DNA Polymorphism and Assessment of Genetic Relationships in Walnut Genotypes Based on AFLP and SAMPL Markers

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ABSTRACT. Turkey has more than 4 million walnut trees (*Juglans regia* L.), most of which are derived from seedlings, and are nongrafted trees. This characteristic leads to a huge opportunity to select superior walnut genotypes from natural populations for cultivation and for breeding programs. Several selection studies have been performed in the last decades and few genotypes were selected. The goal of this study was to characterize and determine genetic relationships among 21 walnut genotypes with potential in walnut production using amplified fragment length polymorphism (AFLP) and selective amplification of microsatellite polymorphic loci (SAMPL) techniques. Eight primer combinations (six for AFLP and two for SAMPL) were applied to 21 walnut genotypes and a total of 230 bands of which 50.4% of them were polymorphic were obtained. The SAMPL technique was more effective than AFLP in the separation of very closely related genotypes. Genotypes of the pairs 'Maras-18' with 'Maras-46', 'KSU-5' with 'Sutyemez-1', 'Maras-12' with 'Sutyemez-2', 'Kaman-3' with 'Kaman-4', and 'KSU-11' with 'Maras-10' were the most closely related.

The genus *Juglans* includes 21 species (Manning, 1978). However, *J. regia* is the only commonly cultivated species in Europe. Turkey, with 125,000 Mt of nuts, is one of the countries of origin of this species, and is the fourth largest walnut producing country in the world (FAO, 2003). In Turkey, ≈4 million walnut trees are in production and most of them are derived from seedlings (i.e., they are nongrafted trees) (Akca, 2003; Sen, 1986). Although this characteristic provides a very good opportunity for the breeders to select the best genotypes for production as well as walnut breeding programs, it has the disadvantage of giving nonuniform nuts that are unsuitable for market.

Until the beginning of 1970s, nongrafted walnut seedlings were used in Turkey for new walnut plantations. The first selection study from natural populations of the Marmara region was done by Olez (1971) and four walnut genotypes ('Yalova-1', 'Yalova-2', 'Yalova-3', and 'Yalova-4') were selected. Afterwards, Celebioglu (1985) selected the genotypes 'Sebin' and 'Bilecik' according to their yield and nut characteristics. All these genotypes, except 'Yalova-2' (because of alternate bearing), are still used to produce grafted walnut plants in the nurseries. After Celebioglu, a few selections from natural populations were performed in different walnut-producing provinces.

The knowledge of the genetic relationships among walnut genotypes and their pomological characteristics will be very useful in walnut cross-breeding programs. Isozymes were used in different studies to characterize walnut species and cultivars, and to detect interspecific hybrids (Aleta et al., 1990, 1993; Arulsekar et al., 1985, 1986; Fornari et al., 2001; Germain et al., 1993; Louskas

et al., 1984; Malvoti et al., 1993; Solar et al., 1993; Wenheng, 1984). RFLP markers were also used in walnuts to determine presence of apomixis, to establish phylogenetic relationships in the genus *Juglans*, to assess genetic diversity and to characterize cultivars (Aly et al., 1992; Fjellstrom et al., 1994; Fjellstrom and Parfitt, 1994a, 1994b, 1995). With the development of polymerase chain reaction (PCR), the randomly amplified polymorphic DNA (RAPD) technique has been employed in walnut to find markers linked to hypersensitivity to the cherry leafroll virus, to identify interspecific hybrids, to assess genetic relationships among the genotypes, and to construct a genetic linkage map (Malvoti et al., 1997, 2001; Nicese et al., 1998; Woeste et al., 1996a, 1996b). Inter simple sequence repeats (ISSRs), instead, were only used to characterize Californian walnut cultivar germplasm (Potter et al., 2002).

Amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995) was applied to analyze intraspecific diversity in different fruit and nut tree species but it was never used in walnut. This technique reveals significant levels of DNA polymorphism and appears to be most promising for fingerprinting, mapping, and genetic diversity studies. In addition, one of the main advantages of AFLP technique is its high multiplex ratio, which means that a larger number of amplified products are generated in a single reaction (Powell et al., 1996).

Selective amplification of microsatellite polymorphic loci (SAMPL) is one of the microsatellite-based marker systems and a modification of AFLP methodology. The same template is used as in the case of conventional AFLP. Restriction fragments resulting from the digestion of genomic DNA with two endonucleases ligated with adaptors and preamplified using primers designed on the basis of the synthetic adaptor plus the restriction site and carrying one selective base. The selective amplification is achieved using one of the standard AFLP primers with a SAMPL

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primer (Rakoczy-Trojanowska and Bolibok, 2004). The use of a SAMPL primer in combination with an AFLP primer results in the amplification of clear and reproducible fingerprint patterns: SAMPL analysis is particularly suitable for studies where low genetic variation is expected since the primers target the hypervariable microsatellite loci (Witsenboer et al., 1997). This technique, therefore, is theoretically able to detect more polymorphic loci than AFLP markers (Singh et al., 2002).

As mentioned above, only three attempts were made in the past to assess the genetic diversity of walnut cultivars, and all of them were limited to the characterization of the Californian walnut germplasm by using RFLP (Fjellstrom et al., 1994), RAPD (Nicese et al., 1998), and ISSR (Potter et al., 2002) markers. To date, neither a report regarding the extent of genetic diversity of Turkish walnut genotypes that is based on molecular markers nor a characterization of walnut germplasm by AFLP and SAMPL markers were found. Hence, the present study was carried out to assess the genetic diversity and to establish the relationships between different walnut genotypes from different parts of Turkey by using AFLP and SAMPL molecular markers.

Materials and Methods

PLANT MATERIAL AND DNA EXTRACTION. After leaves of 21 *J. regia* genotypes (Table 1) were collected from Nut Research Center of Sutcuimam Univ. in Kahramanmaras, a province of Turkey, they were frozen in liquid nitrogen and stored at -70 °C until use. Genomic DNA was extracted from leaf tissue by the CTAB method of Doyle and Doyle (1987) with minor modifications (Kafkas and Perl-Treves, 2001).

AFLP AND SAMPL REACTIONS. For AFLP and SAMPL analysis, 250 ng of genomic DNA from each sample was digested with 5 units of *EcoRI* and 5 units of *MseI* in a final volume of 25 µL. The reaction buffer was 10X RL-buffer (100 mM Tris HAc pH 7.5, 100 mM MgAc, 500 mM KAc, 50 mM DTT). This mixture was incubated for 2 h at 37 °C. To the double-digested DNA sample,

a total volume of 5 µL of ligation reaction mixture including 5 pmol of *EcoRI* adaptor and 50 pmol of *MseI* adaptor, 10 mM ATP, 1 unit of T4-DNA ligase, and the same reaction buffer as the above mentioned were added and incubated for 5 h at 37 °C.

The preselective amplification reaction mixture contained 2.5 µL restricted-ligated DNA as PCR template, 50 ng µL⁻¹ of each of the preselective amplification primers (*EcoRI*+A and *MseI*+C), 2 mM dNTPs, PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 2.5 mM MgCl₂, and 1 unit of *Taq* polymerase (Fermentas, Vilnius, Lithuania). The reaction volume was 25 µL. PCRs were performed as described by Vos et al. (1995) with minor modifications: 20 cycles of 30 s at 92 °C, 30 s at 60 °C, and 60 s at 72 °C. After preamplification, the PCR products were diluted 1:20 with ddH₂O. The adaptor sequences, preselective amplification primers and selective primers are listed in Table 2.

The labeling reaction contained 5 ng of *EcoRI* adaptor binding selective primers, 0.05 units T4 DNA kinase, 1 X T4 DNA kinase buffer, and 0.05 µL of [γ ³³P]-ATP (3000 Ci/mmol) in a final volume of 0.25 µL per selective amplification PCR. The labeling was performed for 40 reactions of selective-amplification PCRs at 37 °C for 1 h and the enzyme was inactivated by 10 min of incubation at 70 °C.

The selective amplification reaction was conducted in a final volume of 10 µL containing 2.5 µL of diluted preselective amplification product as a template, 3 ng labeled *EcoRI* site primer or SAMPL-6 primer, 15 ng *MseI* site primer, 2 mM dNTPs, PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 3 mM MgCl₂, and 1 unit of *Taq* polymerase. The cycling conditions were 11 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C (0.7 °C/cycle) for 30 s, extension at 72 °C for 60 s, and additional 24 cycles of denaturation, annealing and extension at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s, respectively. Ten microliters of the AFLP selective amplification product was mixed with 10 µL of loading buffer (98% formamide, 10 mM EDTA, 0.25% each of bromophenol blue and xylene cyanol FF), then denatured at 94 °C for 5 min and placed immediately on ice. Electrophoresis was performed on an EC160 standard sequencing unit (Thermo Elec-

Table 1. Walnut genotypes and their origins examined for genetic relationship using AFLP and SAMPL markers in this study.

Code	Genotype	Province	Region
1	Sebin	Giresun	Black Sea
2	Kaman-1	Kirsehir	Central Anatolia
3	Kaman-3	Kirsehir	Central Anatolia
4	Kaman-4	Kirsehir	Central Anatolia
5	Yalova-1	Istanbul	Marmara
6	Yalova-3	Kocaeli	Marmara
7	Yalova-4	Yalova	Marmara
8	Tokat-1	Tokat	Black Sea
9	KSU-5	Kahramanmaras	Southeast Anatolia
10	KSU-11	Kahramanmaras	Southeast Anatolia
11	Bursa-95	Bursa	Marmara
12	Van-4	Bitlis	East Anatolia
13	Kaplan-86	Yalova	Marmara
14	Sutyemez-1	Kahramanmaras	Southeast Anatolia
15	Sutyemez-2	Kahramanmaras	Southeast Anatolia
16	Maras-10	Kahramanmaras	Southeast Anatolia
17	Maras-18	Kahramanmaras	Southeast Anatolia
18	Maras-19	Kahramanmaras	Southeast Anatolia
19	Maras-46	Kahramanmaras	Southeast Anatolia
20	Maras-12	Kahramanmaras	Southeast Anatolia
21	Bilecik	Bilecik	Marmara

Table 2. Sequences of oligonucleotide adaptors and primers used in the characterization of 21 walnut genotypes by the AFLP and SAMPL markers.

Adaptor/primer	Code	Sequence
Adaptors		
<i>EcoRI</i> adaptors		
		5'-CTC GTA GAC TGC GTA CC-3'
		3'-CAT CTG ACG CAT GGT TAA-5'
<i>MseI</i> adaptors		
		5'-GAC GAT GAG TCC TGA G-3'
		3'-TA CTC AGG ACT CAT-5'
Preselective amplification primers		
<i>EcoRI</i> primer + A	E _A	5'-GACTGCGTACCAATTC+A-3'
<i>MseI</i> primer + C	M _C	5'-GATGAGTCCTGAGTAA+C-3'
Selective amplification primers		
<i>EcoRI</i> + 3-ACG	E _{ACG}	5'-GACTGCGTACCAATTC+ACG-3'
<i>MseI</i> + 3-AAG	M _{AAG}	5'-GATGAGTCCTGAGTAA+AAG-3'
<i>MseI</i> + 3-ACA	M _{ACA}	5'-GATGAGTCCTGAGTAA+ACA-3'
<i>MseI</i> + 3-AGA	M _{AGA}	5'-GATGAGTCCTGAGTAA+AGA-3'
<i>MseI</i> + 3-AGC	M _{AGC}	5'-GATGAGTCCTGAGTAA+AGC-3'
<i>MseI</i> + 3-AGG	M _{AGG}	5'-GATGAGTCCTGAGTAA+AGG-3'
<i>MseI</i> + 3-AGT	M _{AGT}	5'-GATGAGTCCTGAGTAA+AGT-3'
<i>MseI</i> + 3-ATA	M _{ATA}	5'-GATGAGTCCTGAGTAA+ATA-3'
SAMPL primer		
SAMPL-6		5'-ACACACACACACATATAA-3'

tron Corp., Milfort, Mass.). Two PCR reactions were performed for each primer combination.

About 3 μ L of mixture was loaded onto a 4.5% (w/v) polyacrylamide denaturing gel with 0.5 X TBE buffer after a prerun electrophoresis at 60 V for 30 min, and then were run at 60 V until the loading dye reached to the bottom of the gel. The gels were dried at 80 °C for 3 h. Hyperfilm-Multi Purpose (Amersham Biosciences, Buckinghamshire, England) was exposed to the gels for 2 d.

BAND SCORING AND DATA ANALYSIS. The AFLP and SAMPL bands were scored manually as present (1) or absent (0). Only the clearest and strongest bands were scored and used for the analysis. The ability of the most informative primer pairs to differentiate between the genotypes was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (1999) using $Rp = \sum Ib$, where $Ib = 1 - [2 \times (0.5 - p)]$ and p is the proportion of the 21 genotypes containing the I band.

Parsimony analysis was performed using phylogenetic analysis using parsimony program (PAUP 4.0b; Swofford, 1998) with different Heuristic search-options. A consensus tree was constructed from 500 bootstrap replicates of the same data. Genetic distances between all pair-wise combinations of genotypes were calculated by the same program. The distance values were based on the proportion of different bands between all the possible pairs of genotypes. The same binary data was also used to construct a neighbor-joining tree based on the Nei and Li (1979) distance matrix using the same package software.

Results and Discussion

LEVELS OF POLYMORPHISM AND RESOLVING POWERS OF THE PRIMER PAIRS. Six AFLP and two SAMPL primer combinations were used in the molecular characterization of 21 walnut genotypes originated from different regions of Turkey. Fingerprinting patterns of walnut genotypes using E_{ACG}/M_{AGT} primer combination in AFLP and SAMPL-6/ M_{AGT} primer pair in SAMPL are shown in Fig. 1A and 1B, respectively. A total of 230 bands were obtained and 116 of them (50.4%) were polymorphic with an average of 28.8 total bands and 14.5 polymorphic markers per primer pair (Table 3). The six AFLP primer sets produced in total 179 bands and 49.9% of them were polymorphic with an average of 29.8 bands per primer pair. In SAMPL analysis, the two primer combinations amplified 51 fragments and 50.9% of them pointed out that there is polymorphism among walnut genotypes. The average number of total and polymorphic bands per primer in SAMPL was slightly lower than AFLP, whereas the percentage of polymorphisms per primer was slightly higher in SAMPL than in AFLP. The maximum number of bands was 36 for primer combination E_{ACG}/M_{ATA} , whereas the lowest number of bands (23) was obtained with primer pair E_{ACG}/M_{AGC} (Table 3). The highest level of polymorphism was found with primer pairs E_{ACG}/M_{AGA} (55.3%), SAMPL-6/ M_{AGG} (53.8%), and E_{ACG}/M_{ACA} (53.6%), whereas the lowest one (43.5%) was generated with E_{ACG}/M_{AGC} primer combination. The low number of AFLP and SAMPL bands that were detected in this study was compared to

Fig. 1. Fingerprinting of 21 walnut genotypes (A) by AFLP using $E_{AAC} \times M_{AGT}$ primer combination and (B) by SAMPL using SAMPL-6 $\times M_{AGT}$ primer pair. Lanes are: 1) 'Sebin', 2) 'Kaman-1', 3) 'Kaman-3', 4) 'Kaman-4', 5) 'Yalova-1', 6) 'Yalova-3', 7) 'Yalova-4', 8) 'Tokat-1', 9) 'KSU-5', 10) 'KSU-11', 11) 'Bursa-95', 12) 'Van-4', 13) 'Kaplan-86', 14) 'Sutyemez-1', 15) 'Sutyemez-2', 16) 'Maras-10', 17) 'Maras-18', 18) 'Maras-19', 19) 'Maras-46', 20) 'Maras-12', and 21) 'Bilecik'.

Table 3. Number of total and polymorphic bands, percentage of polymorphism, and resolving powers of primer pairs detected in the DNA fingerprinting of 21 walnut genotypes with AFLP and SAMPL markers.

Primer combinations	Total bands (no.)	Polymorphic bands (no.)	Poly-morphism (%)	Resolving power (Rp)
E _{ACG} /M _{AAG}	34	17	50.0	15.3
E _{ACG} /M _{ACA}	28	15	53.6	18.9
E _{ACG} /M _{AGA}	29	16	55.3	17.5
E _{ACG} /M _{AGC}	23	10	43.5	10.3
E _{ACG} /M _{AGT}	29	15	51.7	18.1
E _{ACG} /M _{ATA}	36	17	47.2	17.0
Subtotal/mean	179/29.8	89/14.8	49.9	97.1/16.2
SAMPL6/M _{AGG}	26	14	53.8	13.7
SAMPL6/M _{AGT}	25	12	48.0	16.1
Subtotal/mean	51/25.5	26/13.0	50.9	29.8/14.9
Total/mean	230/28.8	116/14.5	50.4	126.9/15.9

previous reports in different plant species (Paglia and Morgante, 1998; Roy et al., 2002; Singh et al., 2002; Tseng et al., 2002; Witsenboer et al., 1997). The findings can be explained by the use of selective primers with three nucleotides and by the scoring of only clear and strong bands in the gel. One of the problems emerging while utilizing multiplex fingerprinting techniques such as AFLP and SAMPL is the high complexity of amplification profiles. To deal with this problem, an option is to increase the number of selective nucleotides on the primers (Hayden and Sharp, 2001). In this study, we followed this approach both for AFLP and SAMPL analysis, therefore, lower numbers of bands were amplified.

By using 18 RAPD primers, Nicese et al. (1998) observed 23 polymorphic fragments that correspond to $\approx 25\%$ polymorphism, with an average of 1.3 loci per primer. In our study, using six AFLP and two SAMPL primer-pairs, we observed 230 fragments with an average of 14.5 polymorphic loci per primer pair. This indicates that AFLP and SAMPL primer pairs on average detected 11 times more polymorphisms per primer pair than RAPD primers.

Potter et al. (2002) recorded 31 polymorphic bands out of a total of 54 ISSR fragments scored, with an average of 3.9 polymorphic bands per primer. In this study, eight AFLP and SAMPL primer pairs detected, on average, 3.7 times more polymorphisms per primer pair, even though the proportion of polymorphic bands per total number of bands detected was lower for AFLPs and SAMPLs in walnut.

The walnut genotypes characterized in these studies were from different countries and continents. In the RFLP study (Fjellstrom, et al., 1994), walnut genotypes from 12 different origins all over the world were assessed; in the RAPD analysis (Nicese et al., 1998), the authors fingerprinted 19 genotypes from California, France, Afghanistan, and China; and in the ISSR experiment (Potter et al., 2002), 47 genotypes originated from different countries in America, Europe and Asia continents were characterized. With regard to these, in this study, 21 walnut genotypes, all from Turkey, were analyzed. Therefore, the effectiveness of different methodologies used in these walnut genetic diversity studies can be compared. These results also demonstrate the usefulness of AFLP and SAMPL techniques in germplasm diversity studies for walnut when compared to PCR-based techniques such as RAPDs and ISSRs.

The resolving power (Rp) of the primer combinations (Table 3) ranged between 10.1 (E_{ACG}/M_{AGC}) and 18.9 (E_{ACG}/M_{ACA}).

According to the Rp values of all primer pairs, AFLP primer combinations seemed to discriminate 21 walnut genotypes better than the SAMPL primer pairs. However, the SAMPL technique was more effective than AFLP in the separation of very close genotypes. This is apparent in the closest genotype pairs of 'Maras-12' and 'Sutyemez-2' that are different by only 12 bands. Five of them were produced by two SAMPL primer sets and seven from six AFLP combinations. Similar results were obtained from another similar genotype pair of 'KSU-5' and 'Sutyemez-1', which differed only in 10 fragments. The SAMPL technique was superior to AFLP in resolving differences between closely related genotypes as in previous studies which were performed with different plant species (Porceddu et al., 2002; Singh et al., 2002; Tosti and Negri, 2002).

In cultivar identification, AFLP and SAMPL techniques are more reliable in walnut than other methods and especially RAPD which has reproducibility problems. In plants, the efficiency of different types of markers was compared in many studies and AFLP was found to have higher reproducible bands and polymorphism when compared to the RAPDs and ISSRs. Nonetheless, the high costs and the need of skilled technicians are mentioned as the drawbacks of AFLP analysis (Patzak, 2001).

Our results suggest the existence of high genetic variation in walnut at the intra-specific level as revealed by AFLP and SAMPL analysis. Previous studies in walnut using ISSR, RAPD, and RFLP markers also reported sufficient polymorphism within *J. regia*. This result may be explained by the high heterogeneity and out-crossing rate of this species, and consequence of its monoecious and dichogamous reproduction habit.

GENETIC RELATEDNESS AMONG WALNUT GENOTYPE. The data in this study were analyzed using two different approaches. The parsimony method, in which only phylogenetically informative characters are used to construct the shortest tree by stepwise addition of taxa and characters, yielded four shortest trees with 368 steps long: Fig. 2 depicts one of them as unrooted. The data were also analyzed with a distance matrix method, and the resulting neighbor joining tree was identical to the parsimony tree (data not shown).

Figure 2 shows that the 21 walnut genotypes are clearly separated from each other based on AFLP and SAMPL fingerprintings. A branch of 6 steps separated the walnut genotypes into two groups. The first group included nine genotypes from Kahramanmaraş province. This group also collected 'Kaman-3', 'Kaman-4', 'Van-4', and 'Tokat-1' genotypes, although they were separated from the Kahramanmaraş ones by a branch of four steps. The second group gathered mainly the genotypes originated from the Marmara region. 'Sebin' and 'Kaman-1' genotypes clustered in the second group as well. The pair-wise genetic distance values based on 230 AFLP and SAMPL markers ranged from 0.013 to 0.232 (genetic distance units). The genotypes 'Maras-18' and 'Maras-46', 'KSU-5' and 'Sutyemez-1', 'Maras-12' and 'Sutyemez-2', 'Kaman-3' and 'Kaman-4', and 'KSU-11' and 'Maras-10' were the closest pairs. According to the average distances between a genotype and all the other genotypes, 'Bilecik' (0.182 unit), 'Yalova 3' (0.182 unit), 'Van-4' (0.178 unit), and 'Yalova-4' (0.176 unit) appeared to be the most diverged genotypes, whereas 'Maras-12' had the lowest average genetic distance.

Efficient conservation and utilization of plant genetic resources, and successful breeding programs rely on a deep knowledge of the amount and distribution of the genetic diversity present in the germplasm, and on the genetic divergence and relatedness existing among breeding materials. Besides providing a much needed

Fig. 2. Parsimony analysis of AFLP and SAMPL fingerprinting data from 21 walnut genotypes. The data-base included 179 AFLP and 51 SAMPL bands. A heuristic search was conducted by the phylogenetic analysis using parsimony (PAUP) software using tree bisection reconnection (TBR) swapping resulting in four trees of 368 steps. One (consistency index = 0.310) of the trees is depicted. Numbers indicate the length (number of steps) of each branch. Percentage values of 500 bootstrap replicates of the same data are shown in parenthesis.

genetic background, such studies also facilitate the planning of new breeding approaches for cultivar improvement.

In conclusion, the results in this study are significant for further walnut breeding programs and development of the walnut crop in Turkey, helping the growers in the identification of walnut genotypes, and assisting walnut breeders in parent selection. Furthermore, the findings of this study will facilitate further molecular studies in walnut by means of AFLP and SAMPL techniques.

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