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Original scientific article

MOLECULAR CHARACTERIZATION OF WILD CAROB (*Ceratonia Siliqua* L.) GENOTYPES BY SEQUENCE-RELATED AMPLIFIED POLYMORPHISM (SRAP) TECHNIQUES IN TURKEY

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Carob (*Ceratonia siliqua* L.) with limited widespread in Turkey is considered as secondary forest tree. In this study, molecular characterizations were made for 508 genotypes of seven different carob populations colelcted from Eagean, Western and Eastern Mediterranean regions of Turkey with the aid of sequence-related amplified polymorphism (SRAP) technique. Identification of wild carob genotypes, relativity levels and genetic variations among them were performed. Genetic similarities among 508 wild carob genotypes collected from Eagean, Western and Eastern Mediterranean regions of Turkey varied between 0.20-1.00 and there was a large variation among the genotypes. The genetic similarities among 250 wild carob genotypes collected from Aegean region varied between 0.36-1.00. The genetic similarities among 154 wild carob genotypes collected from Western Mediterranean region varied between 0.23-1.00. The genetic similarities among 102 wild carob genotypes collected from Eastern Mediterranean region varied between 0.21-1.00. Through the molecular analyses conducted with SRAP primers, besides the large variations among the entire genotypes, large variations were also observed between the genotypes of different regions. With this study, genetic

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variations were put forth among the wild carob genotypes naturally growing in different regions of Turkey. It was concluded based on present findings that marker system could reliably be used to put forth genetic variations among wild carob genotypes.

Key words: Carob*; Ceratonia siliqua*, wild carob trees, genetic characterization, SRAP marker

INTRODUCTION

Carob (*Ceratonia siliqua* L.) with limited widespread is considered as secondary forest tree (Non-Wood Forest Products). Thus, scientific researches on carob are genereally neglected. Despite such a limited widespread, fruits play a significant role in local and national economy. Trees have recently been used in rehabilation of infertile agricultural lands.

Turkey is very rich for horticulture plant genetic resources (DOGAN *et al*., 2014; IKINCI *et al*., 2015; BOZHUYUK *et al*., 2020; CAVUSOGLU *et al*., 2021; BAHAR *et al*., 2022). Carob (*Ceratonia siliqua* L.) with various local names in Turkey including harnup, karabe, harap, haraç or kırat belongs to Caesalpiniodieae sub-family of Fabacea family (Leguminosae). Ever-green trees have broad leaves and grown in shrub and samll tree forms. Branchy carob trees have quite hard stems and wide tops. Flowers are generally dioic, sometimes monoic and rarely hermrophredic. Carob is a member of Mediterrnean maquis formation (BATLLE and TOUS, 1997; BOLARIC *et al*., 2021). Carob trees with edible fruits are among the economic value-added plant taxa of Turkey (SECMEN, 1973; KELES *et al.*, 2014).

Carob seeds have an impervious, waterproof testa, thus they have been used to weigh diamonds for centuries. Then, they named the measure of "kırat" or "karat" (ANONYMOUS, 2012; ALEXANDER and SHEPPERD, 1974). Carob seeds are processed into a natural galactomannon-containing polysaccharide (carob gum) through grounding the seed endosperms and resultant polysaccharide has a stabilizer effects and thus is used in food, cosmetics, die, textile, film and drug industries as a stabilizer (PEKMEZCI *et al.,* 2008).

Carob is originated from Eastern Mediterranean and has a natural widespread in Mediterranean costs and California. Carob has the best growth and the greatest widepread in Mediterranean area. In Turkey, it has a spread from İzmir- Urla to Southern Anatolia, Antalya, Mersin, Adana and Hatay (Samandağ) porivinces. Trees grow over stony and dry places in groups or individually (SECMEN, 1974; ANONYMOUS, 1991). As the new natural spread of this species in Turkey, wild carob trees were encountered at the furthest northern latitudes in İzmir-Karaburun (between 38°39’ and 26°28’) and the furthest southern latitudes in Hatay- Yayladağ - Denizören (between 35°57’ and 35°55’) (KELES, 2015).

Despite quite much health impacts and wide range of use, carob production worldwide is continuously decreasing. For instance, annual production was about 650,000 tons in 1945, 656,877 tons in 1961 and decreased continuously until the year 2007. Annual production was 181,830 tons in 2005. A slight increase was observed recently (about 6%) and annual production reached to 193,250 tons in 2017. Of this total annual production worldwide, 37,2% comes from Spain. Spain has been the leading carob producer of the world for centuries, but country production is also continuously decreasing. The annual production of Spain was 299.600 tons in 1970, 197.000 tons in 1980 and 64,100 tons in 2005. Productions are performed economically in

6 provinces of Turkey and annual production from 284.789 fruiting trees of 326.229 trees was 14.413 tons in 2017 (FAO, 2017).

Turkey has a great position worldwide with regard to plant diversity and genetic resources. Different climate conditions contribute a lot to such a genetic diversity. Diverse range of climate allows cultivation and culture of several species. Diverse genetic sources have brought the country to a significant position in world pomiculture (ERCISLI, 2004). Among these genetic sources, carob has an important place.

Molecular markers quite facilitate the collection and characterization of genetic sources. Morphological characteristics are widely used in generation of germplasm. Because of low heredity and polymorphism, environment has limited effects (SMITH and SMITH, 1992). However, there aren't such restrictions in DNA markers. DNA markers are quite efficient in finding out the separation between the close genotypes. Different types of molecular markers are commonly used to identify genetic diversity in different species. However, each technique has its own specific advantages (BENJAK *et al*., 2005; KAFKAS *et al.,* 2008; PAVLOVIC *et al.,* 2012; ERDINC *et al*., 2021; HASANBEGOVIC *et al*., 2021).

In the past few decades, many investigations in the field of plant biology have employed selectively neutral, multilocus, dominant markers such as inter-simple sequence repeat (ISSR), random-amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP). Molecular markers are also used in resistance analysis and resistance genes to pests and diseases generally exist in wild forms of the plants (BOERMA and HUSSEY, 1992).

More recently, sequence-related amplified polymorphism (SRAP) markers have been developed, which are used to amplify coding regions of DNA with primers targeting open reading frames. These markers have proven to be robust and highly variable, on par with AFLP, and are attained through a significantly less technically demanding process. SRAP markers have been used primarily for agronomic and horticultural purposes, developing quantitative trait loci in advanced hybrids and assessing genetic diversity of large germplasm collections (ROBARTS and WOLFE, 2014).

There are several reports about successful use of Sequence-related amplified polymorphism (SRAP) markers in genetic diversity studies in different countries. Studies were carried out for the first time with recombinant breeding and diploid lines of *Brassica oleracea* L.; following the sequence analyses, it was observed that 45% of gel-isolated bands matched with the known-genes of the genebank. Following the sequence analyses, it was also determined that 20% of SRAP markers were co-dominant markers (LI and QUIROS, 2001).

The studies about identification of genetic structure and variation, interactions of regeneration and growth characteristics and pollination variation in carobs are quite limited both in Turkey and in the world. Therefore, this study was conducted for molecular characterization of natural-wild carob populations of Turkey. The primary target was to put forth the genetic differences between the populations.

MATERIAL AND METHODS

For DNA analyses, fresh shoots-leaves were collected from 23 wild populations in Aegean, Western and Eastern Mediterranean regions of Turkey. Aegean and Mediterranean region are one of the warmest regions in Turkey with an average daily high temperature of 22 ^{0}C.

In these area summers are usually hot and dry, whereas winters are warm and rainy. A total of 508 individual materials were used. Of these shoot-leaf materials, 455 were taken from female and 53 were taken male trees. DNA isolations were performed from the leaves of 508 wild carob genotypes in accordance with CTAB (Cetyltrimethyl ammonium bromide) method. For DNA extractions, fresh leaves of each individual sample were collected and preserved at -80ºC until DNA extraction procedures (BARRACOSA *et al.*, 2008). Sample DNA densities were measured with a NanoDrop microscale spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE).

In SRAP analyses, 208 SRAP primer combinations as of 13 forward (from Me-1 to Me-13) and 16 reverse (from Em-1 to Em-16) were tested. Among the tested SRAP primer combinations, 16 SRAP primer pairs yielding high number of bands and able to be scored the best were selected. PCR components and conditions were generated with the aid of modified method GULSEN *et al.* (2005). PCR products were electrophoresed in 1X TAE buffer and 2% agarose gel, stained with ethidium bromide and imaged in gel-imaging (Kodak Imaging System 440 CF) (Kodak, Rochester, NY, USA).

Following the gel electrophoresis and imaging, images were scores as (1) for existing bands, (0) for absence of bands and (9) for none-amplified ones. Data were gathered with the aid of NTSYS (Numerical Taxonomy and Multivariate Analysis System) software. A similarity matrix was generated with the aid of Dice method and a dendrogram of carob trees was generated with the aid of UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. For each marker used in this study, number of bands, number of polymorphic bands and polymorphism ratios were determined. For polymorphism ratio, the equation (number of polymorphic bands x 100/Total number of bands) was used.

RESULTS AND DISCUSSION

For SRAP analyses on natural-wild carob populations collected from Aegean, Western and Eastern Mediterranean regions, 16 different primers were used. SRAP primer names, base sequences, polymorphic band lengths (PBL), number of polymorphic bands (NPB), total number of bands (TNB) and polymorphism ratios (PR) are provided in Table 1.

Number of polymorphic bands (NPB) varied from 3 to 8 and there were not any primers without bands. Total number of bands (TNB) was 109; total number of polymorphic bands (NPB) was 87; average number of bands was 5.4; average total number of bands was 8.81. The greatest number of bands was obtained from the primers 2 and 15 (9 bands) and the lowest number of bands was obtained from the primers 8, 13, 14 and 16 (5 primers). Polymorphic band lengths (PBL) varied between 180 bp and 1800 bp. Average polymorphism ratio (PR) was 80.91% with the greatest ratios from the primers 1, 4, 11, 13 and 16 (100%) and the lowest ratios from the primer 3 (50%). KONATE *et al.* (2007) carried out a study with 10 different carob genotypes grown in Morocco for characterization of genotypes with the aid of RAPD molecular marker system and obtained about 65% polymorphism among the carob genotypes. AFIF *et al.* (2008) used 7 RAPD markers to assess the genetic diversity and structure of 10 different carob genotypes collected from different geographical and climate zones of Tunisia and reported a polymorphism ratio of 76.31%. Similarly, KONATE *et al.* (2009) used ISSR marker system for molecular characterization of 10 carob genotypes grown in Morocco and reported a

polymorphism ratio of 77.27% among the carob genotypes. KACAR *et al.* (2009) used RAPD and SRAP marker techniques for molecular characterization of 15 different carob genotypes collected from Mediterranean region and Cyprus. Researchers used 18 polymorphic RAPD primers and 17 polymorphic SRAP primers. RAPD analyses revealed a polymorphism ratio of 63.2% and SRAP analyses yielded a polymorphism ratio of 55.2%. Based on results from both molecular markers, it was concluded that genotypes of Turkey and Cyprus were separated from each other, but they both had a narrow genetic diversity.

Table 1 SRAP primer names, base sequences, polymorphic band lengths (PBL), number of polymorphic bands (NPB), total number of bands (TNB) and polymorphism ratios (PR)

No	Primers	PBL	NPB	TNB	PR (%)
1	ME2EM6	200-1100	7	7	100
2	ME2EM7	180-1300	7	9	77,7
3	ME2EM14	400-1200	4	8	50
4	ME2EM15	600-1200	8	8	100
5	ME3EM16	350-1400	6	8	83,3
6	ME7EM18	600-900	5	6	83,3
7	ME8EM15	400-1300	4	7	57,2
8	ME6EM1	300-1800	3	5	60
9	ME9EM1	600-1100	7	8	87,5
10	ME10EM11	350-1100	5	6	83,3
11	ME1EM2	180-1300	6	6	100
12	ME2EM13	300-1600	6	7	85,7
13	ME8EM9	400-1000	5	5	100
14	ME8EM10	500-1000	3	5	60
15	ME10EM10	500-1600	6	9	66,6
16	ME6EM1	400-1400	5	5	100
	Total		87	109	50-100
	Average	5.4	6.81	80.91	

According to molecular analyses with SRAP markers on 508 wild carob genotypes collected from Aegean, Western and Eastern Mediterranean regions of Turkey, a wide variation was observed among all genotypes and the was also a large variation among the regions. (average 79%). Although present findings comply with the results of KONATE *et al.* (2007), AFIF *et al.* (2008) and KONATE *et al.* (2009), KACAR *et al.* (2009) reported a lower polymorphism ratio. Such differences were mainly attributed to genetic differences between the genotypes used in different studies. Since present genotypes were collected from the natural spread zones of the carob trees, there was a larger variation among them.

The dendrogram for wild 508 carob genotypes collected from Aegean, Western and Eastern Mediterranean regions is presented in Figure 1. Molecular marker analyses with the aid of 16 SRAP primers revealed that genetic similarity among the genotypes varied between 0,20-1,00 and there was a large variation among the natural carob genotypes. Similar genotypes were not encountered.

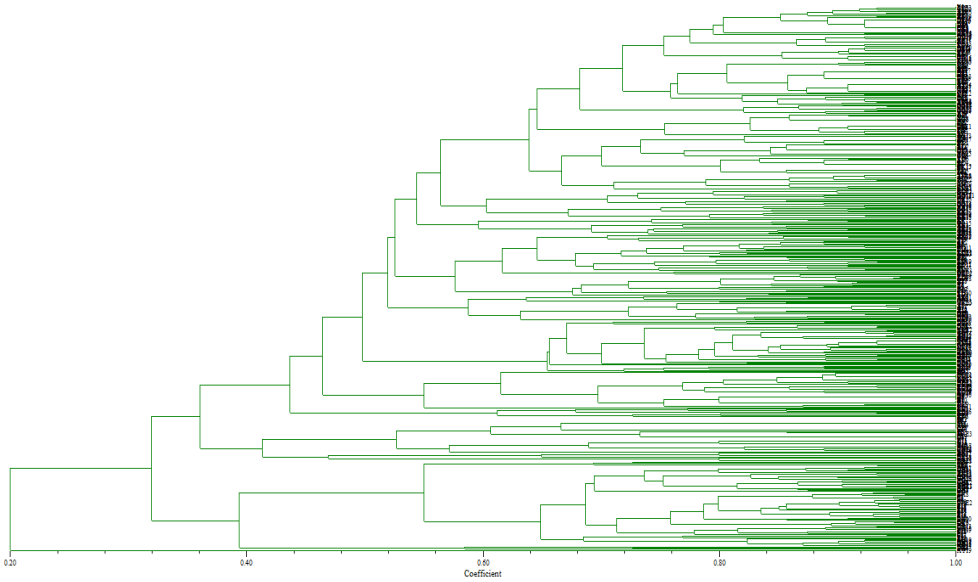

Fig. 1 The dendrogram obtained for 508 wild carob genotypes with the use of 16 SRAP primer pairs

The genotypes collected from three different regions were assessed separately. The dendrogram for 154 wild carob genotypes collected from Western Mediterranean region is presented in Figure 2. The genetic similarity among the wild carob genotypes collected from Western Mediterranean region varied between 0.23-1.00 and the genotype E12 was identified as the furthest genotype. Similarity ratios were close to the ratios observed for entire regions.

The dendrogram for 102 wild carob genotypes collected from Eastern Mediterranean region is presented in Figure 3. The genetic similarity among the wild carob genotypes collected from Eastern Mediterranean region varied between 0.21-1.00 and the genotype A119 was identified as the furthest genotype. There were any similar genotypes among these 2012 wild carob genotypes.

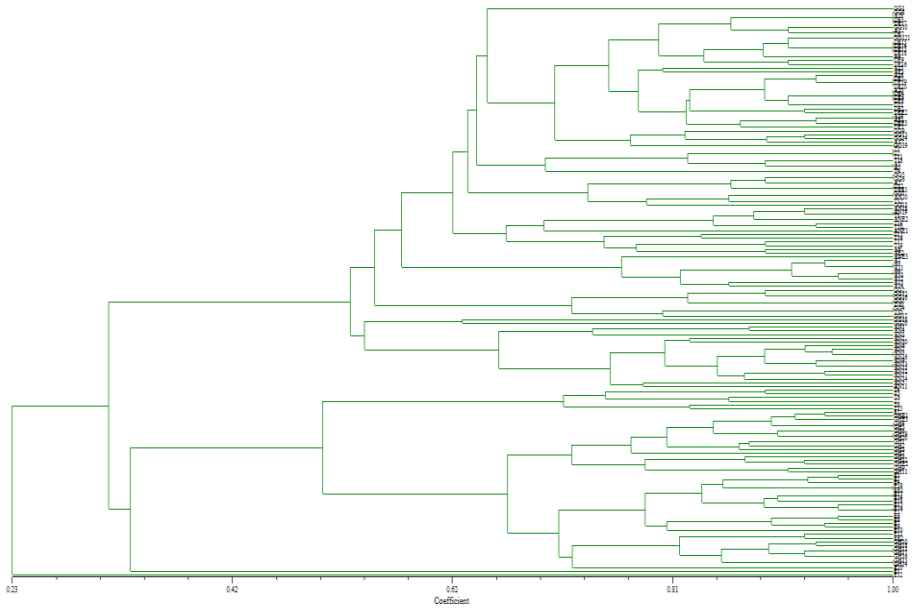

Fig. 2 The dendrogram for 154 wild carob genotypes with the use of 16 SRAP primer pairs collected from Western Mediterranean region

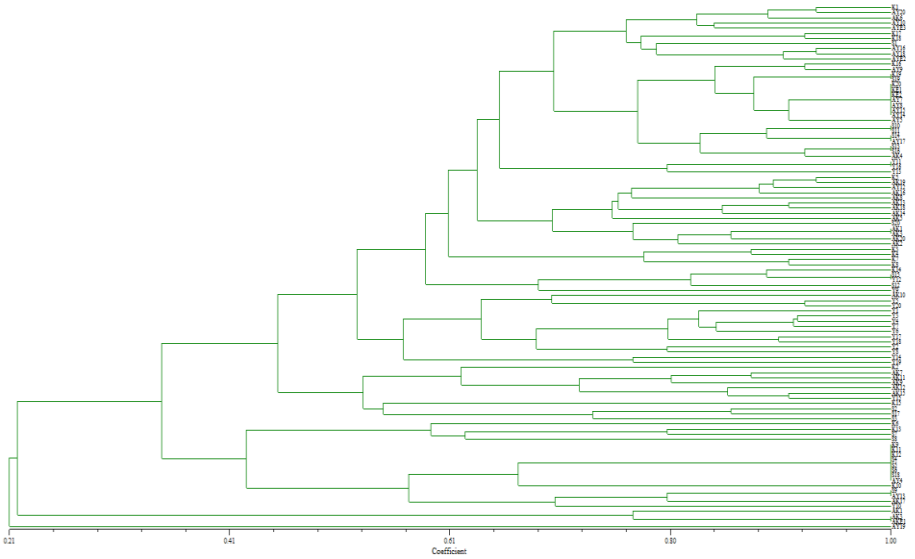

Fig. 3 The dendrogram for 102 wild carob genotypes with the use of 16 SRAP primer pairs collected from Eastern Mediterranean region

The dendrogram for 250 wild carob genotypes collected from Aegean region is presented in Figure 4. The genetic similarity among these 250 wild carob genotypes varied between 0.36-1.00 and the genotype G5 was identified as the furthest genotype. As compared to the genotypes collected from Eastern and Western Mediterranean regions, there was a greater genetic similarity among the genotypes collected from Aegean region. As it was in the other regions, similar genotypes were not observed in Aegean region.

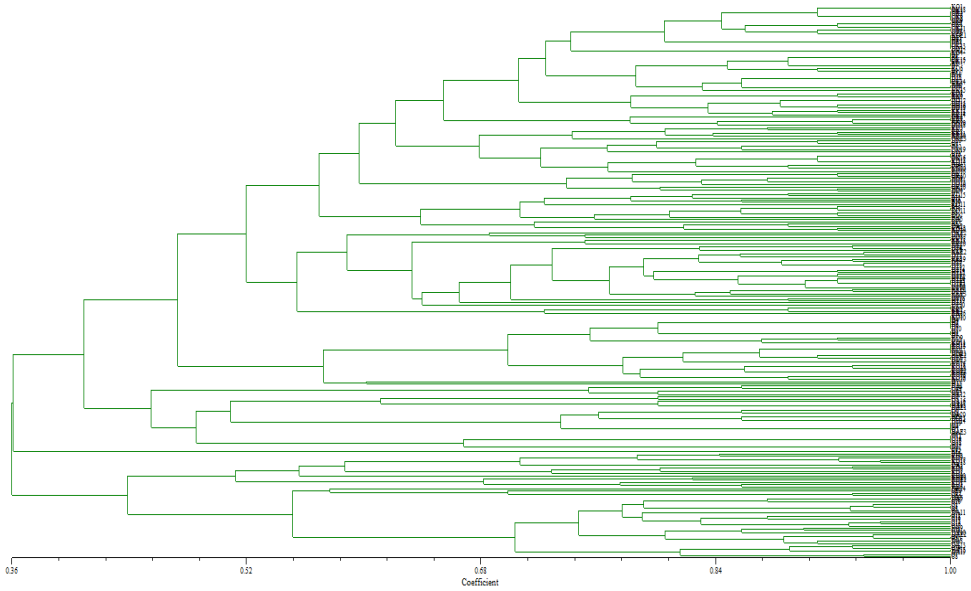

Fig. 4 The dendrogram for 250 wild carob genotypes with the use of 16 SRAP primer pairs collected from Aegean region.

Biochemical analyses on carob genotypes haven't revealed any polymorphic loci representing the genome. Low polymorphisms were observed among the genotypes in some studies (TOUS, 1992; BATTLE and TOUS, 1997). BARRACOSA *et al.* (2008) genetically identified 68 carob genotypes collected from southern sections of Portugal with the aid of morphological characters, RAPD and AFLP techniques. Researchers reported 40% polymorphism with the analyses performed with 18 RAPD primers and 41% polymorphism with the analyses performed with 4 selective primer combination of AFLP. KONATE *et al.* (2009) worked with ISSR (Inter-Simple Sequence Repeat) markers and reported 77,27% polymorphism for the resultant bands and identified 16 primers. KONATE *et al.* (2007) investigated genetic diversity in 10 carob populations with the aid of RAPD markers and identified 67 primers.

CARUSO *et al.* (2009) carried out genetic identifications on carob genotypes collected from Sicilia region of Italy and from Spain with the aid of AFLP method and reported 36% polymorphism among the genotypes. In that study, researchers were able to separate all individual, except for 'Latinissima' and 'Racemosa' genotypes, with the aid of AFLP technique.

CONCLUSIONS

Previous studies revealed that genetic-breeding studies conducted for identification of genotypic structure with the aid of molecular markers, interactions of regeneration and growth characteristics and pollination variation were quite limited. In present study, through the molecular analyses with the aid of SRAP primers, a large variation was observed among 508 wild carob genotypes collected from Aegean, Western and Eastern Mediterranean regions and a large variation was also observed between the genotypes of different regions. With this study, genetic variations were put forth among the wild carob genotypes naturally growing in different regions of Turkey. It was concluded based on present findings that marker system could reliably be used to put forth genetic variations among wild carob genotypes.

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MOLEKULARNA KARAKTERIZACIJA GENOTIPOVA DIVLJEG ROGAČA (*Ceratonia Siliqua* L.) SRAP TEHNIKOM U TURSKOJ

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Izvod

Rogač (*Ceratonia silikua* L.) sa ograničenom rasprostranjenošću u Turskoj smatra se sekundarnim šumskim drvetom. U ovoj studiji, molekularna karakterizacija je urađena za 508 genotipova sedam različitih populacija rogača prikupljenih iz regiona Egeja, zapadnog i istočnog Mediterana Turske uz pomoć tehnike amplifikovanog polimorfizma povezanog sa sekvencom (SRAP). Izvršena je identifikacija genotipova divljeg rogača, stepena relativnosti i genetičkih varijacija među njima.

Genetičke sličnosti među 508 genotipova divljeg rogača prikupljenih iz regiona Egeja, zapadnog i istočnog Mediterana Turske varirale su između 0,20-1,00 i postojala je velika varijacija među genotipovima. Genetičke sličnosti između 250 genotipova divljeg rogača prikupljenih iz Egejskog regiona varirale su između 0,36-1,00. Genetičke sličnosti između 154 genotipa divljeg rogača prikupljenih iz regiona zapadnog Mediterana varirale su između 0,23-1,00. Genetičke sličnosti između 102 genotipa divljeg rogača prikupljenih iz regiona istočnog Mediterana varirale su između 0,21-1,00. Kroz molekularne analize sprovedene sa SRAP prajmerima, pored velikih varijacija među genotipovima, uočene su i velike varijacije između genotipova različitih regiona. Ovom studijom otkrivene su genetičke varijacije među genotipovima divljeg rogača koji prirodno rastu u različitim regionima Turske. Na osnovu sadašnjih nalaza zaključeno je da se sistem markera može pouzdano koristiti za utvrđivanje iznošenje genetičkih varijacija između genotipova divljeg rogača.

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