pmol of each primer, 0.5 mM dNTP, 0.5unit GoTaq DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 2 µL 5X buffer. The forward primers were "labelled" with WellRED fluorescent dyes D2 (black), D3 (green) and D4 (blue) (Proligo, Paris, France). Reactions without DNA were included as negative controls. PCR amplification was performed using the Biometra® PCR System. The amplification conditions consisted of an initial denaturation step of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 52-56 °C and 2 mins at 72 °C with a final extension at 72 °C for 10 mins. Determination of polymorphisms, the PCR products were run on CEQTM 8800 XL Capillary Genetic Analysis System (Beckman Coulter, Fullerton, CA). The analyses were repeated at least twice to ensure reproducibility of the results.

Data analysis and genetic relationships

The genetic analysis program "IDENTITY" 1.0 (Wagner and Sefc, 1999) was used according to Paetkau *et al*. (1995) for the calculation of number of alleles, expected and observed heterozygosity. Genetic similarity was determined by the program "MICROSAT" (version 1.5) (Minch *et al*., 1995) using proportion of shared alleles, which was calculated by using "ps (option 1 - (ps))", as described by Bowcock *et al*. (1994). The results were then converted to a similarity matrix, and a dendrogram was constructed with the UPGMA method (Sneath and Sokal 1973) using the software NTSYS-pc (Numerical Taxonomy and Multiware Analysis System, version 2.0) (Rohlf 1988). Polymorphic Information Content (PIC) was calculated according to Kalinowski *et al*. (2007). The principal coordinate analysis (PCoA) was performed to show the relationships and differentiation of the mulberry genotypes in a three-dimensional array of eigenvectors, using the DCENTER and EIGEN modules of NTSYS-pc 2.10e software.

Results and Discussion

In this study sixteen published primer pairs flanking nuclear microsatellites were employed to investigate the level of genetic variation among the 73 mulberry genotypes that belongs to *Morus nigra*, *Morus rubra* and *Morus alba* sampled different parts of eastern Anatolia in Turkey.

In total, 83 alleles were obtained at the 16 SSRs loci analysed. Results showed that the number of alleles per locus ranged from 2 (SS01 and SS05) to 11 (MulSTR3) with a mean number of 5.19 alleles per locus (Table 1). The highest number of alleles was observed at loci MulSTR3 as 11 and followed by MulSTR5 and MulSTR6 as 8 alleles, respectively (Table 1). In literature a few reports are available on SSR analysis of mulberry genotypes. Garcia-Gomez *et al*. (2019) used thirty-seven mulberry genotypes from nine different countries including Cuba, Costa Rica, Brazil, South Korea, China, Japan, Italy, Ethiopia and Spain. In SSR analysis, they obtained the number of alleles from 2 (SS19) to 19 (MulSTR3), with a total of 68 alleles for the 9 loci and an average of 7.55 bands per locus. Wangari *et al*. (2013) used ten mulberry accessions, which included five *Morus alba* and five *Morus indica* genotypes in SSR analysis. They used 15 SSR primers and obtained a total of 35 alleles with an average of 6.3 per locus. They obtained the highest number of alleles from SS09 primer as 15, while the lowest number of alleles generated by SS19, SS20, MulSTR2, MulSTR6 and MulSTR5 primers as 2. Aggarwal *et al*. (2004) reported mean number of alleles as 18.6 in mulberries while Zhao *et al*. (2007) reported average 5.13 alleles per locus by using SSR markers in mulberry genotypes. Wani *et al*. (2013) characterized 17 mulberry genotypes by 6 SSR markers. The molecular analysis of those genotypes showed significant variability. They obtained a total of 17 alleles across the 17 mulberry genotypes using 6 SSR markers. They determined the number of alleles per loci generated by these SSR markers varied from 2 (MulSTR3) to 4 (MulSTR4) with an average of 2.84 per locus indicating lower values than our study. The existence of the variability in the number of alleles reported by other studies could be due to difference in selected genotypes and primers.

Table 1. Number of detected alleles, observed heterozygosity (*Ho*), expected heterozygosity (*He*) and polymorphism information content (PIC) of 16 SSR markers on 73 genotypes of mulberry species

SSR Primers	Number of alleles	Observed heterozygosity (*Ho*)	Expected heterozygosity (*He*)	PIC
MulSTR1	6	0.65	0.52	0.56
MulSTR2	6	0.56	0.45	0.67
MulSTR3	11	0.71	0.49	0.84
MulSTR4	4	0.55	0.50	0.56
MulSTR5	8	0.69	0.71	0.77
MulSTR6	8	0.77	0.60	0.72
SS01	2	0.50	0.50	0.45
SS02	5	0.55	0.46	0.66
SS04	7	0.64	0.60	0.70
SS05	2	0.53	0.39	0.55
SS06	3	0.57	0.44	0.52
SS09	3	0.60	0.55	0.56
SS17	5	0.70	0.51	0.60
SS18	3	0.62	0.50	0.54
SS19	3	0.55	0.45	0.43
SS20	7	0.64	0.60	0.64
Average	5.19	0.61	0.52	0.61

The observed and expected heterozygosity were found between 0.53 (SS05) and 0.77 (MulSTR6), 0.39 (SS05) and 0.71 (MulSTR5), respectively (Table 1). The average observed and expected heterozygosity were found as 0.61 and 0.52, respectively. Results clearly indicating that our outcrossing mulberry genotypes have shown high degree of heterozygosity. Garcia-Gomez *et al*. (2019) used thirty-seven mulberry genotypes from nine different countries in the SSR analysis and they observed of SSR markers heterozygosity ranged from 0.0 (SS19) to 0.91 (MulSTR3), with a mean of 0.52. Wangari *et al*. (2013) used ten mulberry accessions, which included five *Morus alba* and five *Morus indica* genotypes in SSR analysis. They used 15 SSR primers and obtained mean observed (Ho) and expected (He) heterozygosity as 0.37 and 0.36, respectively. Zhao *et al*. (2005) reported Ho and He as 0.43 and 0.51 in 27 mulberry genotypes. Aggarwal *et al*. (2004) also reported a higher Ho value of 0.59 across 45 mulberry genotypes from species of diverse origin using SSR markers. Our results are in general comparable even indicating higher observed and expected heterozygosity than above studies and our findings reveals a high genetic variability among the mulberry genotypes. This implies, there is a high genetic variability among mulberry genotypes grown in Turkey.

Polymorphic information content (PIC) is a widely used measure of the usefulness of a molecular marker and therefore the PIC values were calculated for all primers (Table 1). The average PIC value was obtained as 0.61. The most informative loci were MulSTR3 (0.84), while the least informative loci were SS19 (0.43), respectively. According to our results ten loci as informative markers (PIC>0.5) and four loci as suitable for mapping (PIC>0.7) (Table 1). These results indicate that all the markers could contribute substantial information to mulberry genetics and breeding research. The results also show that the mulberry populations of eastern Anatolia have relatively high levels of genetic diversity. Wani *et al*. (2013) characterized 17 mulberry genotypes by 6 SSR markers and they obtained PIC values between 0.26 (MulSTR3) and 0.62 (MulSTR4), with an average of 0.44 per locus indicating lower values than our study. However same 6 SSR markers utilized by Ramesh *et al*. (2004) produced a polymorphic information content from 0.85 to 0.90 among the 43 mulberry genotypes indicating higher values than our study. Zhao *et al*. (2007) used twenty-seven mulberry genotypes representing 11 species and three varieties of genus *Morus* and found PIC values from 0.20 to 0.68, with an average of 0.45. The main propagation system of mulberries through seed should be the responsible of this great diversity (Orhan, 2009).

The dendrogram, in general, separated 73 genotypes into 3 main clusters, which indicates the heterogeneity of the seed propagated mulberry genotypes/landraces in the eastern Anatolia region in Turkey (Figure 1). Cluster I represent 26 *Morus nigra* genotypes and clearly separated from the other species and further divided 2 subgroups. *Morus nigra* genotypes showed close genetic relationships and lower genetic diversity. The similarity ratio among *Morus nigra* genotypes was between S7 and S11 (0.95) and S2 and S9 (0.69), with an average of 0.77 (Figure 1). Cluster II consisted of 21 *Morus rubra* genotypes and could be further divided 2 sub-clusters. The genetic diversity among *Morus rubra* genotypes higher than *Morus nigra* (Figure 1). In cluster II the closest *Morus rubra* genotypes were K2 and K5 with 0.88 while the most distant genotypes were K4 and K7 with 0.55 similarity ratio. The average similarity ratio among *Morus rubra* genotypes was 0.62. Cluster III consisted of 26 *Morus alba* genotypes and local cultivars (landraces). In cluster III local cultivars and genotypes formed mixed population and cluster III further divided 2 sub-cluster. Cluster III is the most diverse group compared cluster I and II (Figure 1). In another word among 3 *Morus* species the genetic diversity was the highest among *Morus alba* followed by *Morus rubra* and *Morus nigra*. The closest genotypes/landraces in cluster III were Angut 3 and Angut 4 with 0.80 similarity ratio whereas the most distant genotypes in cluster III were Kastamonu and Kemaliye 3 with 0.42 similarity ratio. The average similarity ratio among 26 *Morus alba* genotypes/landraces was 0.46. Such a wide interval of similarity values indicates a wide range of genetic diversity in the mulberry genotypes analysed in this study. Furthermore, the genotypes originating from the seedlings also affect the present results. In Turkey most of the mulberry trees non-grafted, which increases the genetic variation in mulberry germplasm, and offers an opportunity for breeders to select the best genotypes.

Genotypes collected from various parts of eastern Anatolia showed diverse clustering; this may be due to out-crossing nature of mulberry.
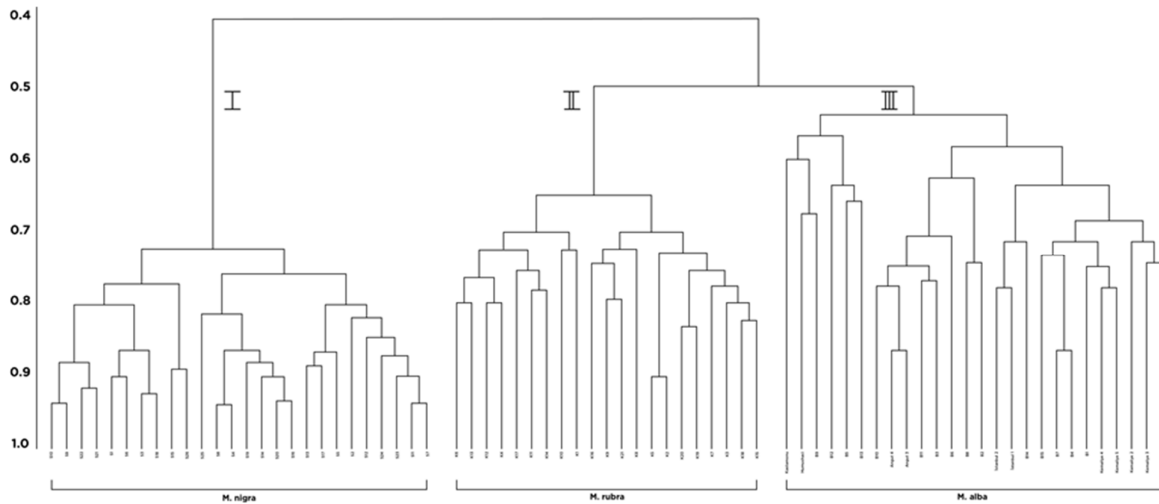

Figure 1. The UPGMA dendrogram based on simple matching similarity matrix obtained using 16 SSR markers, illustrating the relative similarity among 73 genotypes, of different *Morus* species

Principal coordinate analysis (PCoA) indicates that the three first 3 principal coordinates explain 45.1, 24.4, and 9.5%, respectively, accounting for 79.0% of the genetic similarity variance (Figure 2).
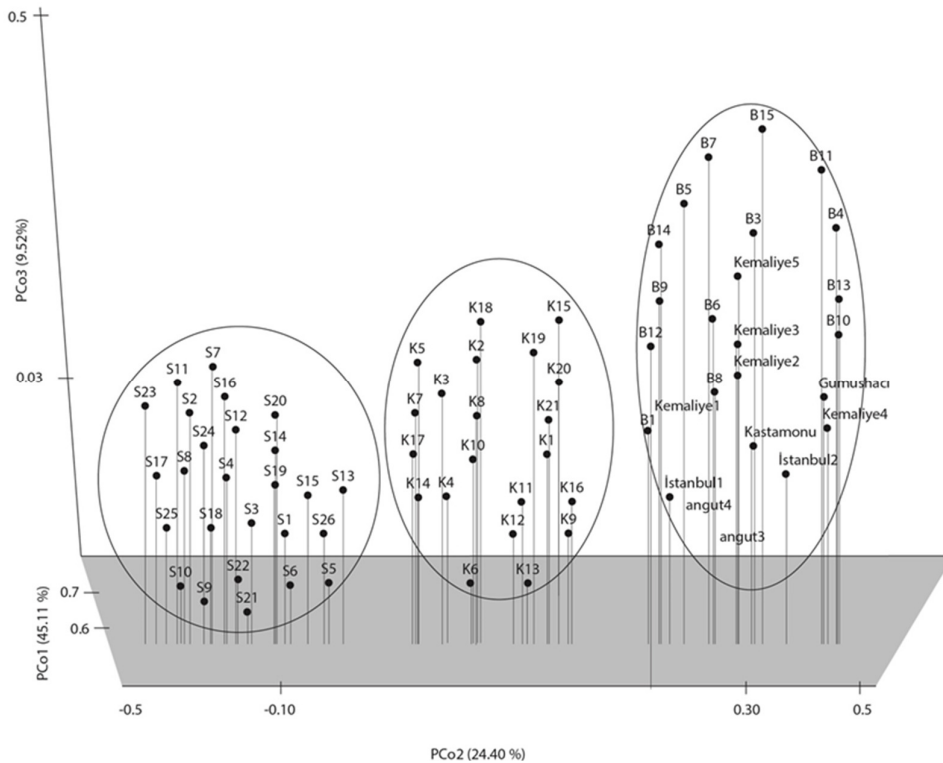
Figure 2. PCoA plot for the *Morus nigra* L, *Morus rubra* L. and *Morus alba* L. genotypes (S: *Morus nigra*; K: *Morus rubra*; B: *Morus alba*)

These mulberry genotypes samples were partitioned into 3 distinct groups. PCoA Group 1 included only *Morus nigra* samples. PCoA Group 2 included *Morus rubra* samples and PCoA Group 3 included only *Morus alba* samples and genetic diversity was lowest among *Morus nigra* genotypes and was the highest among *Morus alba* genotypes (Figure 2) and in general consistent with the results of cluster analysis. The group 1 characterized by early ripening time. The genetic diversity higher in Group 2 than in Group 1. The markers employed here should be useful for the characterization and comparison of mulberry germplasm collections and for the detection of propagation errors. The evaluation of the molecular diversity of mulberry genetic resources is important for the optimal development of programs aimed at conservation. These markers were able to identify uniquely all the mulberry species and genotypes studied.

Conclusions

In conclusion, the current study found the existence of reasonable variability among the tested 73 *Morus nigra, Morus rubra* and *Morus alba* genotypes/landraces, which could be exploited for future breeding. The results revealed that 16 selected SSR markers were highly polymorphic and sufficiently distinguished the tested mulberry species, genotypes/landraces. The cluster analysis classified the 73 mulberry genotypes into three major distinct genetic groups respective of the source of species. Hence, the information generated will contribute significantly to mulberry improvement in Turkey.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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