DETERMINATION OF THE GENETIC DIVERSITY OF WALNUT (*JUGLANS REGIA* L.) CULTIVAR CANDIDATES FROM NORTHEASTERN TURKEY USING SSR MARKERS

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Most of the walnut trees grown in Turkey have been obtained from seeds and show great variability. Humans selected the best seed-propagated ones and kept them in the field. This precious walnut germplasm could be potentially interesting either to directly register them as cultivar or to use them in walnut cross-breeding studies as parent to improve some characteristics of cultivars. This study assessed the genetic diversity of 28 seed propagated walnut genotypes grown in Gumushane province in Turkey using 21 polymorphic walnut SSR markers. Among the used germplasm 129 alleles were detected. Allele number ranged from 2 to 13 and the average allele number was 6.15 alleles per locus. There were no identical genotypes in the dendrogram. Present results revealed that the studied walnut germplasm is genetically variable. This could be important for future cross-breeding activities.
Keywords: molecular characterization, genetic diversity, *Juglans regia* L., SSR

Bestimmung der genetischen Vielfalt von Walnusssorten (*Juglans regia* L.) aus dem Nordosten der Türkei unter Verwendung von SSR-Markern. Die meisten in der Türkei angebauten Walnussbäume wurden aus Samen gewonnen und weisen eine große Variabilität auf. Menschen wählten die besten aus Samen vermehrten Bäume aus und behielten sie auf dem Feld. Diese kostbaren genetischen Ressourcen könnten möglicherweise interessant sein, um daraus entweder direkt Sorten zu registrieren oder sie in Walnusskreuzungsversuchen als Elternsorten zu verwenden, um einige Eigenschaften von bestehenden Sorten zu verbessern. In dieser Studie wurde die genetische Vielfalt von 28 samenvermehrten Walnuss-Genotypen, die in der türkischen Provinz Gumushane angebaut wurden, unter Verwendung von 21 polymorphen Walnuss-SSR-Markern bewertet. Bei dem verwendeten genetischen Material wurden 129 Allele nachgewiesen. Die Allelzahl lag zwischen 2 und 13, und die durchschnittliche Allelzahl betrug 6,15 Allele pro Locus. Es gab keine identischen Genotypen im Dendrogramm. Die vorliegenden Ergebnisse zeigten, dass das untersuchte Genmaterial genetisch variabel ist. Dies könnte für zukünftige Kreuzungsaktivitäten wichtig sein.
Schlagwörter: molekulare Charakterisierung, genetische Diversität, *Juglans regia* L., SSR

Interest in walnut production in Turkey increases and the main focus is on improving quality and on acceleration of the growth of walnut seedlings (Keles et al., 2014). In fact each agricultural region in Turkey has valuable seed propagated walnuts that are adapted to local conditions. They are material selected by humans and have on the one hand a high economic value with a long history of cultivation. On the other hand these walnut genetic resources show resistance to pests and diseases, late bud break, lateral fruit bearing and good nut quality (Kirca et al., 2014).

Determining their genetic diversity is very important for crop improvement programs. Distribution of modern varieties from crop improvement and/or replacement of old landraces with new resistant/tolerant cultivars coupled with urbanization, climate change, population growth and intensive farming are the primary factors contributing to genetic erosion (Adu et al., 2019).

Walnut genotypes, accessions and cultivars have been characterized by morphological and molecular markers (Keles et al., 2014; Vahdati et al., 2015). Morphological markers characterizing accession on a visual basis have some advantages, e. g. they are easy to use, cheaper and sometimes they are more informative than molecular markers, especially when the latter are not connected to traits. However, morphological markers can be influenced by environmental conditions and plant growth stages (Ercisli et al., 2008). Molecular markers are evaluated with high reliability in determining the variation in plant population or the relationships between plant genotypes within a population. Simple Sequence Repeats (SSRs) are widely used in genetic analyses of plants. SSR fingerprints are inherent in genomes and are not affected by external and internal environments, including growth and development time. In addition SSR markers are cheap, fast (within hours), accurate, and reliable (Fazenda et al., 2019; Guney et al., 2019). Molecular characterization of the genotypes is a necessary activity in the management of any germplasm aiming to identify and select individuals with characteristics of economic interest, since this characterization consists of evaluating data to describe, identify and differentiate accessions within species (Eyduran et al., 2016; Guney et al., 2019).

There are no studies reported on SSR analysis of seed propagated walnut grown in Northeastern Turkey. We aimed to provide theoretical knowledge for the construction of DNA fingerprints and the identification (authenticity) of 28 seed propagated walnut genotypes.

MATERIALS AND METHODS

PLANT MATERIAL

In total, 28 seed propagated walnut genotypes along with standard cultivar 'Sebin' from Gumushane province of Turkey were used and analyzed using SSR markers (Table 1).

DNA EXTRACTION, SSR AND DATA ANALYSIS

The Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used to extract genomic DNA from young leaf meristematic tissues according to the instructions provided by the manufacturer. Afterwards, eluted DNA samples were treated with RNAse solution. Purity and concentration of the DNA were controlled both on 1 % (w/v) agarose gels and by NanoDrop® ND-1000 Spectrophotometer (ThermoFisher, Wilmington, USA) (Sarikamis et al., 2010).

A total of 21 walnut SSR loci were used (Table 2). Polymerase Chain Reaction (PCR) was conducted in a final volume of 10 µl and contained 15 ng genomic DNA, 5 pmol of each primer, 0.5 unit GoTaq DNA polymerase (Promega), 0.5 mM dNTP, 1.5 mM MgCl2 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. Biometra® PCR System was used to perform PCR amplification. 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 to 56 °C and 2 mins at 72 °C with a final extension at 72 °C for 10 mins. The PCR products were run on CEQTM 8800 XL Capillary Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) to determine polymorphisms. In order to ensure reproducibility of the results the analyses were repeated at least two times (Sarikamis et al., 2010).

Table 1: Some important morphological traits of studied walnut genotypes

Genotypes	Growth habit	Bearing habit	Tree vigor	Bud break	Nut weight (g)	Kernel ratio (g)	Kernel color
GUM-1	Spreading	Intermediate	Strong	Mid Early	11.40	55.3	Light
GUM-2	Spreading	Intermediate	Medium	Late	8.87	60.2	Light
GUM-3	Semi upright	Lateral	Medium	Early	9.44	51.2	Light amber
GUM-4	Spreading	Lateral	Medium	Early	9.83	50.9	Light
GUM-5	Spreading	Lateral	Strong	Early	13.21	45.0	Light
GUM-6	Semi upright	Lateral	Medium	Late	10.60	60.4	Light dark
GUM-7	Spreading	Intermediate	Medium	Late	8.56	58.3	Light amber
GUM-8	Upright	Lateral	Weak	Late	10.90	47.6	Light amber
GUM-9	Semi upright	Lateral	Medium	Late	10.22	44.1	Light amber
GUM-10	Spreading	Lateral	Medium	Early	9.11	57.3	Light dark
GUM-11	Spreading	Lateral	Strong	Early	11.22	55.0	Light amber
GUM-12	Upright	Lateral	Weak	Late	9.04	55.4	Light
GUM-13	Spreading	Lateral	Weak	Mid Early	10.77	57.2	Light
GUM-14	Spreading	Lateral	Medium	Late	11.25	46.8	Very light
GUM-15	Semi upright	Lateral	Medium	Late	12.59	60.3	Light
GUM-16	Spreading	Lateral	Medium	Early	12.04	60.5	Light
GUM-17	Spreading	Intermediate	Strong	Late	9.50	55.3	Light amber
GUM-18	Semi upright	Intermediate	Medium	Early	11.08	58.7	Light
GUM-19	Spreading	Lateral	Weak	Late	10.26	50.0	Light
GUM-20	Spreading	Lateral	Strong	Late	10.39	44.4	Very Light
GUM-21	Semi upright	Lateral	Medium	Late	8.49	61.4	Light
GUM-22	Spreading	Lateral	Strong	Late	11.19	53.6	Light
GUM-23	Upright	Lateral	Medium	Early	9.61	55.5	Light
GUM-24	Semi upright	Lateral	Medium	Late	9.83	50.3	Light amber
GUM-25	Spreading	Intermediate	Strong	Late	11.50	44.8	Light amber
GUM-26	Semi upright	Lateral	Medium	Late	10.30	54.2	Light
GUM-27	Semi upright	Intermediate	Medium	Early	9.00	52.0	Light
GUM-28	Spreading	Lateral	Medium	Mid Early	11.25	50.7	Light amber
Sebin	Semi upright	Lateral	Medium	Late	11.00	57.7	Light amber

Table 2: Genetic diversity statistics for 21 SSR markers studied in 28 walnut genotypes and one standard cultivar (number of detected alleles, observed heterozygosity (*Ho*), expected heterozygosity (*He*) and Polymorphism Information Content (PIC))

SSR Primers	Number of alleles	Observed heterozygosity (*Ho*)	Expected heterozygosity (*He*)	PIC
WGA001	6	0.55	0.71	0.45
WGA004	6	0.60	0.56	0.51
WGA005	5	0.75	0.85	0.73
WGA009	4	0.52	0.60	0.50
WGA027	2	0.60	0.55	0.65
WGA032	9	0.70	0.73	0.61
WGA054	8	0.50	0.55	0.52
WGA069	7	0.65	0.74	0.50
WGA071	5	0.72	0.56	0.58
WGA072	6	0.70	0.57	0.59
WGA079	3	0.74	0.50	0.43
WGA089	3	0.68	0.72	0.78
WGA118	6	0.63	0.75	0.50
WGA202	7	0.70	0.70	0.82
WGA225	2	0.55	0.60	0.57
WGA276	13	0.62	0.55	0.86
WGA321	9	0.70	0.63	0.43
WGA331	4	0.55	0.50	0.61
WGA332	5	0.65	0.62	0.49
WGA349	9	0.59	0.67	0.75
WGA376	10	0.64	0.58	0.71
Average	6.15	0.64	0.62	0.60

The genetic analysis software program IDENTITY 1.0 (Wagner and Sefc, 1999) was used for the calculation of number of alleles as well as the observed and expected heterozygosity. Genetic similarities were determined by the software program MICROSAT (version 1.5) (Minch et al., 1995). NTSYS-PC software program was used to construct a dendrogram with the UPGMA (Unweighted Pair-Group Method Analysis with Arithmetic Average) method (Sarikamis et al., 2010). Polymorphic Information Content values were calculated according to Kalinowski et al. (2007).

RESULTS AND DISCUSSION

In total, 129 alleles were obtained at the 21 SSR loci analyzed. Results showed that the number of alleles per locus ranged from 2 (WGA027 and WGA225) to 13 (WGA276) with a mean number of 6.15 alleles per locus (Table 2). Along with the WGA276 locus, WGA376 (10 alleles per locus), WGA032, WGA321 and WGA349 (9 alleles per locus) gave high allele numbers per locus (Table 2). Walnut cultivars and genotypes more recently have been characterized by a reliable set of SSR markers in different parts of the world and the number of alleles were found between 2 and 13 (Dangl et al., 2005; Karimi et al., 2010; Ruiz Garcia et al., 2011; Kim et al., 2012; Mahmoodi et al., 2013; Pop et al., 2013; Vahdati et al., 2015; Khokhlov et al., 2018). These results confirm that the genotypes were diverse and need more detailed identification in the future. The average number of each microsatellite allele indicates that each locus is suitable for estimating genetic variability. Ehteshamnia et al. (2009), Aradhya et al. (2010), Ebrahimi et al. (2011) and Dogan et al. (2014) reported that the highest number of alleles was generated by WGA202, WGA276 and WGA376 locus too in walnut cultivars and genotypes. They also reported that WGA027 locus gave the lowest numbers of alleles. The existence of the variability in the number of alleles reported by other studies could be due to difference in selected genotypes and primers. The observed and expected heterozygosity levels were found between 0.50 and 0.74 (WGA079), 0.75 and 0.85 (WGA005) and 0.50 and 0.55 (WGA054), respectively (Table 2). The average observed and expected heterozygosity were found as 0.64 and 0.62, respectively. Results clearly indicate that our seed propagated outcrossing walnut genotypes are characterized by a high degree of heterozygosity. We found observed heterozygosity values close to expected heterozygosity. The average PIC value was obtained as 0.60. The most informative loci were WGA276 (0.86), while the least informative loci were WGA079 and WGA321 (0.43), respectively. These results show that all of the microsatellite primer pairs we used in this study could contribute considerable information to walnut genetics and breeding research. The dendrogram obtained after processing the experimental data divides the analyzed samples into three major clusters (Fig. 1), which indicates the heterogeneity of the seed propagated walnut genotypes in the Gumushane province in Turkey. The first cluster included 8 seed propagated genotypes and 'Sebin' cultivar. The second cluster consisted of 9 seed propagated genotypes and the third cluster included 12 seed propagated genotypes (Fig. 1). The genotypes have spreading growth habit, lateral bearing habit, medium tree vigor and late bud break characteristics clustered in Cluster I (Fig. 1). However, the second and third main cluster also include genotypes, which had semi upright growth habit, lateral bearing habit, medium tree vigor and late bud break characteristics as well. PCA Group 1 included 4 genotypes and 'Sebin' cultivar, PCA Group 2 included 11 genotypes and PCA Group 3 included 10 genotypes (Fig. 2). The genetic variation was higher in the PCA Group 2 than in other groups. The range of similarity values of walnut genotypes characterized in this study indicated a wide range of genetic diversity.

CONCLUSION

Identifying and characterizing genetic resources and protecting them are necessary issues in plant breeding. The use of molecular markers, like microsatellites is very important to build a database for cultivar analysis and plant breeding. In this research, Turkey's northeastern walnuts were characterized by microsatellite markers successfully. Research results supported that used SSR markers are a powerful tool for walnut germplasm characterization. Heterozygosity values and allelic richness showed a high genetic diversity for these valuable walnut genetic resources.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

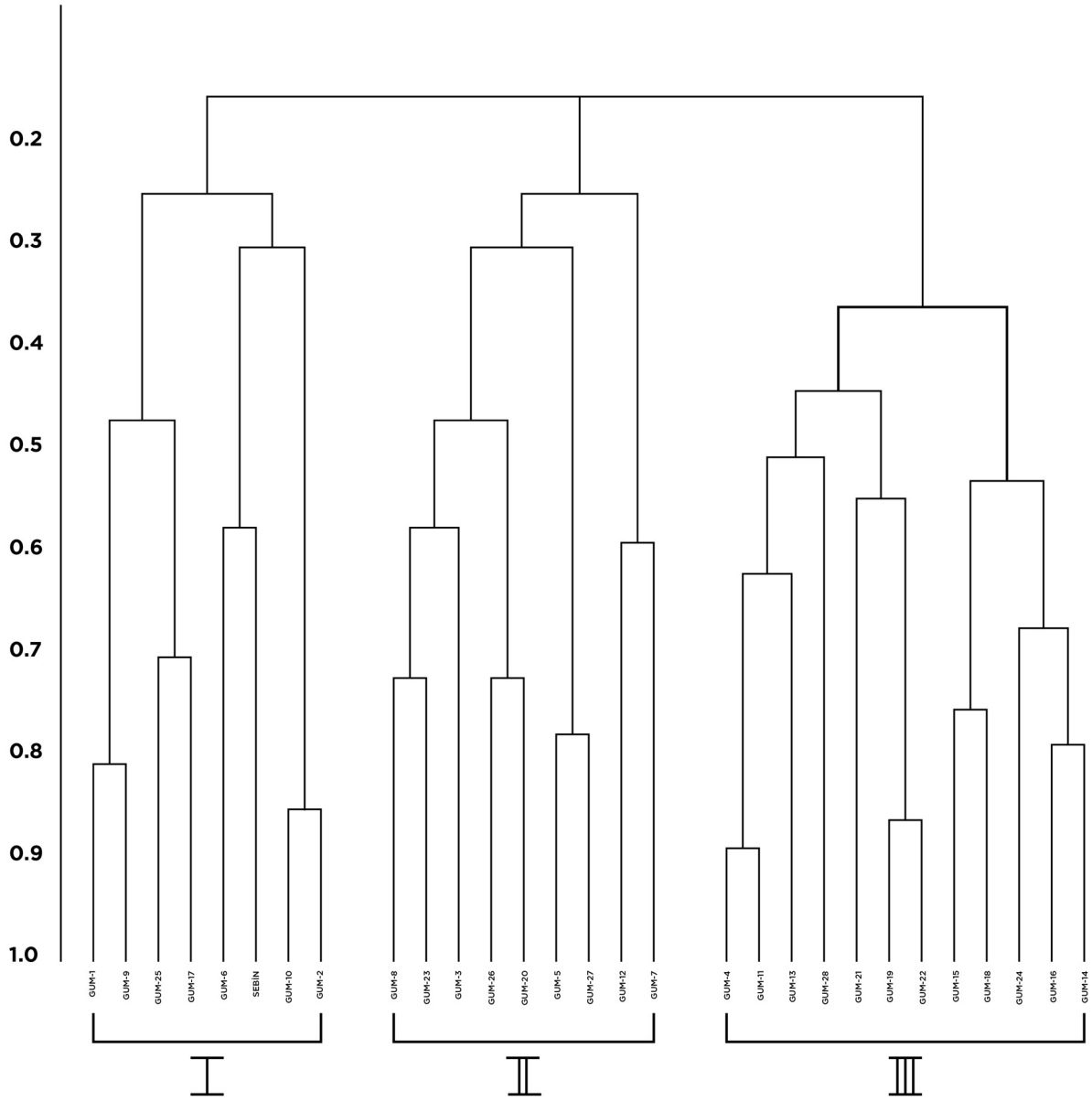

Fig. 1: The UPGMA dendrogram based on simple matching similarity matrix obtained using 21 SSR markers, illustrating the relative similarity between 28 genotypes and the cultivar 'Sebin'.

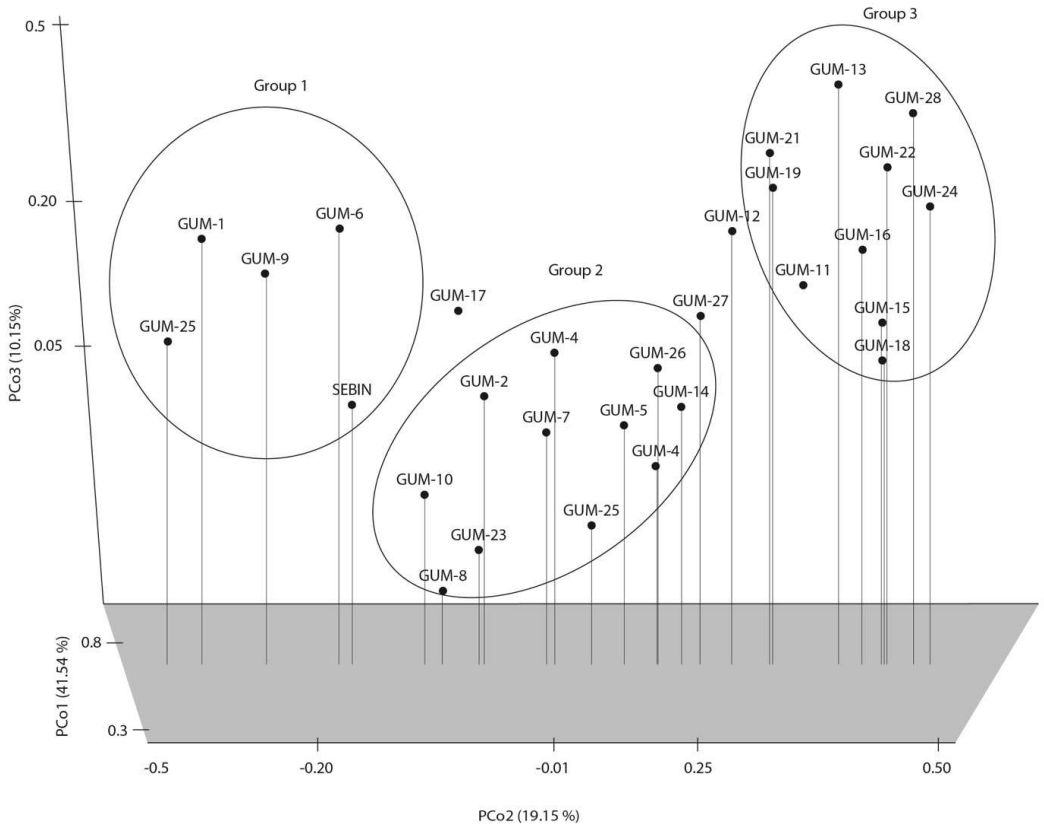

Fig. 2: PCoA plot for the 28 analysed seed propagated walnut genotypes and cv. 'Sebin' (The genotypes number codes on the plot refer to Table 1.)

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