

MOLECULAR CHARACTERIZATION OF *Prunus mahaleb* L. ROOTSTOCK CANDIDATES BY ISSR MARKERS

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Prunus mahaleb is widely used as rootstocks particularly on calcareous and dry soils for both sweet and sour cherry cultivars in Turkey. Genetic diversity and relationships among members of *Prunus mahaleb* including 29 pre-selected rootstock candidate accessions from Tokat region in Turkey were investigated by using 15 ISSR markers. The study revealed high genetic diversity among accessions, detecting 138 fragments, of which 103 (75%) were polymorphic. The number of polymorphic bands per primer was between 3-13, with average of 6.86. The primers 890 and 891 gave the highest polymorphism ratio (100%). The UPGMA dendrogram and the principal coordinate analysis revealed a clear differentiation among accessions. Reference rootstock, SL-64 clustered separately. The study demonstrates that ISSRs provide promising marker tools in revealing genetic diversity and relationships in *Prunus mahaleb* rootstock candidate accessions and can contribute to efficient identification, conservation, and utilization of germplasm for rootstock improvement through conventional as well as molecular breeding approaches.

Key words: Genetic diversity, ISSR, Mahaleb, Molecular characterization

INTRODUCTION

Deciduous fruit trees including cherries are diverse group (RADICEVIC *et al.*, 2012) and commonly propagated onto rootstocks (ERCISLI *et al.*, 2006; ZORIC *et al.*, 2012). In contemporary tree fruit production, the selection of rootstocks is an important long-term

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management decision, which may influence fruit production and quality (OGNJANOV *et al.*, 2012; TURKOGLU *et al.*, 2012). Rootstocks are selected in order to accomplish specific management objectives such as: controlling size; adaptability to the soil of a specific site; adaptability to the climate in a specific location; to confer pest resistance to the rootstock; to increase growth efficiency [precocity, flower formation, fruit set, alternate bearing, partitioning between shoot and fruit growth]; to effect fruit size, quality, or time until harvest; to reduce suckering; to overcome incompatibility to scion cultivars; and to ease propagation of rootstocks and grafting (ROM and CARLSON, 1987; WEBSTER, 2001).

Rootstocks are generally either of seedling origin or asexual propagation of clonally selected stocks. Seedlings are cheap and easy to produce, generally more vigorous, but may delay bearing and not be uniform. The seeds used for seedling stock production are typically produced at fruit processing plants or widely obtained from wild grown trees (ERCISLI *et al.*, 2006; MRATINIC *et al.*, 2012).

Prunus mahaleb is one of the leading rootstocks for sweet cherries in Turkey (MISIRLI, 1991). *Prunus mahaleb* trees from seed are drought tolerant and produce few root suckers (ROM and CARLSON, 1987). In the natural populations there are many mahaleb types overall Turkey. In Turkey, several Gisela rootstocks currently being used extensively to reduce the size of sweet cherry trees and improve early fruit production (ERCISLI *et al.*, 2006). However in calcareous and dry soil conditions Gisela rootstocks may be the tendency toward poor production and small fruit size. Management techniques can mitigate those problems, but it is likely that *Prunus mahaleb* will maintain a position in the Turkey's cherry industry as one of the preferred rootstocks for cherry growers (BOLSU and AKCA, 2011).

Prunus mahaleb trees grows abundantly particularly inner Anatolia region of Turkey, which prefers warm and dry climate. It is well known that there are many mahaleb types in Turkey, but they have not been critically examined properly. Some of them previously evaluated for their some morphological characteristics that strongly influenced by environmental conditions (AYDIN *et al.*, 2002; ERCISLI and ORHAN, 2008; EROGUL and HEPAKSOY, 2008). Knowledge of genetic variation and relationships between accessions or genotypes is important: (i) to understand the genetic variability available and its potential use in breeding programs, (ii) to estimate any possible loss of genetic diversity, (iii) to offer evidence of the evolutionary forces shaping the genotypic diversities, and (iv) to choose genotypes to be given priority for conservation (THORMANN *et al.*, 1994). Characterization of genetic resource collections has been greatly facilitated by the availability of a number of molecular marker systems. Morphological traits were among the earliest markers used in germplasm management, but they have a number of limitations, including low polymorphism, low heritability, late expression, and vulnerability to environmental influences (SMITH and SMITH, 1992). On the other hand, DNA markers do not have such limitations. They can be used to detect variation at the DNA level and have proven to be effective tools for distinguishing between closely related genotypes. Different types of molecular markers have been used to assess the genetic diversity in crop species, but no single technique is universally ideal. Therefore, the choice of the technique depends on the objective of the study, financial constraints, skills and facilities available (KAFKAS *et al.*, 2008; PAVLOVIC *et al.*, 2012).

The major advantage of ISSR markers is the fact that they do not require the time-consuming and expensive step of genomic or other library construction; do not need prior knowledge of DNA sequence for primer design (RAKOCZY-TROJANOWSKA, 2004).

The main goal of this work was to use ISSR markers for *Prunus mahaleb* accessions; to obtain individual identification of all the 29 seed propagated accessions and one international vegetative propagated reference rootstock, SL-64.

MATERIALS AND METHODS

Plant material

29 pre-selected accessions that selected according to their better tree development, productivity and health characteristics and one reference rootstocks SL-64 belonging to species *Prunus mahaleb* were examined. Accessions of *P. mahaleb* which grown on high calcareous soils were collected from the Tokat province located Middle Black Sea region of Turkey. The site characteristics of pre-selected material are shown in Table 1.

Table 1. Site characteristics of Prunus mahaleb genotypes

Genotypes	Coordinates	Altitude
TM01, TM02, TM03, TM17, TM18, TM20, TM23	40°20'54,65" N 36°31'20,75" E	619
TM04, TM05, TM06, TM07, TM10, TM11, TM12, TM13, TM14, TM16	40°20'38,74" N 36°31'23,60" E	598
TM08, TM09, TM31	40°20'30,85" N 36°32'10,00" E	615
TM21, TM26, TM32	40°18'58,40" N 36°32'02,30" E	761
TM15, TM28, TM29, TM30	40°18'57,90" N 36°30'56,65" E	895
TM22, TM24	40°20'38,99" N 36°31'52,12" E	608

DNA isolation and ISSR primers

Total DNA was extracted from leaves using the protocol of DOYLE and DOYLE (1987). The leaves stored at -86 °C for later use. Fifteen ISSR primers that shown in Table 2 was used. Those primers selected for polymerase chain reaction (PCR) amplification due to their previously determined high level of polymorphism, repeatability, and the best readability (scorability).

ISSR analysis

The PCR amplification procedure was performed at 94 °C for 1 min, followed by 35 cycles at 50–54 °C for 1 min, and at 72 °C for 1 min and finally at 72 °C for 5 min. Amplification products were characterized on 3% (w/v) agarose gels (immersed) at 100 V for 3 h and visualized with ethidium bromide (0.5 g/mL) under UV light, then photographed.

Data analysis

In ISSR analysis, the band patterns were scored as present (1) or absent (0) for each primer pair. Only strong, reproducible, and clearly distinguished bands were used in the analysis.

ISSR primers were analysed by using NTSYS-pc (Numerical Taxonomy and Multivariation Analysis System, Version 2.11V) programme as previously described (ROLF, 1992). The genetic distance among genotypes was determined by using RST-22 programme (GOODMAN, 1997).

RESULTS AND DISCUSSION

Levels of polymorphism

The used 15 ISSR markers generated bright amplification products and polymorphisms, and were used in further analysis (Table 2). A total of 138 reliable fragments were obtained and among them 103 were found to be polymorphic with a average polymorphism ratio of 72.86%. The number of fragments per primer ranged from 6 to 15 with the average number of bands per primer being 9.20. The number of polymorphic fragments per primer ranged from 3 to 13 with the average number of polymorphic bands per primer being 6.86. The results of PCR amplification are given in Figure 1. Some of polymorphic bands produced by ISSR primers were unique and could be used to discriminate the *Prunus mahaleb* genotypes. In this study it was possible to distinguish *Prunus mahaleb* genotypes by using totally 15 specific ISSR markers, which showed either presence or absence of bands generated by those primers (Table 2; Figure 1). The ISSR primer 881 gave the lowest number 869 of total bands while ISSR primer 842 gave the highest number of total bands (15). The lowest and highest polymorphic bands are obtained from primer 811 (3) and primer 842 (13), respectively. The highest polymorphism ratio were obtained from ISSR primers 890 and 891 (100%) while the lowest obtained from primer 811 (30%) (Table 2).

Table 2. Primers, and polymorphism of ISSR markers used among 29 Prunus mahaleb genotypes

ISSR primer code	The number of total bands	The number of polymorphic bands	Polymorphism ratio (%)
807	13	9	49
808	7	5	71
810	9	8	88
811	10	3	30
826	9	6	66
835	7	5	71
841	10	7	70
842	15	13	86
844	10	7	70
856	7	5	71
881	6	4	66
888	8	6	75
889	10	8	80
890	8	8	100
891	9	9	100
Average	9.20	6.86	72.86
Total	138	103	

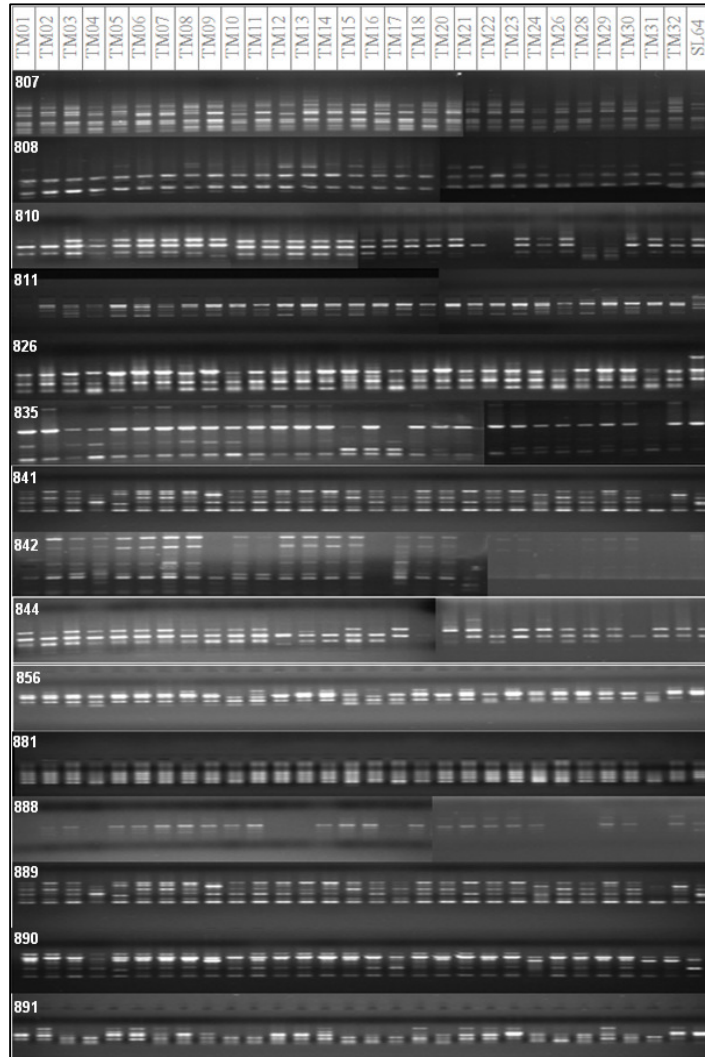


Figure 1. ISSR amplification products and schematic of the bands pattern of genotypes with the 15 primers used

EROGUL (2009) sampled 60 *Prunus mahaleb* genotypes from Turkey and used them in both SSR and AFLP analysis and found that polymorphism ratio were 82% in SSR analysis and 88% in AFLP analysis, which indicate slowly higher polymorphism ratio than our study. When we compared our results to above literature, we can say that our accessions originate from a small geographic area, therefore the genotypes could be close each other. On the other hand we used different marker system that could be effect the polymorphism ratio.

Genetic variation and cluster analysis of *Prunus mahaleb* genotypes

Using the data from all PCR amplification bands shown by 15 ISSR markers, the genetic similarity matrix among all sources used in the present work was obtained by multivariate analysis using Nei's coefficient. Similarity coefficients ranged from 0.62 to 0.87 with an average of 0.73. The highest genetic similarity coefficient (0.87) was found between TM05 and TM06; TM26 and TM28, indicating that they are closely related. The lowest genetic similarity coefficient 0.62 was found between SL-64 and TM07, indicating that they are relatively remote in relationship. In general reference rootstock SL-64 showed distant genetic relationships with all 29 *Prunus mahaleb* genotypes. TURKOGLU *et al.* (2012) used SSR analysis to characterize 40 *Prunus mahaleb* genotypes sampled from Black sea region of Turkey and they showed that genetic relationships of 40 *Prunus mahaleb* genotypes were different and the range of distance varied from 0.05 to 1.00. They did not find identical genotypes according to SSR results. However, ABEDIAN *et al.* (2012) used 58 *Prunus mahaleb* genotypes in Iran and based on cpSSR analysis the lowest genetic distance was showed between "T143" and "T96" genotypes (0.0). KAVAKLI (2006) reported genetic similarity between 0.21 and 0.88 among 11 *Prunus mahaleb* genotypes by using 32 RAPD primers.

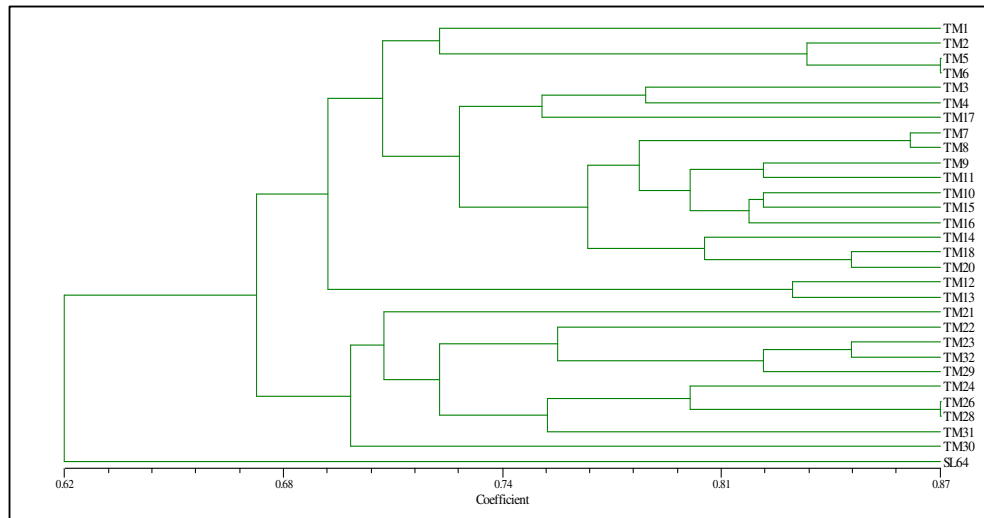


Figure 2. A dendrogram obtained by UPGMA for 29 *Prunus mahaleb* genotypes based on ISSR markers

A tree constructed from the ISSR data divided the genotypes into two main clusters (Figure 2). The first cluster included the reference *Prunus mahaleb* rootstock SL-64. The rest of the genotypes clustered together within cluster 2. The cluster 2 further divided 2 subcluster. Genotypes TM01, TM02, TM05, TM06, TM03, TM04, TM17, TM07, TM08, TM09, TM11, TM10, TM15, TM16, TM14, TM18, TM20, TM12, and TM13 clustered together in subcluster 1 within cluster 2 and TM21, TM22, TM23, TM32, TM29, TM24, TM26, TM28, TM31 and

TM30 formed subcluster 2 within cluster 2 (Figure 2). ABEDIAN *et al.* (2012) used 58 *Prunus mahaleb* and 6 *Prunus avium* genotypes and according to cpSSR analysis results, genotypes places into two main clusters and four groups. Cluster I is the biggest cluster, comprised of 58 *Prunus mahaleb* genotypes which divided in the three groups and Cluster II consisted of six sweet cherry accessions that clustered in one group, separately.

Principal Coordinate Analysis

The genotypes were plotted on three dimensions based on their PCA results (Figure 3). The first three principal axes of PCA analysis explained 15.51%, 10.08% and 8.09% of the total variation, respectively. Sum of first three PCAs could be represented most of (33.68%) the total variation in the original dimensions and confirmed the results of cluster analysis. As indicated before, in ISSR dendrogram TM01, TM02, TM05, TM06, TM03, TM04, TM17, TM07, TM08, TM09, TM11, TM10, TM15, TM16, TM14, TM18, TM20, TM12, and TM13 were clustered together. In PCA analysis those genotypes plotted together again. In addition in ISSR dendrogram, TM21, TM22, TM23, TM24, TM26, TM28, TM29, TM30, TM31 and TM32 were clustered together and they showed similar trend in PCA analysis. Another interesting finding was that reference rootstock, SL-64 were found more close to second group (Figure 3). OZYURT *et al.* (2012) reported that some genotypes placed second group has easily propagated by vegetative methods. The all seed propagated genotypes were easily separated from the reference rootstock SL-64.

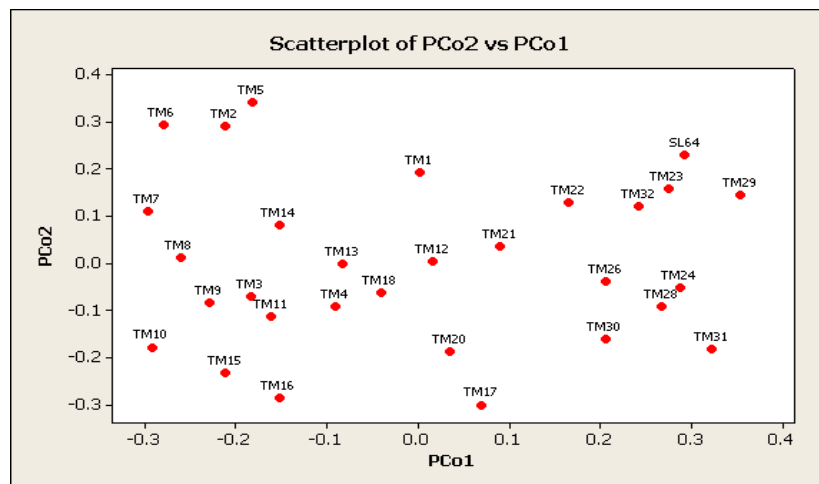


Figure 3a. Scatter plots of 29 *Prunus mahaleb* genotypes based on the first, and second components of principal coordinate analysis using ISSR data

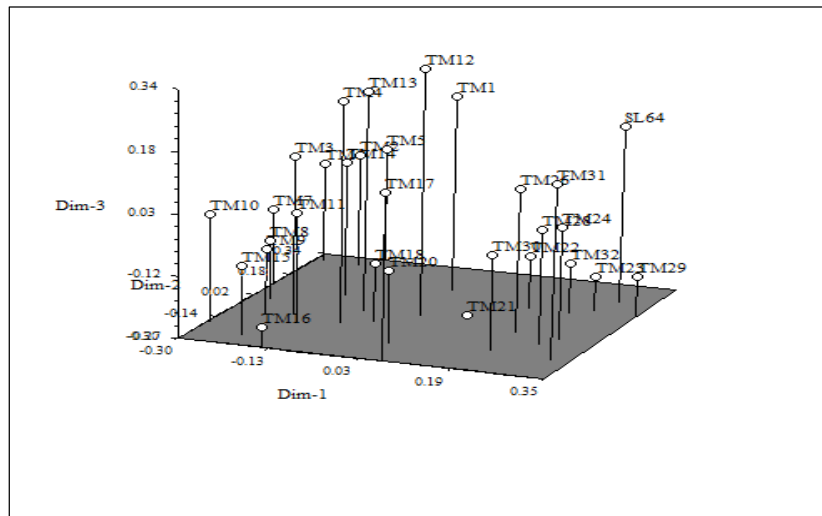


Figure 3b. Scatter plots of 29 *Prunus mahaleb* genotypes based on the third components of principal coordinate analysis using ISSR data

CONCLUSION

In the study we did not find identical *Prunus mahaleb* genotypes that indicate diverse origin of the studied materials. All genotypes separated easily from reference rootstocks, SL-64. The genetic variability among the twenty-nine *Prunus mahaleb* genotypes was relatively high. Our data also showed the relevance of molecular studies for management and *Prunus mahaleb* genetic resources conservation. The results of this study indicated that ISSR technique constitute a useful tool allowed us to identify individually all the 29 *Prunus mahaleb* genotypes.

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**MOLEKULARNA KARAKTERIZACIJA *Prunus mahaleb* L. KANDIDATA ZA
PODLOGU KORIŠĆENJEM ISSR MARKERA**

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Izvod

Prunus mahaleb je veoma korišćena kao podloga posebno na kalcifikovanim i suvim zemljištima kako za slatku tako i za kiselu višnju u Turskoj. Vršena su ispitivanja genetičke divergentnosti i odnosa članova *Prunus mahaleb*, uključujući 29 preselekcionisanih kandidata podloge u region Tokaja u Turskoj korišćenjem 15 ISSR markera. Utvrđena je velika genetička divergentnost ispitivanih podloga detekcijom 138 fragmenata od kojih su 103 (75 %) polimorfna. Broj polimorfnih fragmenata po prajmer sekvenci je 3 – 13, sa prosekom 6.86. Prajmeri 890 i 891 su dali najveći odnos polimorfnosti (100 %). The UPGMA dendrogram i analiza osnovne koordinate su potvrdili jasnu diferencijaciju uzoraka. Referentna podloga, SL-64 se grupisala odvojeno. Dobijeni rezultati su pokazali da je korišćenje ISSR markera pouzdan metod u utvrđivanju genetičke divergentnosti *Prunus mahaleb* kao i u efikasnoj identifikaciji, konzervaciji i korišćenju germplazme za poboljšanje podloga u konvencionalnim i molekularnim tehnologijama oplemenivanja.

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