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ORIGINAL PAPER

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Recovery and characterisation of DNA from virgin olive oil

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Abstract The characterisation of the genetic identity of DNA recoverable from olive oil could facilitate the assessment of its place of origin and conformity to type thanks to the particular regional spread of olive cultivars and to their different contributions to the olive oil mixture as stated by each Protected Designation of Origin regulation. This requires that intact DNA should be recovered from virgin olive oil. In an attempt to recover DNA from virgin olive oil, the performance of three different oil production methods was compared. The recorded data demonstrate that only olive oil obtained by using proteinase K treatment during the malaxation process contains DNA amenable to random amplified polymorphic DNA (RAPD)-PCR amplification. This important result achieved represent the first unambiguous and reproducible RAPD-PCR amplification of DNA recovered from virgin olive oil.

Keywords DNA extraction · Polymerase chain reaction · *Olea europaea* L. · Olive oil

Introduction

In Europe, several traditional products are protected by two regulations, namely Council Regulation (EEC) N. 2081/92 on designations of origin and geographical indication and Council Regulation (EEC) N. 2082/92 about specific product types. These regulations, specifying in the appendix the products to which they apply,and including olive oil, introduced the Protected Designation of Origin (PDO) of traditional products. There is therefore a need to assess the place of origin of olive oils under PDO control. Moreover, since olive oil is often identified by its PDO, whereas European law dictates olive cultivar composition, the identification of olive genotypes used for oil production are of paramount importance for the final characteristics of virgin olive oil.

Though multivariate statistical analyses may be useful to discriminate among monovarietal virgin olive oils [1], the characterisation of the genetic identity of DNA recoverable from olive oil could facilitate the assessment of its place of origin and conformity to type, thanks to the particular regional spread of olive cultivars and to their different contributions to the olive oil mixture as stated by each PDO regulation. Several studies [2, 3, 4, 5], carried out by using molecular methods have demonstrated the usefulness of these techniques in obtaining the identity of the genotypes of horticultural species and products. This requires that DNA suitable for amplification by PCR and/or for hybridisation by molecular probes should be recovered from olive oil. In a literature survey of previous work, only one report [6] dealing with this topic was found but very poor experimental information and results arise from it. Very recently [7], some enzymatic mixtures have been tested to prevent DNA damage that occurs during crushing and malaxation. Enzymes [8], enzymatic inhibitors [9] and/or additives are widely used in the food industry but they are not legally recognised for use in olive processing, although they are frequently employed by oil millers to enhance extraction yields or to improve olive oil quality. In Spain the use of talc was regulated by Ministry of Health, and talc addition gave rise to a small increase in the oil stability and a slight decrease in oxidised triglyceride levels [10].

This paper presents the results of an experiment whose aim was to prevent DNA damage during crushing and malaxation, and to recover DNA from two different single cultivar olive oil by three different oil production methods.

Materials and methods

Plant material. Samples of olive (*Olea europaea* L.) leaves and fruits were harvested by hand at the end of December 1999 and November 2000 from single olive trees of Carolea and Cassanese

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cultivars respectively, growing in the germ plasm collection of the Istituto Sperimentale per l'Olivicoltura in Rende (CS), Italy.

Work-up of plant material. Olive drupes (10 kg) were crushed with an Oliomio hammer mill (Toscana Enologica Mori, Tavernelle Val di Pesa, Italy) and the oil obtained by centrifugation at 5000×*g* for 20 min after 15 min of malaxation at RT in the following conditions.

1. In the presence of proteinase K (PK), EC 3.4.21.64, (30 mg/kg, lyophilised powder containing minimum 90% protein; Sigma, St. Louis, USA)
2. In the presence of pronase (Pr) (500 mg/kg, molecular biology grade, Boehringer Mannheim, Mannheim, Germany)
3. Without any enzyme mixture

Olive oil samples were produced immediately after harvest at the end of December 1999 and November 2000, respectively. The virgin olive oil samples from commercial sources were produced in the same years from Carolea and Cassanese olives respectively, by a two-phase continuous system without any filtration step.

DNA-containing sediments were recovered by centrifugation (7000×*g* for 30 min at 4 °C) of freshly obtained virgin olive oil and commercial olive oil, respectively. DNA extraction was carried out on leaf tissue and olive oil sediment respectively, using the CTAB method of Doyle and Doyle [11] with minor modifications.

Samples (1 g) of freshly obtained sediment from olive oil and/or rapidly frozen whole leaves were ground in a mortar and pestle in 1 ml of warm (60 °C) extraction buffer [100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% PVP-40, 2% CTAB), with 1% of 2-mercaptoethanol added 30–60 min before use. The product was transferred to a 5 ml centrifuge tube and the mortar was rinsed with an additional 1 ml of extraction buffer. The mixture was then incubated at 60 °C for 30 min, with agitation approximately every 10 min.

After the mixture was cooled to room temperature, an equal volume of dichloromethane/isoamyl alcohol (24:1) was added, and this was centrifuged at 1600×*g* for 10 min. The upper (aqueous) phase was transferred by a Pasteur pipette to a clean 5 ml centrifuge tube and centrifuged at 1600×*g* for 10 min. If the aqueous phase was cloudy, the dichloromethane/isoamyl alcohol extraction was repeated. Once clear, the aqueous phase was transferred to a clean tube and 2 volumes of cold ethanol (–20 °C) and 1/10 volume of ammonium acetate 10 M was added, and the liquid was mixed gently. Samples were left to stand at –80 °C for 30–60 min, or alternatively at –20 °C overnight, and centrifuged at 5000×*g* for 30 min to pellet the DNA. The pellet was washed with 2 ml of 76% cold ethanol, dried at room temperature overnight and resuspended in 0.1 ml of Tris buffer (10 mM Tris-HCl, pH 8.0). Samples were stored at 4 °C for short-term storage or –20 °C for longer.

The total DNA was digested with 50 µg/ml of RNase A (Sigma) for 30 min at 37 °C. Contaminating RNA was completely degraded by the RNase A treatment and no degradation of the genomic DNA was detected. The DNA was extracted with an equal volume of dichloromethane/isoamyl alcohol (24:1) and precipitated with 2 volumes of cold ethanol; it was then dissolved in sterile water. The DNA was analyzed by gel electrophoresis in 1% Seakem LE agarose gel (FMC BioProducts, Vallensbaek Stand, Denmark) in 1×40 mM Tris-HCl, 5 mM sodium acetate, 1 mM EDTA (TAE) buffer (pH 7.7). DNA was then stained in a 0.1 µg/ml GelStar solution (FMC BioProducts) for 30 min, and photographed under UV light using Type 667 Polaroid film. The DNA concentration was determined with a Hoefer DyNA Quant 2000 fluorimeter, diluted to 5 ng/µl and used for PCR amplification.

Random amplified polymorphic DNA analysis. Random amplified polymorphic DNA (RAPD) amplification was carried out by the procedure of Tartarini [12], with some modifications using five random nucleotidic sequence primers (Operon Technologies, Alameda, Calif., USA) to trigger off the reaction. Amplification reactions were carried out in 25 µl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dGTP, dCTP, and dTTP (Boehringer Mannheim), 0.4 µM primer, 0.6 units of *Taq* DNA polymerase (Boehringer Mannheim) and 50 ng of genomic DNA. Each reaction mixture was overlaid with 25 µl of oil mineral to prevent evaporation. To destroy putative carryover products, reaction mixture and mineral oil were placed on a UV (300 nm) transilluminator before the addition of template DNA and DNA polymerase. DNA amplification reactions were performed in a thermal cycler (Gene Amp PCR System 9600, Perkin Elmer Cetus) programmed for 1 cycle of 150 s at 94 °C, 30 s at 36 °C, 120 s at 72 °C; for 21 cycles of 20 s at 94 °C, 15 s at 36 °C, 15 s at 45 °C, 90 s at 72 °C; for 19 cycles of 20 s at 94 °C (increased 1 s/cycle), 15 s at 36 °C, 15 s at 45 °C, 120 s at 72 °C (increased 3 s/cycle); 1 cycle of 600 s at 72 °C. PCR products were stored at 4 °C before analysis. Amplification products were size-fractionated by electrophoresis at about 3.5 V/cm through a 2% Seakem LE agarose gel (FMC BioProducts,) in 1×TAE buffer and stained in a 0.5 µg/ml ethidium bromide solution for 30 min.

Results and discussion

Olive oil samples were produced in a laboratory scale olive mill immediately after harvest at the end of December 1999 and of November 2000, respectively. In general, the agarose gel electrophoresis of nucleic acids extracted from olive oils of the Carolea and Cassanese cultivars do not show any DNA suitable to PCR-amplification procedure. Therefore, in order to prevent DNA damage during olive oil extraction the addition of two different commercial protease mixtures were tested. The enzyme mixtures were added early after the crushing step but before the malaxation process. However, only the PK addition to the crushed olives allowed the detection of intact DNA (Fig. 1) in the agarose gel electrophoresis of nucleic acids extracted from olive oil. The observed DNA from olive oil sediments and olive leaf showed an average size above 13 kb approximately, as determined by comparison with calf thymus DNA (Sigma) detected by GelStar staining (Fig. 1). Only DNA from olive oil sediments obtained by PK treatment was amenable to RAPD-PCR amplification of DNA from Carolea and Cassanese cultivars. In fact, 5 random decamer primers (Table 1), selected beside 50 primers previously successfully tested for DNA amplification from olive leaves [3], gave reproducible amplification fragments. In particular, Fig. 2 and Fig. 3 show the DNA pattern obtained by gel electrophoresis of amplified RAPD-

Table 1 Primers and number of amplified fragments produced by RAPD–PCR of DNA from olive oil sediments extracted from Carolea (*A–D*) and Cassanese (*E–H*) cultivar; *A, E* DNA extracted from olive oil sediments using *PK*; *B, F* DNA extracted from olive oil sediments using Pr; *C, G* DNA extracted from olive oil sediments without enzymatic treatments; *D, H* DNA extracted from commercial olive oil sediments

Primer	A	B	C	D	E	F	G	H
OP A 01 (3′CAGGCCCTTC5′)	3	0	0	0	7	0	0	0
OP A 09 (3′GGGTAACGCC5′)	3	0	0	0	4	0	0	0
OP AA 03 (3′TTAGCGCCCC5′)	4	0	0	0	2	0	0	0
OP AA 11 (3′ACCCGACCTG5′)	2	0	0	0	3	0	0	0
OP AI 05 (3′GTCGTAGCGG5′)	3	0	0	0	3	0	0	0

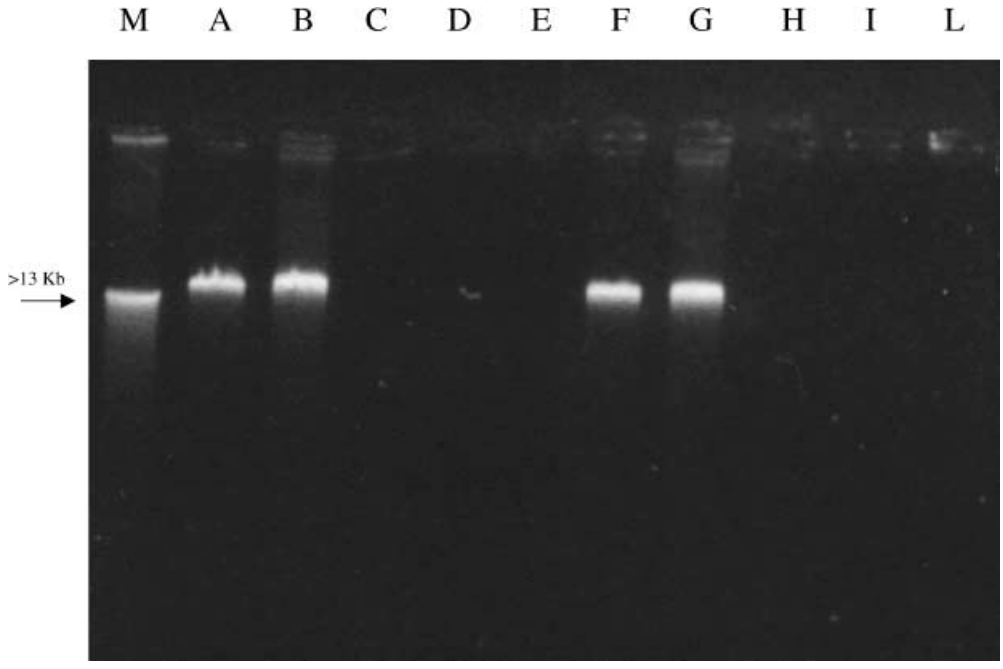
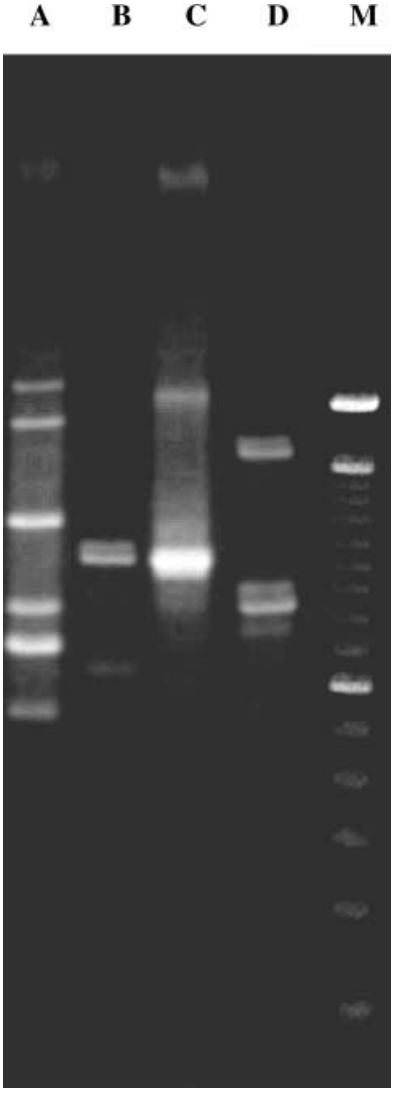


Fig. 1 DNA patterns after gel electrophoresis from cv. Carolea: *A* leaf extract, *B* olive oil extract by using proteinase K (PK), *C* olive oil extract by using pronase (Pr), *D* olive oil extract without enzymatic treatment, *E* commercial virgin olive oil extract; from cv. Cassanese: *F* leaf extract, *G* olive oil extract by using PK, *H* olive oil extract by using Pr, *I* olive oil extract without enzymatic treatment, L commercial virgin olive oil extract. *M* Calf thymus DNA



PCR products with OP A-09 and OP AA-03 primers respectively, from leaf and olive oil sediments of Carolea and Cassanese cultivars respectively. The DNA from olive oil of Cassanese and Carolea cultivars gave 19 and 15 reproducible amplification fragments (Table 1) respectively that may be considered potential useful markers for olive oil DNA fingerprinting.

The number of markers per primer ranged from two to seven (Table 1), and the dimensions from 400 to 2000 bp. DNA fragments were also extracted from a commercial veiled virgin olive oil by the usual treatment. In this case, the agarose gel electrophoresis did not show the band at high molecular weight as observed in the case of oil obtained from PK-treated olives and, as expected, the corresponding RAPD-PCR amplification did not occur.

Surprisingly, the RAPD-PCR amplification of DNA extracted from olive leaves and olive oil sediments from the same tree gave similar but not superimposable RAPD patterns. We don't know the reasons for this unexpected result, but suggest that it is probably attributable to seed DNA. In fact, the olive seed often arises from cross-pollination. In this case, the seed contained different genetic material from another pollinating variety. Therefore, the production of olive oil from destoned olive drupes by PK treatment will be tested.

Finally, the overall chemical composition of (1) olive oil obtained by using PK prior to malaxation and (2) olive oil obtained without any enzymatic treatment were compared. The analytical results were very similar thus confirming the future usefulness of this technique.

◀ **Fig. 2** DNA pattern after gel electrophoresis of amplified RAPD-PCR products obtained from leaf (*A, C*) and olive oil sediments by using PK (*B, D*) from Carolea (*A, B*) and Cassanese cultivars (*C, D*). *M* 100 bp DNA ladder. Primer OP A-09

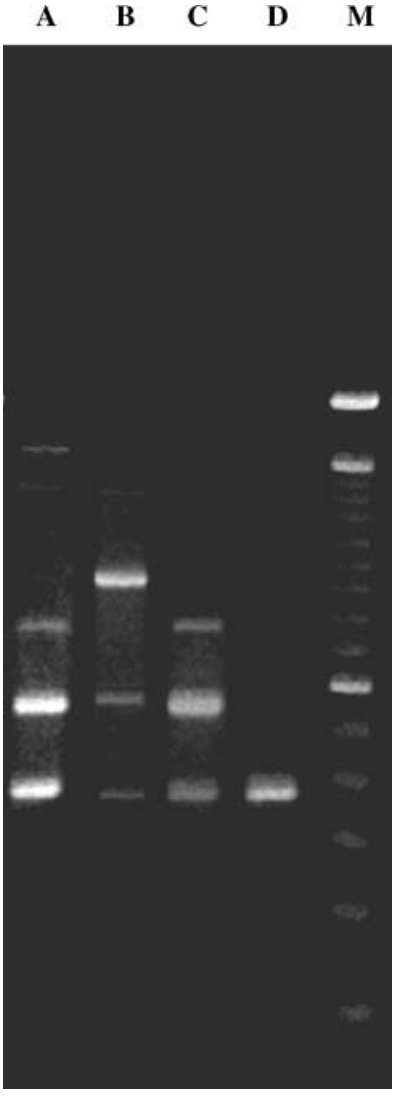


Fig. 3 DNA pattern after gel electrophoresis of amplified RAPD-PCR products obtained from leaf (A, C) and olive oil sediments by using PK (B, D) from Carolea (A, B) and Cassanese cultivar (C, D). M 100 bp DNA ladder. Primer OP AA-03

However, the most important preliminary results achieved was the intact DNA recovery from freshly obtained Carolea and Cassanese cultivar olive oils, and its unambiguous and reproducible PCR amplification by the means of PK treatment.

Thanks to the simultaneous and combined optimisation of the extraction procedure and the use of the PK enzyme, intact DNA from Carolea and Cassanese single cultivar virgin olive oils was recovered, allowing the successful PCR-amplification of DNA. Not enough experience has, however, been accumulated so far to identify all parameters affecting and determining DNA preservation during the oil extraction process. Therefore, new enzymatic protease mixtures should be tested. Finally, this new procedure could be successfully adopted to produce and certify the identity of single cultivar olive oil from destoned olive drupes, which could be of enormous use. Therefore, an investigation of the factors affecting DNA nature and content in virgin olive oils by the investigation of agronomic, technological and enzymatic variables is in progress.

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