

Investigations of Genetic Variation Between Olive (*Olea europaea* L.) Cultivars Using Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

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Characterization and selection of olive clones for the production of olive oil is essential in Turkey because of its profitable exploitation. AP-PCR (Arbitrarily-Primed PCR) is a technique that can distinguish the genetic relationship among plant species and other organisms. In this study, AP-PCR approach was used in order to determine the genetic relationship of different six olive clones. The purity of DNA is one of the most important factors affecting the product of the AP-PCR method. In this respect, modified genomic DNA isolation procedure from *Olea europaea* clones was developed so that this procedure can be used to obtain plant genomic DNA from diverse aromatic plants, which produce essential oils and secondary metabolites. By following the optimized AP-PCR amplification protocol, unique DNA fingerprint profiles for each olive clone were produced. AP-PCR-generated unique DNA fingerprint profiles can be used in the identification, distribution and diversity of various olive cultivars.

Key words: AP-PCR, *Olea europaea*, Olive, Turkey

Introduction

Analyses of the extent and distribution of genetic variation within and among various olives cultivars are essential for understanding genetic relationships among plants for sampling genetic resources for breeding and conservation purposes (Zohary, 1994; Green and Wickens, 1989). Traditionally, genetic variation analyses rely on morphologic and phenotypic markers but these markers have the disadvantage of being environmentally dependent. In recent years, molecular markers have been developed such as restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), arbitrarily-primed polymerase chain reaction (AP-PCR) and simple sequence repeats (microsatellites or SSRs). These markers show their superiority over allozymes showing protein polymorphism, being unlimited in numbers and polymorphisms (Fooland, 1995; Karp *et al.*, 1995).

AP-PCR is a technique, which identify genomic variations within species (Williams *et al.*, 1990; Welsh and McClelland, 1990). Although AP-PCR approach is potentially a very powerful technique for intra-species identification, fingerprint profiles

must be consistently reproduced. This requires careful control of reaction parameters since the methodology is sensitive to minor variations in the primer concentration, and/or the purity and the quantity of DNA (Williams *et al.*, 1990).

High purity DNA is required for PCR and restriction based techniques for genome mapping and DNA fingerprinting, such as AP-PCR, SSR, RFLP, and AFLP (Aljanabi *et al.*, 1999). However, like many other plant species, olive tissues contain high levels of polysaccharides and polyphenolic compounds, which present a major contamination problem in the plant DNA purification. When cells are disrupted, these cytoplasmic compounds can come into contact with nuclei and other organelles (Aljanabi *et al.*, 1999). In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (Katterman and Shattuck 1983; Guillemaut and Marechal-Durouard, 1992; Leutwiller, *et al.*, 1984). Therefore, the need for a rapid and efficient procedure for plants having high amounts of polysaccharides and polyphenols is particularly necessary.

In this study, we have optimized an alternative rapid method that yields polysaccharide and poly-

phenolic free high quality genomic DNA from olive leaves, having tried several published protocols and failed to obtain DNA that was not contaminated with polysaccharides and polyphenolic compounds. AP-PCR reaction parameters were also optimized to establish unique and reproducible DNA fingerprint patterns for various olive cultivars.

Experimental

Plant material

The six *Olea europaea* accessions were obtained from the living collection of the Olive Germplasm institute, Edremit, Balıkesir, Turkey. Portions and young leaves were collected and stored at -80°C until genomic DNA extraction.

Purification of olive genomic DNA

Three different genomic DNA extraction approaches were tested to recover genomic DNA from fresh olive leaves. The first procedure was adapted from Dellaporta *et al.* (1983) and carried out as described in there. The second procedure, also known as CTAB procedure, was taken from Doyle and Doyle (1989). The third procedure was also similar to Dellaporta's method but it was modified by phenol chloroform extraction followed by NaCl extractions which produced obtain polysaccharides and secondary metabolites free genomic DNA suitable for PCR analysis (Do and Adams, 1991). The concentration and purity of DNA was determined at 230 nm, 260 nm, 280 nm, and 320 nm and then visualized on a 0.7% agarose gel.

Optimization of the AP-PCR amplification reaction

An arbitrary oligonucleotide primer designated PA-01, which was previously reported to be effective for AP-PCR genomic fingerprints in some plant species (Besnard *et al.*, 2001), was used in this study. PA-01 primer, 10-mer primer, 5'-CAGGCCTTCA-3, has 60% GC content and a melting point of 34°C .

Each DNA amplification reaction was conducted in a $25\ \mu\text{l}$ volume and included $2.5\ \mu\text{l}$ of $10\times$ PCR reaction buffer [$1\times$ Buffer consisted of 50 mM Tris (Tris[hydroxymethyl]aminomethan)

HCl (pH 8.9), 50 mM KCl, and different MgCl_2 concentrations], $4\ \mu\text{l}$ dNTP mix (200 μM of the each dNTP), 0.2 μM the related primer and 1 unit Taq DNA polymerase (Fermentas, USA). Specific AP-PCR amplifications were optimized with five different olive genomic DNA masses (5 ng, 10 ng, 15 ng, 20 ng and 25 ng) and six different MgCl_2 concentrations (0.5 mM, 1 mM, 1.5 mM, 2.5 mM, 3 mM, and 3.5 mM).

In addition, two different PCR amplification profiles were performed in order to determine the optimum PCR cycling parameters. For the first PCR cycle profile, a step-wise increase in stringency of the reaction was performed through three consecutive series of the amplification of the cycles. Specifically, the DNA samples were initially denatured with stepwise manner; 85°C for 15 s, 95°C for 5 s, 92°C for 1 min and followed by 44 cycles (92°C for 55 s, 35°C for 1 min and 72°C for 2 min) and 72°C for 7 min for final extension procedure. In the second profile, thermal cycles were; 4 min at 94°C followed by 44 cycles of 30 s at 94°C , 30 sec at 35°C and 30 sec at 72°C and finally 1 cycle of 4 min at 72°C for the final extension. The amplifications were carried out using the PROGENE Thermocycler Techne (Cambridge Ltd., UK).

Detection of AP-PCR amplified DNA

The AP-PCR amplified DNA was separated on a 2% (w/v) agarose (Fermentas, USA) gel. Electrophoresis separation was performed in Tris-Boric acid EDTA (TBE) buffer (pH 8.0) at 80V (Maniatis *et al.*, 1982). Following separation, the agarose gel was stained with ethidium bromide, visualized on a UV transilluminator, and photographed using Polaroid type 667 film.

AP-PCR amplification of the various olive cultivars

Six *Olea europaea* cultivars were collected from the living collection of the Olive Germplasm institute, Edremit, Balıkesir. The six olive cultivars were named as UB1, UB3, UB8, UB10, 0108 and 0308. Total genomic DNA from fresh leaves of cultivars was purified by the modified method as described in previously. Three 10-mer primers were used to determine the relationship of the olive cultivars. These primers were PC-01 (5'-TTTCGAGC-

CAG-3'), PB-12 (5'-CCTTGACGCA-3') and wink (5'-CGCTGGCCTA-3'). The optimized AP-PCR reactions were performed and fingerprint profiles were analyzed using 2% agarose gel electrophoresis.

Results and Discussion

Genomic DNA recovery

Three different genomic DNA amplifications were performed in order to obtain polysaccharide-free amplifiable olive genomic DNA, namely, Dellaporta's method (Dellaporta *et al.*, 1983), CTAB procedure (Doyle and Doyle, 1989) and our modified procedure. The first two procedures were common procedures that are routinely used for plant genomic DNA extraction. The concentrations and purity of DNA obtained from three procedures were determined with spectroscopy and gel-electrophoresis. In addition, the suitability of the genomic DNA obtained from these three procedures was also checked in AP-PCR reactions.

The genomic DNA extraction procedure from *Olea europaea* cultivars using the method Dellaporta *et al.* (1983) produced a relatively high yield product. This procedure is a maxi-scale preparation which is very time-consuming and for each extraction over 1 $\mu\text{g}/\mu\text{l}$ DNA with a ratio of A_{260}/A_{280} ranging from 1.2 to 1.45 was obtained suggesting that there is some protein contamination present in the reaction. In addition, the obtained DNA is high in RNA and therefore additional RNase treatment was required (Fig. 1). Most importantly, AP-PCR reaction using olive DNA extracted by this procedure did not give any amplification (data not shown). Thus, the reason for this could be due to high polysaccharide content in the preparation.

The olive genomic DNA prepared by CTAB procedure yielded quite low DNA concentrations that is less than 100 $\text{ng}/\mu\text{l}$ DNA with a A_{260}/A_{280} ratio between 1.3 and 1.6 and there is quite low RNA detectable compared to Dellaporta method however, DNA extracted by this procedure showed inconsistent DNA banding pattern in the AP-PCR reaction, that could be because of contaminants present in the genomic DNA.

For the best genomic DNA purification, we modified the Dellaporta method and performed in the small-scale manner and cleaned by the NaCl

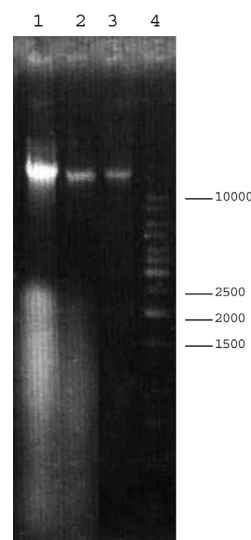


Fig. 1. Agarose gel analyses of the DNA isolated from fresh olive leaves obtained by three different procedures. Lane 1 represents DNA obtained by Dellaporta method (Dellaporta *et al.*, 1983). Lane 2 indicates DNA samples isolated by CTAB procedure (Doyle and Doyle, 1987). Lane 3 represents DNA samples obtained from our modified isolation procedure. Lane 4 indicates 1 kb marker. Some sizes of the 1 kb marker are shown next to the picture.

treatment in order to get rid of the polysaccharides and metabolites present in the plant tissue (Do and Adams, 1991). The quality of the DNA samples were checked on a 0.8% agarose gel and compared to the previous DNA extraction methods (Fig. 1). As seen, no RNase treatment required, as RNA seems to be degraded during extraction. This also reduces sample-handling time. The use of DNA purified by this method as template for AP-PCR amplification produced diverse and reproducible fingerprints profiles. In addition, the DNA remained stable and suitable for fingerprinting for at least two months when it starts generating inconsistent fingerprints patterns.

Optimisation of the AP-PCR parameters of olive cultivars

The oligonucleotide primer, PA-01, was arbitrarily selected using a fixed number of AT and GC nucleotides which were arranged randomly during the custom synthesis (Sigma, Genosys, Cambridge, U. K.). PA-01 generated relatively more diverse and reproducible genomic finger-

prints of a number of plant species and is used for the optimal AP-PCR parameters for olive cultivars (Besnard *et al.*, 2001). Results from the current study showed that $0.2 \mu\text{M}$ of the primer generated distinct and reproducible DNA fingerprints profiles for all accession of the olive cultivars, without any detectable primer artifacts.

From two AP-PCR thermal cycling parameters tested using different cycle conditions, the first set of temperature profiles yielded the most complex AP-PCR fingerprinting profiles. This cycle conditions contain a set gradual increase for the initial denaturation step. However, in second cycle conditions, the use of several single-temperature cycling parameters failed to generate fingerprints of sufficient complexity to allow meaningful discrimination between species and subspecies of the targeted plant species (data not shown). The first cycle parameters were chosen for further analyzing of the clones.

In previous reports, the variability in DNA amount included in the PCR reaction was one of the major reasons for AP-PCR irreproducibility. The optimal DNA concentration is a function of the plant material, DNA extraction method and the polymerase employed. AP-PCR pattern seems to be most affected by very low DNA concentrations. Very high DNA concentrations, however, can also affect banding repeatability probably by inhibiting the reaction due to increased presence of plant-derived contaminants. Fig. 2A represent the effect of five different DNA masses of AP-PCR banding pattern. DNA concentration of $20 \text{ ng}/25 \mu\text{l}$ was used for all subsequent optimization reactions.

Among 6 different MgCl_2 concentrations used in the AP-PCR reactions, 2.5 mM MgCl_2 was optimal for generating the most reproducible and complex fingerprint profiles from all Olive cultivars used in this study (Fig. 2B).

AP-PCR detection of various olive cultivars

Three primers, namely PC-01 (5'-TTCGAGC-CAG-3'), PB-12 (5'-CCTTGACGCA-3') and Wink (5'-CGCTGGCCTA-3') were used for AP-PCR analysis of the cultivars. The fingerprint profiles of the six olive cultivars used in this study are shown in Fig. 3. In order to analyze the intra specific variations of olive cultivars, we evaluated the

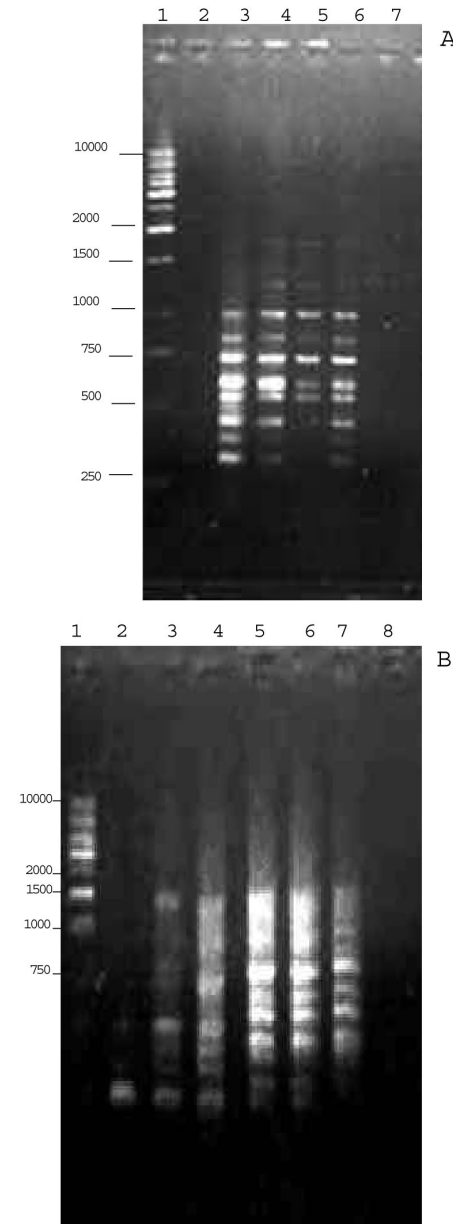


Fig. 2. Optimization of AP-PCR parameters with different masses and concentrations of genomic DNA and MgCl_2 . (A) RAPD profiles of five different DNA masses from the same plant samples obtained by a PA-01 primer using 1% agarose gel. Lane 1: 1 kb marker; lane 2: 5 ng DNA; lane 3: 15 ng; lane 4: 25 ng; lane 5: 35 ng; lane 6: 50 ng, lane 7: negative control. (B) AP-PCR profile of the different MgCl_2 concentrations by the primer PA-01. Lane 1: 1 kb marker, lane 2: 0.5 mM, lane 3: 1 mM, lane 4: 1.5 mM, lane 5: 2.5 mM, lane 6: 3.5 mM; lane 7: 3.5 mM MgCl_2 , lane 8: Negative control. The exact sizes of the marker are shown by the photograph.

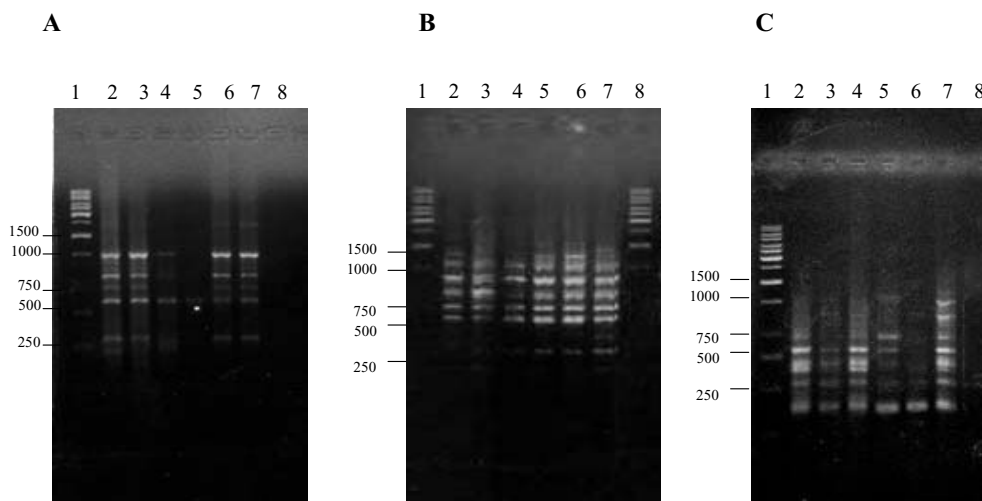


Fig. 3. AP-PCR profile of the different cultivated olive clones obtained by primer PC-01 (A), primer Wink (B) and primer PB-12 (C). (A) Lane 1: 1 kb marker, lane 2: UB1 clone, lane 3: UB3 clone, lane 4: UB8 clone, lane 5: UB10 clone, lane 6: 0108, lane 7: 0308, lane 8: negative control. (B) Lane 1: 1 kb marker, lane 2: UB1 clone, lane 3: UB3 clone, lane 4: UB8 clone, lane 5: UB10 clone, lane 6: 0108, lane 7: 0308, lane 8: 1 kb marker. (C) Lane 1: 1 kb marker, lane 2: UB1 clone, lane 3: UB3 clone, lane 4: UB8 clone, lane 5: UB10 clone, lane 6: 0108, lane 7: 0308, lane 8: negative control. The exact sizes of the marker are shown by the photos.

AP-PCR profile according to existence and absence of individual amplified bands from six cultivars. The representative analysis of primer PC-01 was shown in Table I. Primer PC-01 showed a distinct banding pattern for different olive cultivars suggesting the suitability of the method for the selection of the suitable olive cultivars.

Table I. Analyses of AP-PCR fingerprints of PC-01 primer from six olive cultivars showing the sizes and distribution of the DNA fragments that constitute the intra specific identifications of six *Olea europaea* cultivars.

M of DNA fragment [kb]	UB1	UB3	UB8	UB10	0108	0308
2.0	-	-	-	-	+	+
1.0	+	+	+	-	+	+
0.8	+	+	+	-	+	+
0.6	+	+	-	+	+	+
0.3	+	+	-	-	+	+
0.2	+	-	-	-	-	-

The consistency of the fingerprint profile for each clone was determined from three AP-PCR amplification reactions. The molecular weight of each DNA fragment was determined using a 1 kb ladder as a reference size marker.

kb: kilobase pair.

+: Presence of an amplified DNA fragment.

-: Absence of an amplified DNA fragment.

Conclusion

The information about genetic relationship of *Olea europaea* and its closely related species is very valuable for the taxonomy of the genus, the origin of the cultivated olives (Besnard *et al.*, 2001). AP-PCR, also known RAPD markers, have been demonstrated to be effective in studies on genetic variation, for identifying genotypes, for population analysis and phylogenetic studies in several plant species (Papadopoulo, *et al.*, 2002; Zhou and Li, 2000; Sonnante *et al.*, 2002).

Selection of the appropriate oligonucleotide primer, optimization of the PCR reaction and cycling parameters and purity of the template DNA have been described to be primary factors in order to generate artifact-free AP-PCR amplification (Welsh *et al.*, 1990). In addition, these parameters are important for reproducibility of the AP-PCR based fingerprints. In this study, the modified genomic DNA purification method for olive was developed. The AP-PCR parameters have been optimized and can be used reliably to generate useful DNA fingerprints of olive cultivars. With the application of the optimized AP-PCR protocol using three different primers and analysis of the fingerprinting profile, it was possible to differentiate various olives cultivars. Extensive genomic variations

between cultivars resulted in diverse fingerprint profiles, which can be used to identify, trace the source and geographic distribution of the olive cultivars. Also the AP-PCR fingerprints of olive cultivars can be used to compare and identify specific cultivars.

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