Assessment and characterization of genetic relationships of walnut (*Juglans regia* L.) genotypes by three types of molecular markers

Yıldız Doğan [a], Salih Kafkas [a,*], Mehmet Sütyemez [b], Yaşar Akça [c], Nurgül Türemiş [a]

[a] Department of Horticulture, Faculty of Agriculture, University of Çukurova, 01330 Adana, Turkey
[b] Department of Horticulture, Faculty of Agriculture, University of Sutcuimam, Kahramanmaraş, Turkey
[c] Department of Horticulture, Faculty of Agriculture, University of Gaziosmanpaşa, Tokat, Turkey

ARTICLE INFO

ABSTRACT

Turkey has a rich walnut germplasm that has arisen from populations of naturally grown seedlings over many years. Despite this enormous genetic potential, only a few studies on molecular characterization studies have been carried out so far. In this study, the genetic relationships among 59 walnut genotypes and cultivars of international and Turkish interest were analyzed by 25 RAPD primers, 25 ISSR primers, and 16 SSR primer pairs. Among the molecular assays tested, SSR provided a very high polymorphism rate of 99.1%, while ISSR and RAPD results showed similar to each other but lower polymorphism (71.1% and 69.1%, respectively). The dendrogram constructed divided the genotypes into three main groups: 'Group A' included mainly genotypes originating from the Kahramanmaras and Yalova provinces, while 'Group B' contained genotypes originating from the Kırsehir, Tokat and Corum provinces of Turkey. 'Group C' contained only cultivars originating from the USA and France. According to the clustering in the dendrogram genotypes grouped largely in accordance with their geographical origin, although certain exceptions were present. The pairs of 'Akca-2' with 'Karabodur' and 'Yalova-1' with 'Yalova-3' were the most closely related walnut genotypes in this study. In conclusion, our study indicated that molecular markers are useful tools for use in to determining genetic relationships among walnut genotypes. The results will provide valuable information to walnut cultivar breeding programs for use in parental selection.

1. Introduction

The genus *Juglans* consists of 21 species among which only two, *J. nigra* L. (Eastern black walnut) and *J. regia* L. (Persian walnut), are widely cultivated due to their economic significance as edible nuts or timber (Manning, 1978; Stanford et al., 2000). Central Asia and its neighboring regions are believed to be the origin of certain *Juglans* species (Browicz, 1976). In addition, North and South America are also known as the distribution centers of specific *Juglans* species (Aradhya et al., 2007). In the world, China, the USA, Turkey, and Iran are the leading countries in walnut production. Turkey has a long history of walnut cultivation with a 184.251 metric tons produced annually (Faostat, 2013). The country has more than 4-million walnut trees in production, most of which are derived from non-grafted seedlings. Although this population provides a very good opportunity to the breeders for selecting the best genotypes for production (as well as use in

walnut breeding programs), it has the disadvantage of giving non-uniform nuts, which are unstable for the markets (Akca, 2005). In this respect, reliable knowledge regarding germplasm diversity and the genetic relationship between breeding materials could be an invaluable aid in walnut improvement strategies, as it serves to provide fundamental information about the genetic potential of an existing collection pool. Since conventional methods used walnut germplasm worldwide, researchers have focused on more powerful molecular marker techniques powerful ways. Although EUROGENE (European Forest Genetic Resources Programme) has been dealing with gathering walnut genetic sources, insufficient research on genetic relationships restricts these endeavors.

Knowledge of the genetic relationships among walnut genotypes and their phenotypic characteristics will be very useful in walnut cross-breeding programs. New improvements in molecular tools and DNA markers offer more direct approaches to identifying the biodiversity of walnut genotypes. Several molecular marker assays have already been applied to determine genetic relationships among *J. regia* cultivars and accessions (Fjellstrom et al., 1994; Nicese et al., 1998; Potter et al., 2002; Kafkas et al., 2005; Dangl et al., 2005; Bayazit et al., 2006; Foroni et al., 2005, 2007; Wang

* Corresponding author. Tel.: +90 322 3386030; fax: +90 322 3386030.
E-mail address: skafkas@cu.edu.tr (S. Kafkas).

Table 1
The RAPD, ISSR and SSR primers, their total and polymorphic bands and allele ranges in SSR analysis in the characterization of 59 walnut genotypes originating from Turkey, USA and France.

No	RAPD		ISSR		SSR		
	Primer	Total/Poly bands	Primer	Total/Poly bands	Locus	Total/Poly allele	Allele sizes
1	OPA02	5/3	UBC807	4/3	WGA1	9/9	176–203
2	OPA09	7/5	UBC811	5/3	WGA4	4/4	228–238
3	OPB08	9/7	UBC813	7/6	WGA5	9/9	239–271
4	OPB10	9/6	UBC814	12/9	WGA9	5/5	230–246
5	OPB18	6/4	UBC815	10/8	WGA69	7/6	158–179
6	OPC01	6/4	UBC817	10/4	WGA71	2/2	206–210
7	OPC04	7/4	UBC819	5/4	WGA89	7/7	213–226
8	OPC07	12/10	UBC823	7/2	WGA118	6/6	187–209
9	OPC11	9/8	UBC826	6/4	WGA202	17/17	198–301
10	OPD15	11/10	UBC829	7/4	WGA225	5/5	190–202
11	OPF10	11/6	UBC834	10/8	WGA276	16/16	162–199
12	OPG04	7/4	UBC836	6/5	WGA321	9/9	223–254
13	OPG06	13/7	UBC840	8/7	WGA331	3/3	274–278
14	OPG10	15/11	UBC844	6/5	WGA332	5/5	216–227
15	OPG11	9/7	UBC846	7/7	WGA349	8/8	259–279
16	OPG18	15/6	UBC851	8/6	WGA376	6/6	218–254
17	OPG19	10/9	UBC853	8/4			
18	OPH06	3/3	UBC854	5/3			
19	OPH08	5/1	UBC855	12/10			
20	OPH12	10/9	UBC856	8/6			
21	OPI05	5/4	UBC858	6/3			
22	OPI13	10/3	UBC860	8/6			
23	OPI14	6/5	UBC868	7/5			
24	OPI19	6/4	ISSR 28	5/4			
25	OPJ13	5/4	ISSR 43	4/3			

et al., 2008; Pollegioni et al., 2009; Zhang et al., 2010; Christopoulos et al., 2010; Karimi et al., 2010; Ma et al., 2011; Ebrahimi et al., 2011; Luiz-Garcia et al., 2011; Xu et al., 2012; Ahmed et al., 2012; Kim et al., 2012; Pop et al., 2013).

Kafkas et al. (2005) employed AFLP and SAMPL markers to analyze the genetic relationships among 21 walnut genotypes for the first study in Turkey. Recently, Bayazit et al. (2006) also distinguished 22 Turkish walnut accessions by AFLP markers. Sutyemez (2006) compared AFLP polymorphism in progeny derived from dichogamous and homogamous walnut genotypes. The objective of the present study is to characterize walnut germplasm in Turkey using RAPD, ISSR, and SSR markers and to determine genetic relationships among the genotypes for breeding purposes.

2. Materials and methods

2.1. Plant materials and DNA extraction

Young leaf samples of 59 walnut genotypes originating from Turkey, the USA, and France were collected from the germplasm collections of three universities (Cukurova University in Adana, Sutcuimam University in Kahramanmaras, and Gaziosmanpasa University located in Tokat) and from Kaman County of the Kirsehir province in Turkey. DNA was extracted from leaf tissues by the CTAB method of Doyle and Doyle (1987) with minor modifications (Kafkas et al., 2005). The concentration of the extracted DNA was estimated by comparing the band intensity with λ DNA of known concentrations after 0.8% agarose gel electrophoresis and ethidium bromide staining. DNA was diluted to 5 ng μL^{-1} for RAPD, SSR and ISSR analysis. The walnut cultivars and genotypes used in this study are presented in Fig. 1 and in supplementary file.

2.2. RAPD, ISSR and SSR analysis

RAPD and ISSR analysis were performed according to Williams et al. (1990) and Zietkiewicz et al. (1994), respectively with minor modifications (Kafkas et al., 2006). SSR analysis was done according to Dangl et al. (2005) and Foroni et al. (2005) with minor modifications. The primers used in RAPD, ISSR and SSR analysis are given in Table 1.

Amplification reactions of the three techniques were carried out in a 25 μl total volume containing 10 ng genomic DNA, 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 100 μM each of dATP, dGTP, dCTP, dTTP, 0.2 μM each primer (forward primer in SSR was labeled with either 6-FAM or HEX) and 1.0 unite Taq DNA polymerase.

For RAPD analysis, after 2 min at 94 °C pre-denaturation, 35 cycles of PCR were programmed (45 s at 94 °C for denaturation, 1 min at 36 °C for annealing, and 2 min at 72 °C for extension) followed by a final incubation of 5 min at 72 °C. The ISSR program included 2 min at 94 °C for pre-denaturation and 40 cycles of PCR: 1 min at 94 °C for denaturation, 1 min at a primer specific annealing temperature (40–60 °C), and 2 min at 72 °C for extension followed by 7 min at 72 °C for the final extension. The same cycler was used for the SSR analysis with 3 min at 95 °C for pre-denaturation and 35 cycles of PCR: 45 s at 94 °C for denaturation, 1 min at 58 °C for annealing temperature and 1 min at 72 °C for extension followed by a final extension at 72 °C for 5 min. RAPD and ISSR PCR reactions were repeated at least two times for each tested primer in all the walnut genotypes.

Aliquots of RAPD and ISSR-PCR products were analyzed in agarose gels (1.5% in RAPD and 1.8% in ISSR). After staining with ethidium bromide, the gels were photographed on a UV transilluminator. Electrophoresis of SSR-PCR products were performed in ABI 3130xl Genetic Analyzer [Applied Biosystems Inc., Foster City, CA (ABI)] using 36 cm capillary array with POP7 as the matrix (ABI). The internal size standard used in the sequencer was GeneScan^{TM} 500 LIZ. Injection samples were prepared by mixing 0.5 μl of the amplified product, 0.2 μl of the size standard and 9.8 μl of formamide. The fragments were resolved using ABI data collection software 3.0, and SSR fragment analysis was performed with GeneScan Analysis Software 4.0 (ABI).