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Full Length Research Paper

Standardization of RAPD assay for genetic analysis of olive

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This study aimed at the standardization of PCR conditions for the purpose of determining the genetic similarities and distances in wild and cultivated olives collected from Izmir and Manisa provinces in Aegean Region. Three different PCR mix and three different PCR cycle as well as 20 primers from OP-I series were tried in 13 wild and 12 cultivated samples. A suitable PCR protocol was developed and evaluable bands were derived for further studies. Required result could not be obtained from PCR Mixes I and II and also PCR cycles I and II; however, favorable results were attained by PCR Mix III and PCR cycle III. Evaluable bands were obtained for defining the olive samples by using primers from OP-I. Thus, it was concluded that RAPD profiles are effective in the study of genetic similarities and distances of wild and cultivated olives.

Key words: Olive, RAPD, PCR, standardization.

INTRODUCTION

Olive (*Olea europaea* L.), a member of the family *Oleaceae,* is one of the most economically and agriculturally important fruit tree in the Mediterranean and Aegean Basin. The olive plant has been cultivated in this region for thousands of years and it meets not only the edible olive requirements but also the needs of olive oil, a very valuable vegetable oil, throughout the world (Rallo et al., 2000). The olive plant, estimated to be cultivated on an area of approximately 7 million hectares in the Mediterranean Basin, shows a high genetic variety (Khadari et al., 2003; Martins - Lopes et al., 2007). Rugini and Lavee (1992) noted that more than 2600 cultivars have been identified for olive and Barranco et al. (2000) marked that majority of the same might be synonyms and homonyms (Doveri et al., 2008). Therefore, it is very important to identify the genetics of olive plant accurately.

Determination of genetic structure of plants is significant in terms of cultivation and improvement studies. Use of genetic markers increased with the development of DNA based techniques because of these markers cannot affected by the environmental conditions. Determination of polymorphism is important in identifying the genetic variety, determining the relations with relatives as well as the genetic markers connected to characteristics. It was ensured to reveal the distances or similarities between the plants easily together with the individuals, varities, species and clones in economically significant plants. Phenotypic (morphological) properties influenced by environmental conditions do not provide direct information on the genotype of olive trees. On the other hand DNA based markers allows us to have direct information about the genetic structure of individual without being affected from phenological period or environmental conditions (Doveri et al., 2008; Martins-Lopes et al., 2007; Essadki et al., 2006; La Rosa et al., 2003; Hatzopoulos et al., 2002).

RAPD, which is based on PCR, was developed by Welsh and McClelland and Williams et al. (1990). Today genetic variety or similarities can be determined in a short time and easily and populations can be scanned in the genomic way rapidly by means of RAPD-PCR (La Rosa determining the genetic structure of plants such as wheat, et al., 2003; Wu et al., 2004). RAPD was initially used for

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corn, barley and grapes (D'ovidio et al., 1990; Shattuck et al., 1990; Weining and Landridge, 1991; Gogorcena et al., 1993; Collins and Symons, 1993). Similarly, it was used in water melon (Levi et al., 2001), fig (Papadopoulou et al., 2002) and almond (Mir Ali and Nabulsi, 2003), which are subtropical climate fruits like the olive.

The genetic markers used commonly today for the determination of genetic variety in olive are RAPD's (Fabbri et al., 1995; Besnard et al., 2001; La Rosa et al., 2003; Wu et al., 2004; Ganino et al., 2007; Martins-Lopes et al., 2007); AFLP's (Angiolillo et al., 1999; Bandelj et al., 2004; Owen et al., 2005; Martins-Lopes et al., 2007; Rony et al., 2009); ISSR's (Gemas et al., 2004); SSR's or microsatellites (Martins-Lopes et al., 2007; Doveri et al., 2008; Rony et al., 2009) and SCAR's (Wu et al., 2004; Bautista et al., 2002).

In RAPD, different from polymerase chain reaction, not the oligonucleotides specific to the target region but random oligonucleotide primers are used. In RAPD markers, the bands that are formed as a result of random primer finding a region in which it is the complementary in target genomic DNA and annealing to the same are assessed. Polymerase chain reaction is a simple protocol imitating *in vivo* DNA replication process in *in vitro* setting. In PCR based RAPD method, target DNA isolated from the plant is denatured in high temperature; whereas short, random oligonucleotide primers are annealed to areas they are complementary in the target DNA in low temperature and the complementary of target DNA is elongated through DNA polymerase available in the setting as the temperature arises. Each denaturation ensures the annealing of primers and the multiplication of DNA in a geometric series by repeating the polymerization cycles in required quantities (Soltis et al., 1998).

Presence and absence of bands determines polymorphism. Disadvantages of RAPD method include homozygote dominant characteristic shown by the bands. Reliability of bands is based on the amplifications in PCR phase. PCR amplifications are affected by some conditions. Purity of the genomic DNA, Mg^{+2} rate, primer, dNTP concentrations, different DNA polymerase enzymes affect the success of cycles (Williams et al., 1993). RAPD protocols should be standardized in accordance with the available laboratory conditions in order to acquire reliable and scorable bands (Soltis et al., 1998).

This study aimed at the standardization of PCR conditions for the purpose of determining the genetic similarities and distances in wild and cultivated olives collected from Izmir and Manisa provinces in Aegean Region. Some of the protocols have been unsuccessful in our study and no scorable and reproducible bands were observed; whereas, a protocol produced successful results and scorable bands were obtained.

MATERIALS AND METHODS

Plant material

Saplings of olive were obtained from villages of Manisa and Izmir provinces where olive breeding is very common. Saplings were transferred to a greenhouse; then fresh leaves were collected and stored in liquid nitrogen until DNA extraction. Table 1 shows the types of cultivated and wild olives used in this study, their origins and places where they were supplied.

DNA extraction

Genomic DNA was extracted from young leaves by using Doyle and Doyle method (1987). Young plant tissues were crushed with liquid nitrogen in mortar. Ground tissues were immediately transferred to 1.5 ml Eppendorf tubes, 700 µl preheated CTAB extraction buffer (2% CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 2% β- mercaptoethanol) added onto leaf powder, mixed several times by gentle inversions. Samples with CTAB buffer were incubated for 30 min in 65°C. Tubes were mixed by inversions in every 5 min. Tubes were removed from water bath and waited to be cooled down and then added to 700 µl of cold chloroform : isoamyl alcohol (24:1). Tubes were spun for 10 min at 10.000 rpm/min in a refrigerated centrifuge. Supernatants were poured into new tubes; 600 µl of cold chloroform : isoamyl alcohol (24:1) was added and mixed by gentle inversions for 5 min. Samples were spun for 10 min at 10.000 rpm/min in a refrigerated centrifuge again and supernatants were transferred to fresh tubes including 10 M ammonium acetate and 3 m sodium acetate; 500 µl cold isopropanol was added and mixed by shaking very gently for DNA precipitation. Precipitated DNA was removed with pipette and washed with 70% ethanol. DNA's were dried and re-suspended in 50 µl EDTA. RNA ase (1 µl) was added against the possibility of contamination, in the content of 100 µl DNA solution.

Spectrophotometric analysis

Following steps of DNA extraction, for the determination of DNA quality and concentration of DNA samples, samples were both subjected to spectrophotometric analysis and run in 0.8% agarose gels. In spectrophotometric analysis, each sample of DNA was calculated by their optical density values at 230, 260 and 280 nm. Optical density ratios were evaluated and only good quality DNA samples were used in PCR (Wu et al., 2004).

Standardization of PCR conditions

The OP-I kit (20 primers) was used among the primer sets of 10 base series as supplied by the company Operon Tech. Prior to starting polymerase chain reaction, random primers were prepared by dissolving in 1 X TE as suggested by the company. Since the commercial PCR tampon (Sigma) contained magnesium chloride, no other magnesium chloride was added. Ten microlitres was taken from each dNTP tube for dNTP stock solution, which is one of the reagents of polymerase chain reaction and 90 µl ultra distilled water was added for each dNTP to prepare the stock solution and this stock solution was used. In this study, three different PCR mix and three different PCR cycle were experimented. Table 2 shows the PCR mixes and Table 3 shows the PCR cycles used for the assays. It was determined that optimum study conditions for amounts of PCR reagents and PCR cycles were ensured provided Assay III in Table 2 and Assay III in Table 3, respectively.

Electrophoresis and data analysis

PCR products were separated 1.5 % agarose gels (Sigma) in 0.5 X TBE buffer with 0.5 µg/ml ethidium bromide at 100 V constant voltages. For evaluating the base pair length of bands, DNA ladder

Table 1. Origins of and places of supply for cultivated and wild olives.

Type of olive	Origin	Place of supply	Province	Amount of samples
Cultivated				
Manzanilla	Cordoba, Spain	ORI*	Izmir, Bornova, Turkey	4
Gemlik	Gemlik, Kocaeli	ORI	Izmir, Bornova, Turkey	4
Domat	Akhisar, Manisa	ORI	Izmir, Bornova, Turkey	1
Memecik	Mugla	ORI	Izmir, Bornova, Turkey	1
Edremit	Edremit, Balikesir	Sapling Planters	Akhisar, Manisa, Turkey	1
Uslu	Akhisar, Manisa	Sapling Planters	Akhisar, Manisa, Turkey	1
Wild				
Wild 1	Caglak	Natural setting	Akhisar, Manisa, Turkey	2
Wild 2	Haskoy	Natural setting	Akhisar, Manisa, Turkey	1
Wild 3	Harlak	Natural setting	Akhisar, Manisa, Turkey	1
Wild 4	Sabancılar	Natural setting	Akhisar, Manisa, Turkey	2
Wild 5	Yayakırıldık	Natural setting	Akhisar, Manisa, Turkey	1
Wild 6	Isikkoy	Natural setting	Akhisar, Manisa, Turkey	1
Wild 7	Karacakas	Natural setting	Soma, Manisa, Turkey	2
Wild 8	Bornova	ORI	Izmir, Bornova, Turkey	2
Wild 9	Bademli	Natural setting	Izmir, Dikili, Turkey	1
Total amount of samples = 25				

*ORI: Olive Research Institute-Izmir/Turkey.

Table 2. PCR mixes.

Reagents	Mix I	Mix II	Mix III
Template DNA	25 ng	40 ng	25 ng
Primer	0.2 µM	0.5 µM	1.0 µM
PCR Buffer (with $MgCl_2$)	2.0 µl	2.0 µl	2.42 µl
dNTP	200 µM	200 µM	200 µM
Taq DNA Polymerase	0.5 µl	0.2 µl	0.13 µl
Total volume	25 µl	20 µl	10.5 µl

(Fermentas) was loaded on first lane of each gel. Samples of wild and cultivated olives were implemented simultaneously for each primer.

Genetic polymorphism analysis

Gels were visualized with Photo Print (Vilber Lourmat, France) imaging system and analysis of RAPD bands were performed by BioOne D++ software (Vilber Lourmat, France). The RAPD bands (markers) were scored as 1 if present and 0 if absent. Only clear and reproducible bands were used for binary data matrix and dendrogram was constructed by POPGEN32 program according to coefficient and then UPGMA algorithm (Unweighted Pair-Group Method Using Arithmetic Averages) was chosen for hierarchical clustering analysis method (Sneath and Sokal, 1973; Nei, 1972; Yeh et al., 1997).

RESULTS

In this research, totally 71 bands were derived from OP-I primer set at the wild type olives. Maximum number of bands was observed in OP-I 14 primer with 24 bands and minimum number of bands was observed in OP-I 4 primer with three bands from the electrophoresis results of the wild type olives. It was determined that the molecular size of totally 71 bands varied between 1568 and 304 bp. Evaluable bands in wild olive samples were derived from OP-I 4, OP-I 14, OP-I 15, OP-I 16, OP-I 17 primers.

As for cultivated type olives were obtained from Gemlik, Manzanilla, Domat, Memecik, Edremit and Uslu varieties, totally 141 bands were derived from OP-I primer set. Maximum number of bands was derived from OP-I 2 primer with 29 bands. Minimum number of bands was observed in OP-I 8 primers with three bands. It was determined that the molecular sizes of totally 141 bands varied between 1902 bp and 223 bp. Evaluable bands in cultivated olive samples were obtained from OP-I 1, OP-I 2, OP-I 3, OP-I 4, OP-I 7, OP-I 8, OP-I 9, OP-I 10, OP-I 11 and OP-I 12 primers.

Table 3. PCR cycles.

Step	Temperature	Cycle I		Cycle II		Cycle III	
Initial Denaturation	94°C	2 min		1 min		1 min	
Denaturation	94°C	30 s		20 s		20 s	
Annealing	35°C	1 min	45 cycles	20 s	50 cycles	20 s	35 cycles
Extension	72°C	2 min		30 s		20 s	
Final Extension	72°C	10 min		5 min		5 min	

DISCUSSION

RAPD markers are intensely in studies towards the determination of genotyping and genetic variety of olive tree. As specified by Wu et al. (2004), RAPD marker applications can be used as the initial step for exhibiting a genomic map in plants with unknown or very less known genetic background.

Majority of RAPD studies are based on determining the clustering by applying the cluster analysis on the derived bands and thus determining the genetic similarities and distances. The evaluation capability of bands and nonformation of primer artifacts are dependent on the sensitivity of PCR conditions. Suitable primer and primer concentration, purity of obtained DNA, number of cycles and denaturation, annealing, extension periods and purity and concentration of tampons included in the reaction mix affect the RAPD analysis and correspondingly its products. Evaluation capability and reproducibility of RAPD products is dependent on PCR conditions. Only clear and reproducible bands are used in the evaluation of bands. As demonstrated in previous studies, RAPD analysis is a useful method for studying genetic structure in olives (Fabbri et al., 1995; Besnard et al., 2001; La Rosa et al., 2003; Wu et al., 2004; Ganino et al., 2007; Martins-Lopes et al., 2007).

Increasing the primer concentration in PCR mix results in a decrease in the number of wide bands; however, the number of small particles would increase. It is determined as a result of assays on 0.2, 0.5 and 1 µmol primer concentrations that although more wide bands were observed in 0.2 µmol concentrations, the number of bands with small molecular weight increased in 1 µmol concentration. However, excess amount of primers in the setting causes an increase in nonspecific products; on the other hand there occurs a decrease in the efficiency of bands, because of the primer would be depleted rapidly within PCR cycles in low concentration. Therefore, it is important to determine the best concentration in order to ensure the efficient of band its reproducibility and clarity (Welsh and McClelland, 1990; Williams et al., 1990; Soltis et al., 1998; Bartlett and Stirling, 2003; Dieffenbach and Dveksler, 2003). It is considered that 1 µmol primer concentration is suitable for olive samples.

Also the purity of DNA and DNA amount affects the efficiency of derived bands. The primers can increase the DNA amount in a sample as they are annealed randomly in RAPD analysis; however, at that point, the DNA quality and amount used are important. Good quality, pure and DNAase free buffers should be used to prevent contaminations. It is suitable to add RNAase for the purpose of prevent RNA interaction following the DNA isolation. High DNA concentration may prevent DNA amplification (Welsh and McClelland, 1990; Williams et al., 1990; Soltis et al., 1998; Bartlett and Stirling, 2003; Dieffenbach and Dveksler, 2003).

In our study performed with three different reaction mixes and under three different PCR cycle conditions, the DNA's of wild and cultivated olives supplied from Manisa and Izmir were amplified through PCR method and a suitable PCR protocol was developed and evaluable bands were derived for further studies. Required result could not be obtained from Mixes I and II and also PCR cycles I and II; however, favorable results were attained by Mix III in Table 2 and PCR Cycle III in Table 3. Favorable values were obtained for defining the olive samples by using primers from OP-I. Thus, it was concluded that RAPD profiles are effective in the study of genetic similarities and distances of wild and cultivated olives.

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