DNA Extraction from Olive Oil and PCR Amplification of Microsatellite Markers

RAFFAELE TESTOLIN AND ORIETTA LAIN

ABSTRACT: DNA was extracted from single-cultivar of cold-pressed (virgin) unfiltered and cotton-filtered olive oils that were stored at 4 °C for up to a year using different DNA extraction kits and protocols. DNA was amplified using original and nested primers designed on 6 microsatellites loci of the UDO series. The most consistent results in terms of successful single sequence repeat amplifications were achieved using the Qiagen QIAamp DNA stool extraction kit, slightly modified and applied to oil sample amounts as small as 200 μL without any pretreatment. The kit allowed getting polymerase chain reaction (PCR) amplicons visible on gel and scorable peaks at the automatic sequencer for all 6 markers analyzed. Less consistent results were achieved with other kits, such as the Promega Wizard® Magnetic DNA Purification System for Food, the LB Link-Biotech ExtMan 50-100 Evolution, the Qiagen Plant Mini kit, and the standard cetyltrimethyl-ammonium bromide–based DNA extraction protocol. The integration in the protocols of further tools, such as the hexane-based phase separation, the addition of water or NaCl solutions to the oil, the precipitation and the use of the pellet, and others, did not result in any substantial use. PCR amplifications that gave low DNA yields were improved by adopting the nested PCR technique, which uses the product of the 1st PCR as a template for a 2nd PCR carried out by means of internal primers. Conclusions are drawn as to the applicability of the method to trace the identity of single-cultivar virgin olive oils. Further work is required to check the sensitivity of the method in determining the varietal composition of blended oils, especially in detecting alleles from cultivars present in only small amounts.

Keywords: olive oil, genetic traceability, molecular markers, SSR, microsatellite markers

Introduction

Traceability in food is a recently developed concept of control for the entire chain of food production and marketing that allows food to be traced through every step of its production back to its origin. This widely used concept of food traceability is slightly different from the concept of genetic traceability we use here. By genetic traceability, we mean the determination of the genetic identity of the plant material from which the transformed products have been derived. Establishing the genetic origin of food products allows verification of the authenticity of valuable foods and discourages adulteration with material of lower cost and value.

Olive oil is usually traded as a blend from different cultivars and of different provenances. Yet, some olive oils originating from well-defined geographical areas and cultivars are recognized as being of higher quality, command better prices, and are, in some cases, legally protected (for example, the Protected Designation of Origin regulation of the European Community).

The presence in highly prized olive oils of lower-grade material is sometimes revealed by specific analytical methods (reviewed in Lichan 1994), but when olive oils of the same grade, but different provenances, are blended, then most analytical analyses are of limited value. In such cases, DNA-based technologies may help reveal the different origins of lots that have contributed to the blend (Breton and others 2004).

Significant amounts of DNA are present in olive oil obtained by cold pressing (Cresti and others 1996). However, the filtration process lowers concentrations and then DNA tends to disappear due to degradation by nucleases (Muzzalupo and Perri 2002, De la Torre and others 2004). We report here a protocol developed to extract DNA from olive oil and show how DNA microsatellite fragments up to about 200 bp long can be successfully amplified and used to identify the olive cultivar from which oil was produced.

MS 20040419 Submitted 6/24/04, Accepted 8/19/04, Revised 10/18/04. *Authors are with Dipt. di Scienze Agrarie e Ambientali, Univ. of Udine, Via delle Scienze 208, 33100 Udine, Italy. Direct inquiries to author Testolin (E-mail: raffaele.testolin@uniud.it).*

Materials and Methods

DNA extraction

Samples of filtered and unfiltered virgin olive oil from individual cultivars were obtained from the Istituto Sperimentale per l'Olivicoltura of Rende (CZ), Italy, or from private companies and kept at 4 °C until use.

DNA was extracted from 2 to 40 g oil using different extraction procedures and kits (Table 1). The following modifications of the procedures at various steps were compared: hexane addition to the oil in a ratio varying from 5:1 to 1:1; centrifugation (12000 to 14000 rpm) for 10 min as opposed to ultracentrifugation at 55000 rpm for 2 h; addition of 5 M NaCl in a ratio to oil 1:2 v:v; and others that had no significant effect. In some experiments, the pellet and the supernatant obtained by centrifugation were recovered and tested separately for comparison. After initial experiments, hexane was not used and DNA was extracted directly from 200 μL of oil using the standard protocols of the kits with minor modifications. DNA samples were resuspended in either the solution provided by the kit when available or Tris-EDTA (TE). buffer pH 8.0.

Total DNA was extracted for comparison from 1 g young leaves of the same olive cultivar using either the cetyltrimethyl-ammonium

Table 1—DNA extraction procedures and kits compared

Protocol	Kit name, notes
Promega Wizard	Promega Wizard Magnetic DNA Purification System for Food, based on paramagnetic particles that bind nucleic acids. Protocol C (purification of DNA from vegetable oils) with modifications.[a]
Qiagen QIAamp DNA stool	Qiagen QIAamp DNA stool mini kit, based on resin tablets that absorb PCR inhibitors and silica-gel columns that allow separation of nucleic acids. Protocol for pathogen detection with modifications.[a]
LB Link-Biotech ExtMan	LB Link-Biotech ExtMan 50-100 Evolution formula, based on liquid resin that separates nucleic acids from PCR inhibitors and other compounds.
CTAB (control 1)	Cetyltrimethyl-ammonium bromide–based (Doyle and Doyle 1990) micro-extraction method routinely used for olive leaf tissue (Cipriani and others 2002) and slightly modified for oil.
Qiagen Plant Mini kit (control 2)	Cetyltrimethyl-ammonium bromide–based extraction. Two columns, respectively, are used to purify and elute DNA.

[a]The amount of oil sample was reduced to 200 μL after several preliminary trials. See also Materials and Methods. Other minor modifications are linked to the amount of oil sample adopted.

Table 2—DNA microsatellite regions (SSR) used in this study, primer sequences, and PCR conditions

SSR	Primer forward 5′ → 3′	Primer reverse 5′ → 3′	Length[a] (bp)	$MgCl_2$ (mM)	T anneal (°C)
Original primers					
UDO-008	AAAAACACAACCCGTGCAAT	AAATTCCTCCAAGCCGATCT	159	2.5	57.0
UDO-009	TTGATTTCACATTGCTGACCA	CATAGGGAAGAGCTGCAAGG	119	1.5	57.0
UDO-012	TCACCATTCTTAACTTCACACCA	TCAAGCAATTCCACGCTATG	164	1.5	57.0
UDO-024	GGATTTATTAAAAGCAAAACATACAAA	CAATAACAAATGAGCATGATAAGACA	188	1.5	50.0
UDO-039	AATTACCATGGGCAGAGGAG	CCCCAAAAGCTCCATTATTGT	170	1.5	57.0
UDO-043	TCGGCTTTACAACCCATTTC	TGCCAATTATGGGGCTAACT	174	1.5	52.0
Internal primers					
UDO-008s	AAAAGAAAAGTGGATGCTGGTC	Same as original[b]	94	1.5	52.0
UDO-009s	GGCACACGTACACGCACA	Same as original[b]	76	1.5	52.0
UDO-012s	AAACGAGAATGTATATTAAGCACCA	AACATTTTCTTCCCGCTGTT	80	1.5	52.0
UDO-024s	AAAACTATGTCAAAGTTAGGAATACAC	CTGATATTTCTCTTCTTTACGTGTG	79	1.5	52.0
UDO-039s	CCATGGGCAGAGGAGGAATA	ATCAACATGCACGCACACAC	95	1.5	52.0
UDO-043s	Same as original[b]	GGTGGAAAGATAGAAAACCCTTA	99	1.5	52.0

[a]Length of the amplified fragment in the source sequence.
[b]No space for internal primer.

bromide (CTAB) method (Doyle and Doyle 1990), as modified slightly by Cipriani and others (2002), or the Qiagen Plant DNA Mini Kit. DNA samples were treated with 4 μL RNase (100 mg/mL) for 10 min at 65 °C.

DNA concentrations in samples were determined by fluorimetry (Hoefer DyNA Quant 200) using the Hoechst H 33258 fluorescent dye and human DNA (50 ng/μL) as a standard. DNA degradation was checked by means of 0.8 % agarose gel electrophoresis, ethidium bromide, or Sybr Green staining, and ultraviolet (UV) inspection.

Primer design

Six microsatellites or simple sequence repeats (SSRs) among the 43 isolated from olive in previous work (Cipriani and others 2002) were selected for their high polymorphism, their easily scored patterns, and the small-scale stuttering. New primers were designed internal to the original primer pairs for nested PCR assays (Newton and Graham 1997) using Primer3, available at the site http://www-genome.wi.mit.edu (Rozen and Skaletsky 2000). Original primers and internal primers are reported in Table 2.

Polymerase chain reactions (PCRs) on agarose gels

PCRs to be run on agarose gel were carried out in a 25-μL volume containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM each primer, about 10 to 100 ng genomic DNA (approximation is due to the uncertainty in determining the DNA concentration in DNA samples extracted from oil), and 0.5 U of Taq polymerase (Amersham Biosciences, Piscataway, N.J., U.S.A.) using a PT 100 thermal cycler (MJ Research, Piscataway, Mass., U.S.A.). The PCR profile was as follows: 95 °C for 5 min for 1 cycle; 94 °C for 45 s, the annealing temperature as given in Table 2 for 45 s, 72 °C for 45 s for 30 to 35 cycles, 72 °C for 8 min. In preliminary experiments, the touchdown approach (Don and others 1991) was compared with that based on a constant annealing temperature for all original and internal primer pairs: the touchdown approach was subsequently adopted routinely only for oil samples, for which it slightly improved the sharpness of bands.

PCR products were loaded onto 1% agarose gel containing ethidium bromide (1:20,000 v:v) or Sybr Green (1:10000 v:v), electrophoresed at 1 V cm^{-1} for 50 min and photographed under a UV transilluminator.

Nested PCRs were performed by diluting the product of the 1st PCR 1:50 in water and using an aliquot of 5 μL as a template for the 2nd PCR, carried out with the internal primers. Additional PCR reactions with all compounds of the mix except the DNA template were included as a control for nonspecific PCR amplification and/or cross-contamination.

PCRs on capillary sequencer

PCRs to be run on the capillary sequencer were carried out in 25-μL volume (8-μL for the 1st PCR carried out on leaf samples) containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.3 μM each primer (forward primer was labeled with either FAM or HEX fluorescent dye), about 10 to 20 ng genomic DNA, and

0.3 U of Taq polymerase (Amersham Biosciences). The PCR profile was as above.

One microliter of desalted PCR product was mixed with 2.75 μL of loading solution (70% formamide, 1 mM EDTA), 0.25 μL ETROX–labeled Et400-R size standard (Amersham Biosciences), and 1.0 μL deionized H_2O, centrifuged at 900 rpm for 2 min, denatured at 95 °C for 4 min, cooled in ice, and analyzed on a MegaBACE 500 capillary sequencer (Amersham Biosciences) using Genetic Profiler v2.0 to estimate the allele size.

Nested PCRs were carried out by diluting the products of the 1st PCR 1:50 in water and using 5 μL as a template for the nested PCR.

Results and Discussion

DNA recovery from olive oil

Numerous modifications designed to improve the amount and quality of DNA extracted from olive oil did not lead to substantial improvements, and the DNA recovered seldom exceeded 10 ng/μL (Table 3). Hexane, as recommended in several protocols and in the literature, to separate the aqueous phase and the pellet from the lipid phase did not significantly improve DNA yields, as appreciable amounts of DNA remained in the supernatant. Moreover, PCR amplification was usually unsuccessful when the template DNA was extracted from the oil with hexane added (Table 4). Other modifications to the extraction protocols, such as ultracentrifugation and the use of NaCl solutions, did not improve DNA yields: only addition of proteinase K to oil increased yields (data not shown).

Of the kits assayed, the most consistent results were achieved with the Qiagen QIAamp DNA stool extraction kit; the other kits gave more erratic PCR amplifications (Table 5). Small amounts of DNA were extracted from unfiltered and cotton-filtered olive oil using all methods tested and were, in most cases, below the sensitivity of the fluorimeter. However, the concentration of DNA did not appear to be limiting; rather, successful amplification likely depended on the ability of the different kits to free DNA from inhibitors of PCR present in the samples (Table 5).

Table 3—Influence of hexane pretreatment and phase separation on the amount of DNA recovered from filtered olive oil[a]

Treatment[b]	Qiagen Plant Minikit ng/μL	LB ExtMan ng/μL
No hexane, no phase separation (control)	8.7 ± 2.5	5.7 ± 2.2
Hexane treatment and centrifugation (supernatant)	5.7 ± 2.1	7.7 ± 2.5
Hexane treatment and centrifugation (pellet)	2.7 ± 1.2	4.9 ± 2.6

[a]Data obtained with 2 different extraction kits are reported.
[b]2 to 20 mL cold-pressed olive oil from the cultivar 'Carolea'; oil:hexane 1:5 v:v; centrifugation at 14000 rpm, resuspended in 60 μL buffer.

Table 4—Influence of the hexane pretreatment and phase separation on the amount and quality of DNA recovered from olive oil[a]

Treatment	DNA (ng/μL)	PCR product[b]
Hexane[c]	5.0 ± 0.8	0/4
Control[d]	6.2 ± 2.8	4/4

[a]Quality was evaluated indirectly by PCR amplification of 2 different SSRs.
[b]Number of successful standard PCRs/total number of standard PCR assays.
[c]2 milliters filtered olive oil from the cultivar 'Carolea', oil:hexane 1:5 v:v, DNA extracted with Qiagen QIAamp DNA stool mini kit, PCR as in Materials and Methods, EF on 1% agarose gel.
[d]As above, without hexane added.

PCR amplification of SSRs

All 6 SSR primers tested gave DNA amplicons of the correct size

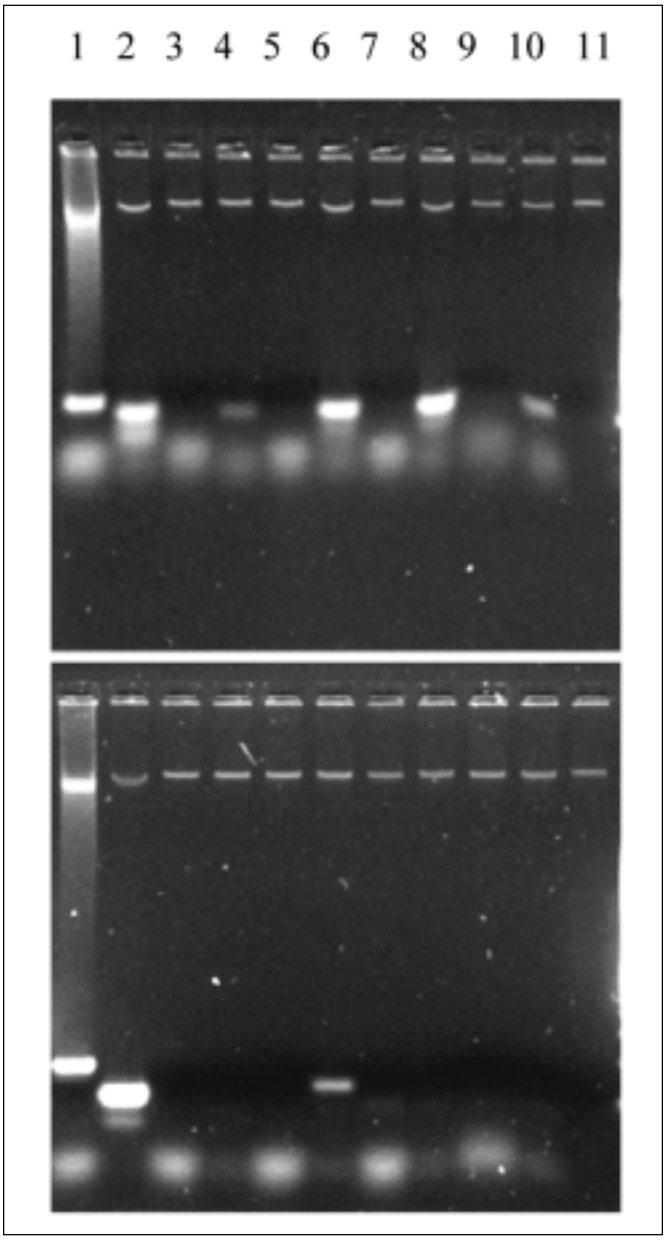

Figure 1—1% agarose gel electrophoresis of PCR products obtained from the olive cultivar "Carolea" with the microsatellites UDO-009 (top) and UDO-043 (bottom). Lanes 1 to 2: leaf sample (control); 3 to 4: oil sample extracted with Promega Wizard kit; 5 to 6: oil sample extracted with Qiagen QIAamp DNA stool kit; 7 to 8: oil sample extracted with LB Link-Biotech ExtMan kit; 9 to 10: oil sample extracted with CTAB; 11 negative control (PCR mix without DNA template). For each pair of lanes, the 1st shows standard PCR and the 2nd, nested PCR. The smear at the bottom of both gels represents a mixture of primers, which are present in all lanes and/or degraded DNA template as well as amplicons of small size produced by the polymerase using short DNA fragments as a template, which increase the intensity of the smear in lanes where standard (= 1st) PCR products were loaded. The irregularity of true bands in the top gel are due to the Sybr green staining.

in unfiltered oil samples for at least 1 extraction procedure (Table 5). The DNA yield of the 1st PCR was sometimes low. In such cases, nested PCR improved the amount of DNA obtained, as shown in Figure 1 and 2. The analysis has now been extended to filtered oils and to oils of cultivars different from 'Carolea,' such as 'Frantoio' and 'Casaliva', and in all cases the results have essentially been the same as those reported for 'Carolea' (data not shown).

Finally, unfiltered oil of 'Carolea' treated or untreated with proteinase K and stored at 4 °C was tested with the Qiagen QIAamp DNA stool extraction kit at 4-mo intervals up to 12 mo after pressing. The analysis was carried out with only 2 primer pairs (UDO-009 and UDO-012) and the results showed that in all cases nested PCR was successful (Table 6).

Conclusions

DNA can be easily found in virgin olive oil immediately after pressing, but only small amounts of DNA can be recovered up to 12 mo after pressing. The amount and quality of DNA can be improved by adding proteinase K to the oil immediately after pressing (Muzzalupo and Perri 2002). Proteinase K inhibits the nucleases that are released into the oil during pressing and are responsible for degradation of DNA in stored oil. Small samples of olive oil can probably be preserved for up to 12 mo for DNA analysis by addition of proteinase K. We have not tested any sample kept for longer periods. A recent paper reports that DNA was extracted and Rapid Amplified Polymorphic DNA (RAPD)-derived Sequence-Characterized Amplified Region (SCAR) markers successfully amplified from olive oil produced the year before the analysis (De la Torre and others 2004).

Our results did not justify using hexane to separate the aqueous from the lipid phases; neither did the use of solutions with high ionic strength or the separation of pellets from the supernatant improve DNA yields. We therefore recommend that extraction protocols be applied directly to oil.

We compared and contrasted a variety of different DNA extraction and cleanup methods routinely used with processed foods and other difficult materials. The amount of DNA extracted from unfiltered and filtered olive oils was low with all methods tested and, in most cases, below the sensitivity of the fluorimeter. Nevertheless, DNA concentrations as low as 4 ng/μL (nominally corresponding to 20 ng DNA used as a template for PCR) were sufficient for consistent amplifications, if DNA was well purified from oil compounds, such as secondary metabolites which could inhibit polymerase activity (Tengel and others 2002; Breton and others 2004, De la Torre and others 2004). Hence, dilution of DNA by using water or buffer in the last step of protocols (elution of DNA from the matrices) could increase the success of PCR amplification.

Use of microsatellite polymorphism to identify the olive cultivars from which oils were obtained appears promising. We have shown that DNA in olive oil, although degraded, has fragments long enough to allow making copies of amplicons up to 188 bp, and this confirms results recently reported by other research groups (Breton and others 2004; Pasqualone and others 2004). Nested PCR, designed to increase the sensitivity of the procedure, also produced detectable fragments when the target template was present in too few copies and the 1st PCR failed to produce scorable or reliable products.

We worked on single-cultivar virgin oils stored at 4 °C after pressing for up to a year and demonstrated that SSRs could be consistently amplified starting from 200 μL oil. Further work is needed to check the sensitivity of the method in detecting the varietal composition of blended oils, especially in detecting alleles from cultivars present in only small amounts. Breton and coworkers (2004), working with mixtures of 2 oils, one of which was below 20% in percent-

Table 5—Influence of DNA extraction procedure on DNA yield and quality as evaluated by suitability as template for 6 SSR PCR-based analyses[a]

Protocol	DNA ng/μL	UDO -008	UDO -009	UDO -012	UDO -024	UDO -039	UDO -043
Promega nWizard kit	2.8 ± 2.2	+	+	0	0	+	0
Qiagen QIA amp DNA stool kit	5.4 ± 0.9	++	++	++	+	++	+
LB Ext Man kit	7.2 ± 1.9	++	++	++	+	0	0
CTAB (control 1)	5.4 ± 2.9	+	+	?	+	0	0

[a]Cold-pressed oil from the cultivar 'Carolea,' results from nested PCRs, electrophoresis on 1% agarose gel. Key: ++ = PCR product of expected size and clearly visible on gel; + = PCR product of expected size, weak band/s; ? = PCR product not consistently scorable; 0 = PCR product not visible on gel.

Table 6—Influence of duration of storage of oil from the cultivar 'Carolea' on the quality of DNA, as measured by successful amplification of 2 SSR loci[a] by nested PCR

SSR/period of oil storage (mo)	0	4	8	12
UDO-009	2/2	2/2	2/2	2/2
UDO-012	2/2	2/2	2/2	2/2

[a]Number of successful PCRs/total number of PCR assays.

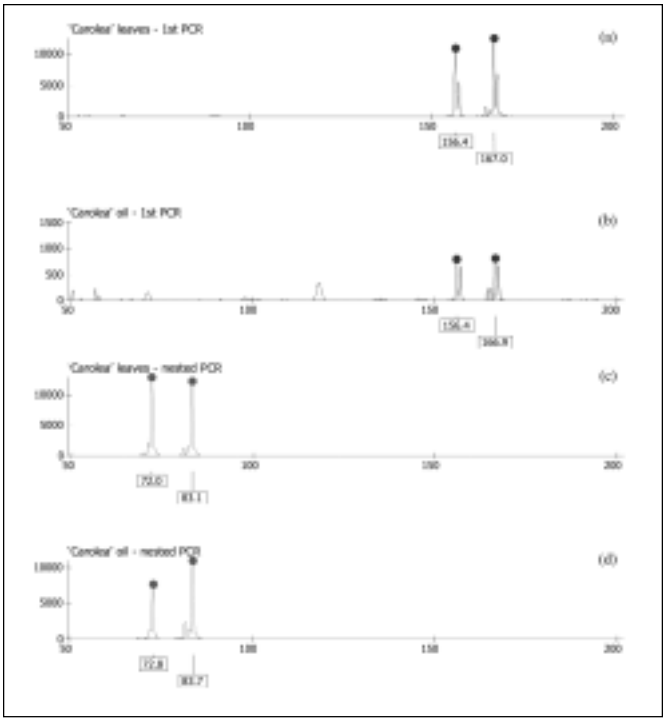

Figure 2—First amplification of the microsatellite UDO-012 from DNA extracted from leaves (a) and oil (b) of the olive cultivar 'Carolea.' Nested amplification of the same microsatellite using internal primers and the 1st PCR product as template: DNA extracted from leaves (c) and oil (d). Peaks corresponding to SSR alleles are marked ●, the corresponding allele size in bp is framed below the x axis in correspondence with the peak. Note the low signal in graph (b), owing to the low amplicon yield of the 1st PCR carried out on DNA extracted from oil. This drawback has been overcome by nested PCR as shown in the graph (d).

age, reported that only in 1 of 12 cases examined, the less represented cultivar was correctly identified using SSR markers. The improvement of the procedure in all steps, from the DNA extraction to the choice of markers, shall make more reliable the identification of the cultivars of origin in oil blends. The adoption of new DNA markers, such as single nucleotide polymorphism (SNP) markers, in which detection makes use of shorter DNA templates (about 20-bp long) could represent even a more promising approach in the future.

In all cases, most foreseen forensic applications of oil fingerprinting do not require the identification of each individual cultivar of the blend. The detection of alien alleles, that is, alleles not compatible with the panel of olive cultivars declared in the blend, could be sufficient for instance as a proof of fraud.

Acknowledgment

This research study was jointly funded by the Ministry of Univ. & Scientific Research and the Ministry of Agriculture of the Italian Government (FISR-MIUR 2000 2.1-VATIPICI Publication nr 18). The authors thank Dr. Silvia Vettore of Promega for her valuable assistance in the improvement of DNA extraction protocols, Dr. Cinzia Guarnieri of Qiagen for kindly providing the kit, and Dr. Enzo Perri of the Istituto Sperimentale per l'Olivicoltura of Rende (CZ), Italy, and Mrs. Laura Turri of the Turri Co. for the oil samples.

References

Breton C, Claux D, Metton I, Skorski G, Berville A. 2004. Comparative study of methods for DNA preparation from olive oil samples to identify cultivar SSR alleles in commercial oil samples: Possible forensic applications. J Agric Food Chem 52(3):531–7.

Cipriani G, Marrazzo MT, Marconi R, Cimato A, Testolin R. 2002. Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. Theor Appl Genet 104:223–8.

Cresti M, Linskens HF, Mulcahy DL, Bush S, Di Stilio V, Xu MY, Vignani R, Cimato A. 1996. Preliminary communication about the identification of DNA in leaves and in olive oil of *Olea europaea*. Adv Hort Sci 10:105–7.

De la Torre F, Bautista R, Cánovas FM, Claros G. 2004. Isolation of DNA from olive oil and oil sediments: application in oil fingerprinting. Food Agric Envir 2(1):84–9.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS. 1991. Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–5.

Lichan E. 1994. Developments in the detection of adulteration of olive oil. Trends Food Sci Technol 5(1):3–11.

Muzzalupo I, Perri E. 2002. Recovery and characterization of DNA from virgin olive oil. Eur Food Res Technol 214(6):528–31.

Newton CR, Graham A. 1997. PCR. 2nd ed. Oxford, U.K.: Bios Scientific Publishers. 192 p.

Pasqualone A, Montemurro C, Caponio F, Blanco A. 2004. Identification of virgin olive oil from different cultivars by analysis of DNA microsatellites. J Agric Food Chem 52(5):1068–71.

Rozen S, Skaletsky HJ. 2000. Primer 3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, editors. Bioinformatics methods and protocols: Methods in molecular biology. Totowa, N.J.: Humana Press. p 365–86.

Tengel C, Schüssler P, Setzke E, Balles J, Sprenger-Haussels M. 2002. Detection of genetically modified soybean and maize in raw and processed foodstuffs. Qiagen News 2:16–8.