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Food Chemistry 83 (2003) 127–134

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

DNA extraction from olive oil and its use in the identification of the production cultivar

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Received 28 January 2002; received in revised form 28 April 2003; accepted 28 April 2003

Abstract

DNA recovery from food samples might be of great importance when the raw material used in the production process has to be traced. We were interested in verifying the presence of nucleic acids in extra virgin olive oil in order to determine the cultivar of origin of the olives used for the production. A reliable DNA extraction method for extra virgin olive oil has been defined, as far as both quantity and quality are concerned, and the possibility of using this DNA for fingerprinting the original cultivar has been demonstrated. DNA extraction was tested on four monovariety oils, plus four commercial extra virgin olive oils. The DNA in the extracted solution was of chloroplast and nuclear origin since we were able to amplify cloned cultivar RAPD and AFLP fragments homologous to nuclear DNA of other species. It has also been shown that DNA purified from oil can be used for AFLP analysis and that the profile of the DNA purified from a monovariety oil corresponds to the profile of the DNA purified from the leaves of the same cultivar.

Keywords: Olive oil DNA; Fingerprinting; Cultivar identification; AFLP; RAPD; SCAR

1. Introduction

Recent crises in the food sector, such as dioxin poultry (Vellinga & Van Look, 2002) and BSE (Willesmith, 1996) have highlighted the need for more stringent food quality control, which should include determination of the origin of the product and the raw materials used in it. Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG) are all important instruments for quality control. The origin of a food product and the typicality of its production procedures, which are the result of centuries of experience, is a guarantee of the product healthiness and safety. This is particularly true for extra virgin olive oil, where origin is an important guarantee of quality.

A new European Regulation has recently been passed (EU Regulation no. 2815/98) establishing that the oil origin is to be determined by the place where the olives are milled, no longer by region where the olive plants are grown. This regulation further reduces the possibility of certifying PDO of olive oil, while olive trade can become very profitable. Furthermore since oil quality is inversely related to the time elapsing from olive harvesting to milling, the characteristic of olive oil are expected to worsen with time. The quality is also determined by the cultivar and the climatic conditions in the growing area, thus the differences in growing areas and cultivars justify the price differences between products. For these reasons the traceability of olive oil is an important issue for consumer protection. Neither chemical analysis of different clusters of compounds, nor the analysis of biomorphological traits have led to cultivar identification, due to environmental effects on the chemical composition and phenotype (Alessandri et al., 1997; Barone, Di Marco, Motisi, & Caruso, 1994). Genetic identity seems to be the only possibility for

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doi:10.1016/S0308-8146(03)00218-8

identifying the cultivar and the products deriving from it. The introduction of polymerase chain reaction (PCR; Mullis & Faloona, 1987) has accelerated the development of new DNA fingerprinting methods, including Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat Polymorphism (SSR), Cleavable Amplified Polymorphic Sequences (CAPS), Amplified Fragment Length Polymorphism (AFLP), Sequence Characterized Amplified Region (SCAR) and Inter-Repeat Amplification (IRA), now available for genetic mapping in plants (Dietrich, Weber, Nickerson, & Kwok, 1999). AFLP technique seems to be the most reliable and informative fingerprinting procedure for classifying and identifying olive cultivars (Angiolillo, Mencuccini, & Baldini, 1999). SSR is also very reliable (Rallo, Dorado, & Martin, 2000; Sefc et al., 2000), followed by RFLP (Baldoni, Angiolillo, Pelligrini, & Mencuccini, 1999) and RAPD (Cresti et al., 1997; Fabbri, Hormaza, & Polito, 1995; Vergari, Patumi, & Fontanazza, 1999).

Difficulties in the naming and recognition of olive germplasm remains a hard task due to the number of varieties described in the species (Bartolini, Prevost, Messeri, & Carignani, 1999), with many synonymous and clonal differences within the same named cultivar. A study of the geographical distribution of the main cultivars will be a first step forward in applying a traceability procedure for the most important Mediterranean olive oil producing regions. The availability of a reliable method for DNA extraction from extra virgin olive oil is crucial for this project to be carried out successfully. This paper reports the development of this extraction method and demonstrates the usefulness of the DNA obtained for fingerprinting.

2. Material and methods

2.1. Plant material

Leaves (250 g) and olives (10 kg) of the four *Olea europaea* cultivars Casaliva, Moraiolo, Leccino and Taggiasca were collected in October 1999 from single plants in specific areas of Italy and used for DNA extraction and oil production. The procedure for monovariety oil production followed the standard methods used in oil factories, including milling, squeezing and centrifugation. Leaves were stored at −20 °C, while oil was stored at room temperature until DNA extraction.

2.2. DNA extraction from leaves

Total DNA extraction from leaf tissue followed the CTAB method of Doyle and Doyle (1987) with few modifications. Five grams of leaves were ground in liquid nitrogen and mixed with 20 ml of CTAB buffer [100 mM Tris–HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 1% (w/v) PVP, 0.2% (v/v) β-mercaptoethanol, 0.1% (v/v) $NaHSO_3$]. Samples were incubated at 65 °C for 1 h, mixed with an equal volume of chloroform–isoamyl alcohol (24:1—v/v) and centrifuged at 2000 × *g* for 15 min. The aqueous phase was extracted twice with chloroform–isoamyl alcohol, recovered and mixed with two-third volume of isopropanol. Precipitated DNA was recovered with a sterile toothpick, washed with 5 ml of 10 mM ammonium acetate, dried and resuspended in 0.5 ml of TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA pH 8). The extracted DNA was quantified on agarose gel with a lambda DNA standard (Amersham).

2.3. DNA extraction from oil

Four monovariety oils, obtained in a small-scale plant (Istituto Sperimentale Elaiotecnica, Pescara, Italy) from 10 kg of olives, plus four commercial extra virgin olive oils were used for DNA extraction. Oil samples (50–100 ml) were centrifuged at 14,000 × *g* for 30 min at 4 °C (the volume of the recovered wet pellet should be 0.5 ml). After centrifugation, the supernatant was discarded and the wet pellet moved to a 2 ml microcentrifuge tube and centrifuged again at 14,000 × *g* for 15 min at 4 °C. The oily supernatant was carefully eliminated and the pellet frozen by means of liquid nitrogen. Then the tube was immediately immersed in a bath pre-heated to 65 °C. The freeze-thaw procedure was then repeated twice and at the end 750 μl of 10X CTAB extraction buffer (10% w/v CTAB, 75 mM Tris–HCl pH 8, 15 mM EDTA pH 8, 1 M NaCl, 1% v/v β-mercaptoethanol) pre-heated to 65 °C were added (the volume of added CTAB extraction buffer should be equal to 1.5 of the recovered pellet). The pellet was resuspended vigorously without using a vortex and incubated at 65 °C for 1.5 h with occasional mixing (every 10 min). After adding 750 μl chloroform:octanol (24:1—v/v), the tube content was mixed by inversion for 5 min at room temperature and centrifuged at 14,000 × *g* for 10 min at room temperature. The supernatant was transferred to a clean tube and the chloroform:octanol extraction repeated once again. 75 μl (0.1 of the supernatant volume) of 10% (w/v) CTAB (in water) were added to the supernatant, mixed for 5 min and added with 1.5 v of CTAB precipitation buffer (1% w/v CTAB, 50 mM Tris–HCl pH 8, 10 mM EDTA pH 8), then mixed again by inversion for 10 min. After centrifuging at 14,000 × *g* for 20 min at room temperature, the supernatant was discarded and the pellet dried at room temperature for 15–30 min. The pellet was then resuspended in 100 μl of 1 M NaCl, added with 2 μl RNasi (stock 20 mg/ml) and incubated at 37 °C for 1 h. Chloroform:octanol (24:1) extraction was performed and 2.5 volumes of ice-cold absolute ethanol were added to the aqueous phase. The solution

was mixed by inversion for 5 min and then centrifuged at 14,000 × *g* for 15 min at 4 °C. The aqueous phase was then discarded and the pellet washed by adding 1 ml of 70% ethanol, then centrifuged at 14,000 × *g* for 5 min and the supernatant discarded. The pellet was then dried at room temperature for a few minutes. The pellet was resuspended in 30–50 μl TE buffer and incubated at 65 °C for 10–30 min, then centrifuged at 14,000 × *g* for 5 min. The DNA solution was then transferred and the pellet discarded.

2.4. AFLP analysis

AFLP analysis was performed with a capillary electrophoresis system following the AFLP™ plant mapping protocol (Applied Biosystems), or using the manual method (see below) where the cloning of specific bands was necessary for SCAR development. Olive genomic DNA from leaves and oil (250 ng) was double-digested with EcoRI/MseI restriction enzymes. Restriction and ligation of the adapters were performed simultaneously in a total volume of 11 μl at 37 °C for 2 h. Pre-selective and selective PCR were performed in an Applied Biosystems 9700 thermal cycler. The pre-selective PCR (72 °C for 2 min; 20 cycles at 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min; 60 °C for 30 min) with EcoRI + A and MseI + C primers was performed in a 10 μl reaction volume consisting of 2 μl 20-fold diluted ligated DNA, 0.25 μl of 10 μM pre-selective primers and 7.5 μl of AFLP Core Mix (Applied Biosystems). The selective PCR (94 °C for 2 min; 1 cycle at 94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min, followed by 10 cycles at 1 °C reduced annealing temperature each cycle and 20 cycles at 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min; 60 °C for 30 min) with EcoRI + AXX and MseI + CXX selective primers was carried out in a 10 μl volume consisting of 2 μl of 20-fold diluted pre-amplified DNA, 0.5 μl of EcoRI (Dye-primer-AXX) at 1 μM, 0.5 μl of MseI (Primer-CXX) at 5μM and 7 μl of AFLP Core Mix. The selective primers were fluorescent dye-labeled and the following primer combinations employed: E-ACA + M-CAG, E-AGC + M-CTG. Amplified products from selective amplification were loaded and run on the ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to AFLP plant mapping protocol and analyzed by means of GeneScan Analysis software (Applied Biosystems).

In the manual AFLP analysis, genomic DNA (250 ng) was double digested with EcoRI and MseI following the protocol of Vos et al. (1995). The Resulting fragments were ligated to adapters specific for the EcoRI and MseI restriction sites. A pre-selective amplification (72 °C for 2 min; 20 cycles at 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min; 60 °C for 30 min) was carried out with EcoRI + A and MseI + C primers and the PCR product was then diluted 15-fold with water and used as template for selective amplifications (94 °C for 2 min; 1 cycle at 94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min, followed by 10 cycles at 1 °C reduced annealing temperature each cycle and 20 cycles at 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min; 60 °C for 30 min) using both EcoRI + 3 and MseI + 3 primers; EcoRi + 3 primers were labeled with ^{33}P.

Amplified products from selective amplification were resolved by electrophoresis on 6% denaturing polyacrylamide gels and visualized by exposing a BioMax film for 24–48 h.

Selected fragments were excised and purified from the acrylamide gel and re-amplified using the same primers and conditions. The single-band AFLP products were ligated into a T-vector (pGEM-T easy, Promega) and then transformed into *Escherichia coli* strain XL1B.

2.5. RAPD analysis

The volume of the final reaction (20 μl) was composed of 1X buffer (buffer A, InCura), 2 mM $MgCl_2$, 150 μM dNTPs, 1 μM primer (PLT253 5′-ACGGCGATGA-3′), 1.5 U Taq DNA Polymerase (InCura) and 4 ng of template DNA. Amplifications were performed in an Applied Biosystems 9700 thermal cycler with an initial denaturating step of 2 min at 94 °C, followed by 45 cycles of 20 s at 94 °C, 20 s at 32 °C, 2 min at 72 °C and a final extension step of 2 min at 72 °C. PCR products were run overnight at 20 V on 2% agarose gel and DNA bands were visualized by ethidium bromide staining.

Selected fragments were excised and purified (geneclean III kit, Bio101) from the agarose gel, then re-amplified using the same primers and conditions. PCR products were run on 2% agarose gel and the bands corresponding to the selected fragments were excised and purified.

The single-band RAPD products were ligated into a T-vector (pGEM-T easy, Promega) and then transformed into *Escherichia coli* strain XL1B.

2.6. SCAR analysis

Conversion of AFLP and RAPD markers to SCAR. Both ends of selected cloned fragments were sequenced following the Automated DNA Sequencing Chemistry Guide (Applied Biosystems). PCR reactions for cycle sequencing were performed in an Applied Biosystems 9700 thermal cycler using the following profile: 94 °C for 2 min; 20 cycles at 96 °C for 10 s, 50 °C for 15 s and 60 °C for 2.5 min. The final volume of the reaction (20 μl) was composed of 4 μl Terminator Ready Reaction Mix, 2 μl 5X sequencing Buffer (400 mM Tris–HCl, 10 mM $MgCl_2$ and pH 9), 3.2 pmol of primers (T7 and SP6) and DNA (200–500 ng). Unincorporated dye terminators were removed by isopropanol precipitation according to the manual. The sequences were re-sus-

pended by adding 15 μl TSR (Template Suppression Reagent, Applied Biosystems) and loaded on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Refer to the ABI Prism 310 Genetic Analyzer User's Manual for electrophoresis procedures.

Sequences were analyzed using Sequencing Analysis Version 3.4.1. PCR reactions (94 °C for 4 min; 40 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min; 72 °C for 5 min.) were performed in an Applied Biosystems 9700 Thermal Cycler. The reaction volume (20 μl) consists of 1X buffer (buffer A, InCura), 2 mM $MgCl_2$, 150 μM dNTPs, 0.5 μM of each primer, 2.5 U Taq DNA Polymerase (InCura) and less than 2 ng of template DNA. PCR products were resolved on 1.5% agarose electrophoresis gel and visualized by ethidium bromide staining.

2.7. PCR analysis

PCR reactions were performed in a 25 μl volume containing: 5–10 ng of DNA, 0.5 μM of forward and reverse primers, 200 μM of each dNTP (Amersham), 1X optimized EXT buffer and 0.5 units of DyNAzyme EXT DNA polymerase (Finnzymes, OY). For PCR amplification of the 364 bp fragment the following primers were used: IGP558 5′-ACGGCGATGATAACAGCC-3′ and IGP559 5′-ACGGCGATGACCAAGCG-3′. The amplified fragment corresponds to a RAPD band cloned from the cultivar Moraiolo. For PCR amplification of a fragment corresponding to a non-coding intragenic region of the chloroplast DNA between the 5′ trnL and 3′ trnL exons, highly conserved in all angiosperms and ranging in size between 298 and 653 bp (Taberlet, Gielly, Pantou, & Bouvet, 1991) the following primers were used: IGP87 5′-CGAAATCGGTAG-ACGCTACG-3′ and IGP88 5′-GGGGATAGAGGG-ACTTGAAC-3′. DNA amplification reaction was performed in a thermal cycler (Mastercycler, Eppendorf) programmed for 5 min at 94 °C, followed by 40 cycles of 40 s at 94 °C, 50 s at 58 °C and 1 min at 72 °C. For the amplification of the 1,172 bp fragment, the following primers were used: IGP383 5′-GATATCT-TGGCAGCATTCCG-3′ and IGP384 5′-TCTAGAG-CTACTCGGTTAGC-3′ taken from sequence information present on GeneBank Accession Number AJ001766 related to *Olea europea* chloroplast ribulose diphosphate carboxylase large subunit. For the 1,942 bp fragment, the following primers were used: IGP321 5′-AATCTTCGTCGTATGTGGGC-3′ and IGP322 5′-GAGTTAATTGGGCTAATCCTC-3′ taken from GeneBank Accession Number AF027288 for *Olea europea* NADH dehydrogenase chloroplast gene. DNA amplification reactions for all the fragments were performed in a thermal cycler (Mastercycler, Eppendorf) programmed for 5 min at 94 °C, followed by 30 cycles of 40 s at 94 °C, 50 s at 60 °C, 2.5 min at 72 °C with an increasing extension time of 5 s every cycle, then 10 cycles of 40 s at 94 °C, 50 s at 60 °C and 5 min at 72 °C.

Amplification products were analyzed by gel electrophoresis in 1% agarose gel in TBE 1X, stained with ethidium bromide and photographed under UV light using an ImageMaster VDS (Amersham).

3. Results and discussion

We have succeeded in defining a reliable DNA extraction method, as far as both quantity and quality are concerned, from extra virgin olive oil. The possibility of using this DNA for tracing the raw material has been demonstrated in fingerprinting the cultivar used for oil production.

Four monovariety extra virgin olive oils, obtained from the *Olea europea* cultivars Casaliva, Moraiolo, Leccino and Taggiasca, as well as four commercial extra virgin olive oils, were tested for DNA extraction. Samples of leaves (500 g) and olives (10 kg) of the four cultivars were collected in fall 1999 in central and southern Italy. The commercial samples were bought at a local grocery store.

The recovery of a sufficient amount of pellet, followed by careful elimination of the oil residues, is a crucial step for successful DNA extraction. Since the deposit (dregs) recovered from each sample varies depending on the technology used for the oil production, in order to obtain sufficient DNA to perform 10 to 30 amplification reactions from each sample, it is necessary to start from 50 to 100 ml of oil and the wet pellet obtained from this oil must weigh at least 0.5 g. Tests carried out using higher extraction volumes did not improve DNA yield. On the other hand, it seems that the use of smaller volumes make DNA extraction easier, facilitating the recovery of a denser pellet, thus reducing material loss.

CTAB method of Murray and Thompson (1980) was used for DNA extraction, as fully described in materials and methods, with the following important modifications. Before re-suspending the pellet in the lysis buffer, the freeze/thaw cycles are crucial; high detergent concentration (10% CTAB) is also important. In the organic solvent extraction the combination chloroform:octanol instead of chloroform:isoamylic alcohol is used to facilitate nuclei isolation. DNA amplifiable with a PCR technique was obtained from the eight samples tested, four of which were from commercial high quality sources and four were lab scale preparations (Fig. 1). Electrophoretic analysis shows that the DNA is, in most of the samples, in the 2000–10,000 bp size range, even if in some of the samples partially degraded DNA in the 100–1000 bp range is also found (lane 1 and 2, Fig. 2). The amount of recovered DNA in some of the samples may be very low and not clearly visualized by ethidium bromide staining (lane 3, Fig. 2).

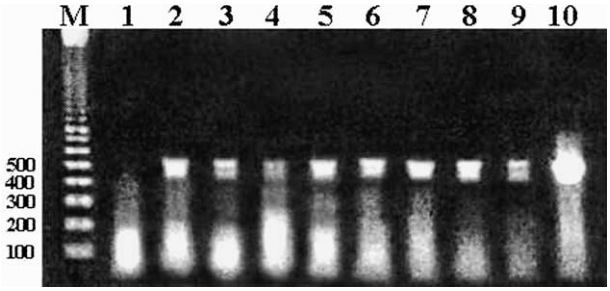

Fig. 1. Electrophoretic agarose gel, stained with ethidium bromide, of the PCR products obtained using the primers IGP87 and IGP88 and the purified DNA extracted from extra virgin olive oil of the following cultivars or commercial sources as template: 2–5 commercial; 6 Casaliva; 7 Moraiolo; 8 Leccino; 9 Taggiasca; 1 negative control; 10 Positive control (DNA from olive leaves); M Molecular marker (100 bp ladder).

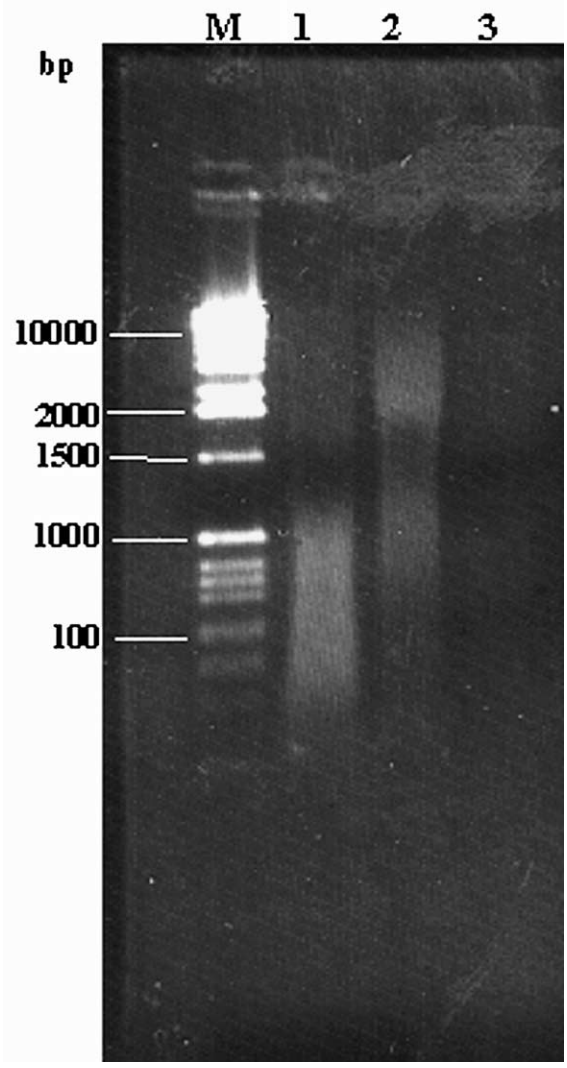

Fig. 2. Electrophoretic agarose gel, stained with ethidium bromide, of the DNA purified from extra virgin olive oil. In sample 1 the majority of the DNA is of low molecular weight (100–1000 bp), in samples 2 and 3 the majority is of high molecular weight (2000–10 000 bp), and in the last one the quantity is significantly lower. M: DNA molecular marker; 1: commercial 1; 2: commercial 2; 3: commercial 3.

In order to demonstrate that the DNA was extracted from all the samples, and that in the purified DNA there were fragments longer than 1000 bp, a degradation limit which ensures the fingerprinting reproducibility, we designed three primer sets. The first set was for the non-coding intragenic region of the chloroplast DNA (Taberlet et al. 1991); with this set of primers the dimension of the fragment amplified in *Olea europea* is about 500 bp. The second set was for a fragment of 1172 bp (chloroplast ribulose diphosphate carboxylase large subunit gene) and the third one was for a fragment of 1942 bp (NADH dehydrogenase chloroplast gene). These primers allowed us to amplify the three fragments from all the DNA samples extracted from the oils (Fig. 3).

Since the analyses of DNA of nuclear origin is essential to fingerprint the plant cultivar, it is crucial to demonstrate its presence in the DNA extracted from the oil. To this aim we cloned several cultivar specific (unique) and cultivar nonspecific (present in more then one cultivar) RAPD fragments which we had identified in fingerprinting experiments performed on several olive cultivars. After cloning and sequencing, these fragments were transformed into more reliable SCAR markers to be used in cultivar identification.

As little or no sequence information of the olive genome is available, RAPD is a simple and reliable source

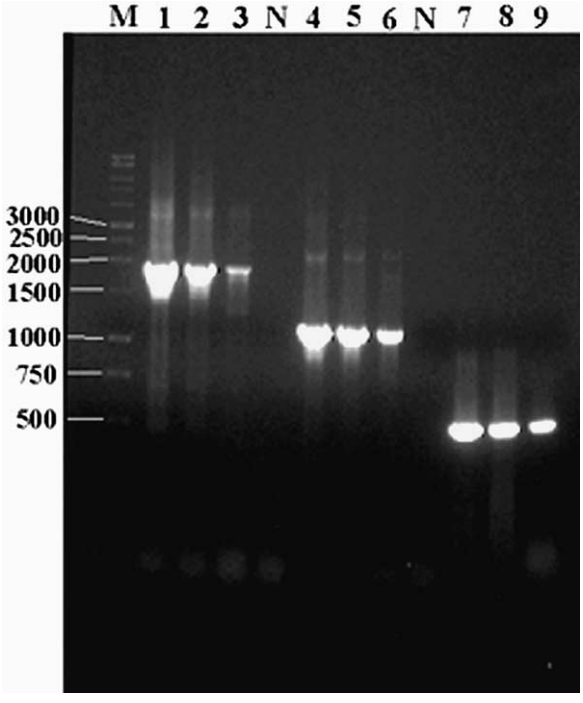

Fig. 3. Electrophoretic agarose gel, stained with ethidium bromide, of the PCR products obtained using the primers (see in Section 2 the PCR analysis) set IGP321-IGP322 (lanes 1–3), IGP383-IGP384 (lanes 4–6) and IGP87-IGP88 (lanes 7–9) and the purified DNA, shown in Fig. 2, extracted from oil of the following sources as template: lanes 1, 4 and 7 commercial 1; lanes 2, 5 and 8 commercial 2; lanes 3, 6 and 9 commercial 3; M Molecular marker (1 kb ladder); N Negative control without DNA.

of polymorphism which has already been used for olive cultivar identification (Fabbri et al., 1995). Fig. 4 shows an electrophoretic agarose gel with the RAPD fingerprinting obtained using the decameric primer PLT253 on the DNA of 15 different olive cultivars. The two fragments indicated by the arrow, after excision and cloning, were sequenced and the primers designed on them were used as sequence characterized amplified regions (SCAR; Hernàndez et al., 2001) in order to demonstrate the presence of nuclear DNA in the oil. The band that we consider cultivar nonspecific, since it is present in 8 out of 15 cultivar tested, has a length of 364 bp (sequence not reported). The set of primers for the cultivar nonspecific band are those indicated as IGP558 and IGP559 (see Material and methods) and their ability to amplify the band, using the DNA extracted from oils as template is reported in Fig. 5. The evidence that the 364 bp fragment is likely to be of nuclear origin came from the results of a BLAST homology search against this sequence that gave homology hits with *Arabidopsis thaliana*, *Oryza sativa* and *Sorghum bicolor* nuclear DNA (data not shown).

Furthermore, we were able to demonstrate that the DNA extracted from oil can be used for AFLP analysis and that the profile of the DNA purified from a monovariety oil shows high correspondence with the profile obtained from DNA purified from the leaves of the same cultivar (Figs. 6 and 7). This finding makes it possible to identify the cultivar used for the oil production either by means of a SCAR specific band or of the fingerprint of the AFLP analysis.

The ultimate possibility for recognizing the identity of a living organism is to operate at genome level since the proteome (analysis of the protein array) or the metabolome (analysis of the chemical components) are influenced by the environment and are meaningless in terms of fingerprinting of the food components. Analysis at genome level requires the availability of purified DNA even in trace quantity, which, with the available techniques, can be used to identify an individual or a larger systematic entity like a cultivated variety (cultivar) or species.

Given the importance of DNA recovery from food samples in terms of traceability of the raw material used in the production process, we were interested in verifying the presence of nucleic acids in extra virgin olive oil in order to determine the cultivar of origin of the olives used for the production and to guarantee the high quality producing areas in Italy, like Tuscany, Umbria, Liguria, Lazio and many others. An important aspect, in terms of reproducibility of the fingerprinting technique, is also the length and quantity of the recovered DNA.

It is worth reminding that, at least in Italy, the tendency is to use a production technique that does not include centrifugation and filtration of the cold pressed oil in order to obtain high quality extra virgin olive oil. This allows good DNA recovery from commercial products and easier identification of the cultivar from which the oil has been extracted. The most difficult task

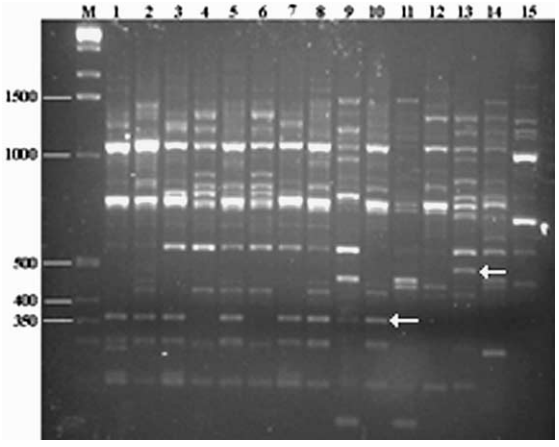

Fig. 4. Example of an electrophoretic agarose gel, stained with ethidium bromide, of the RAPD products obtained using the random primer PLT253 on 15 olive cultivars (1 Lezzo; 2 Leccio; 3 Casaliva; 4 Leccino; 5 Augellina; 6 Frantoio; 7 Taggiasca; 8 Carolea; 9 Favarolo; 10 Coratina; 11 Grappolo; 12 Lazzero; 13 Moraiolo; 14 Cassanese; 15 Marzio). Indicated by the arrows are a cultivar specific band of around 500 bp (present only in the sample 13) and the cultivar nonspecific band of 364 bp (present in 8 of the 15 samples) used, after cloning and sequencing, to demonstrate the nuclear origin of the sequence.

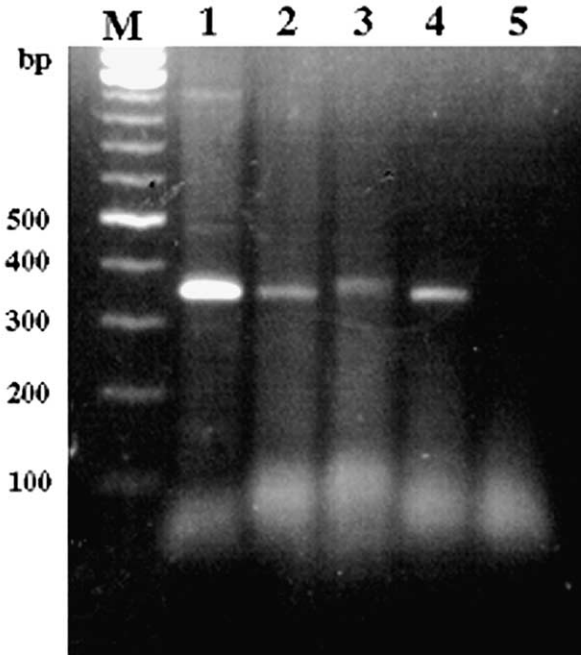

Fig. 5. Electrophoretic agarose gel, stained with ethidium bromide, of the PCR products obtained using the primers IGP558 and IGP559, designed on the sequence of the cultivar nonspecific band of Fig. 4, and the purified DNA extracted from oil of the following commercial sources as template: lane 2 commercial 1; lane 3 commercial 2; lane 4 commercial 3; lane 1 positive control (DNA from leaves); lane 5 negative control; lane M Molecular marker (100 bp ladder). The apparent difference in size between the sample 3 and the others can be real and due to the unknown cultivar present in the commercial oil.

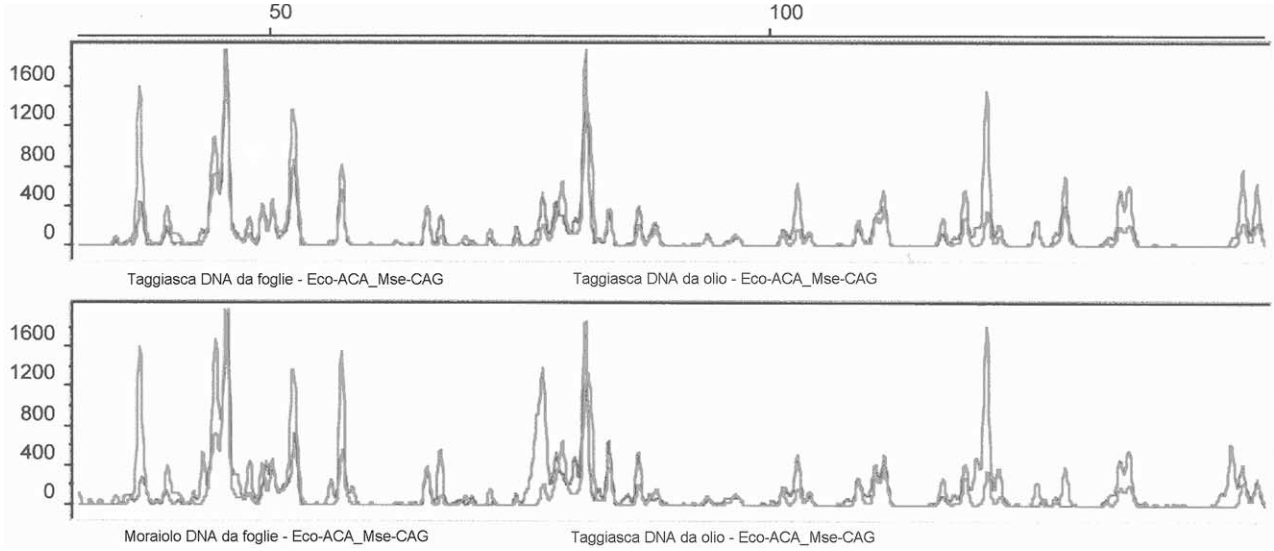

Fig. 6. Capillary electrophoresis electropherogram showing the AFLP profile generated by the following primer combinations: *Eco*RI + ACA and *Mse*I + CAG. In the upper panel there is the superimposition of the profiles of DNA of the cultivar Taggiasca, isolated from leaves (blue) and oil (red). The profile represents the region between 50 and 150 bp. The high coincidence of the two profiles for the fragments present can be seen. In the lower panel there is the superimposition of the AFLP profile of the leaves DNA of the cultivar Moraiolo (blue) with the AFLP profile of the oil DNA of the cultivar Taggiasca (red). The profile represents the region between 50 and 150 bp and differentiating bands are present. In the *x* axis is reported the size of DNA fragments in base pairs, while the *y* axis refers to the fluorescence intensity of the signal.

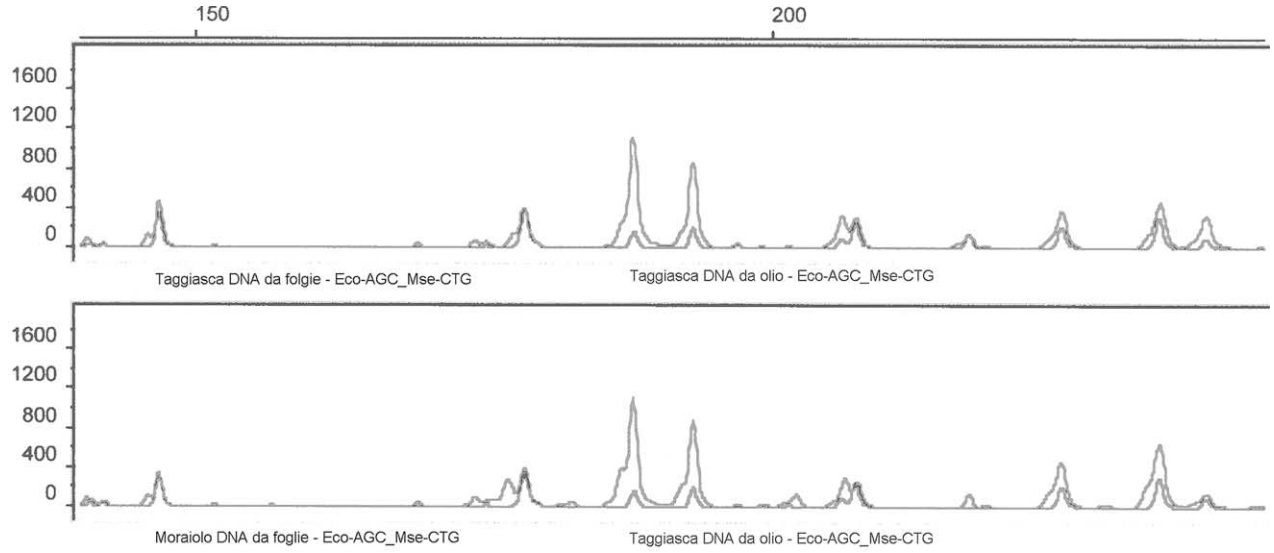

Fig. 7. Capillary electrophoresis electropherogram showing the AFLP profile generated by the following primer combinations: *Eco*RI + AGC and *Mse*I + CTG. In the upper panel there is the superimposition of the profiles of DNA of the cultivar Taggiasca, isolated from leaves (blue) and oil (red). The profile represents the region between 145 and 240 bp using a different primer combination of that reported in Fig. 6. The high coincidence of the two profiles for all the fragments present can be seen. In the lower panel there is the superimposition of the AFLP profile of the leaves DNA of the cultivar Moraiolo (blue) with the AFLP profile of the oil DNA of the cultivar Taggiasca (red). The profile represents the region between 145 and 240 bp and differentiating bands are present.

remains the determination of the geographical distribution of the clonal varieties even if, for important producing areas in Italy, the germoplasm distribution and variability are compatible with full traceability, starting from the raw material up to the final product (Fogher et al., in preparation).

Acknowledgements

This work is part of the MIUR FAR project named: "Messa a punto di metodi di analisi per il controllo delle origini (rintracciabilità) e della tipicità negli oli di oliva extravergini".

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