Simple sequence repeats amplification: a tool to survey the genetic background of olive oils

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Abstract
A reliable DNA extraction method for use on extra virgin olive oil based on a commercial kit was defined, and the possibility of using this DNA for fingerprinting the original cultivar was demonstrated. The genetic traceability of single-cultivar virgin olive oil from two cultivars (Carolea and Frantoio) was achieved by identifying the varieties from which they were produced. This involved the analysis of DNA sequences using a panel of seven simple sequence repeats (SSRs) to provide genotype-specific allelic profiles. The amplified SSR fragments and the DNA profiles from the monovarietal oil corresponded to the profiles from the leaves of the same cultivar. The most reliable SSR in providing correct allele sizing in distinguishing either single-cultivar olive oil samples or the different ratios of their blends are DCA3, DCA4, DCA16, DCA17, and GAPU101, while DCA9, GAPU59 produced less concordance against data obtained by the genetic analysis of leaf samples. To have reproducible results, PCR product purification and selection of a set of markers with a highly robust amplification pattern is suggested.
Keywords: DNA fingerprinting; Genetic traceability; Olive oil; Simple sequence repeats (SSRs)

INTRODUCTION

Differentiation among olive-tree cultivars is traditionally supported by numerous phenotypic traits related to trunk, leaf, flower and fruit shape (Tous and Romero, 1993). More recently, allozymes have been added as a further tool for discriminating varieties (Ouazzani *et al*., 1993). However, both approaches to identify oils and cultivars are limited by their inability to accommodate the chemical and morphological changes in olive trees and other plants that result from environmental conditions (Massei and Hartley, 2000).

The verification of olive oil authenticity has witnessed significant research to develop methods for characterizing the oils by analyzing their chemical constituents, such as sterols, phenols, fatty acids and alcohols (Bianchi *et al*., 2001; Caponio *et al*., 2001; Guillén and Ruiz 2001; Pasqualone and Catalano 2000). However, the evaluation of DNA nucleotide sequences can provide more precise information, which can be obtained through traditional morphological markers or chemical composition analysis.

Utilization of DNA markers in plants has interesting potential in population genetics, germplasm characterization, analysis of varietal identity and kinship and food traceability (Rafalski *et al*., 1996).

Currently all DNA-based methods for determining the authenticity of food make use of molecular markers that are produced by means of the polymerase chain reaction (PCR). However, there are several technical considerations specific to the use of PCR for amplifying DNA derived from food (Woolfe and Primrose, 2004). For example Sefc *et al*., (2000) described the use of microsatellites in characterizing olive cultivars from various regions of Spain and Italy.

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Since there is little sequence information available for the olive genome (Bautista *et al*., 2003; Hernández *et al*., 2001), most recent studies have been performed using "arbitrary" primers. Some attempts at DNA fingerprinting of olive oils were unsuccessful due to degradation of the isolated DNA, which led to inconsistent electrophoretic patterns (Sanz-Cortés *et al*., 2001; Besnard and Berville´ 2000; Claros *et al*., 2000; Rallo *et al*., 2000). Conversely, the best differentiation has been obtained using specific molecular markers that have been isolated from repeat-enriched genomic libraries (Bautista *et al*., 2003).

Several methods of DNA preparation and immobilization for subsequent sample analysis have been developed. These techniques, utilizing supports such as, silica, hydroxyapatite, magnetic beads and spun columns, enable the DNA to be amplified and analyzed using various amounts of oil. In particular, magnetic beads in conjunction with additional processing have proved useful. However, the defined protocol requires 2 to 40 ml of virgin olive oil and DNA preparation routinely required 5 h (Breton *et al*., 2004).

DNA purified from oil has also been shown to be useful for amplified fragment length polymorphism (AFLP) analysis, a separate DNA technique; the profile of DNA purified from a monovarietal oil has been found to correspond to the DNA profile of leaves of the same cultivar (Pafundo *et al*., 2005; Busconi *et al*., 2003). The AFLP technique is generally regarded as the most reliable and informative fingerprinting procedure for classifying and identifying olive cultivars (Angiolillo *et al*., 1999). However, simple sequence repeats (SSRs) are also very reliable (Hannachi *et al*., 2009; Hannachi *et al*., 2008; Poljuha *et al*., 2008; Rallo *et al*., 2000; Sefc *et al*., 2000), as are restriction fragment length polymorphism (RFLP) (Baldoni *et al*., 1999) and random amplification of polymorphic DNA (RAPD) (Vergari *et al*., 1999; Cresti *et al*., 1997; Fabbri *et al*., 1995).

In the present study, we used SSR polymorphisms to analyze DNA recovered from olive oil and thus survey the genetic background of the oil, i.e., the cultivar(s) from which it was made. In fact, the certification of olive oil leads to the definition of protected denomination of origin (PDO) producing regions in European countries. Hence, PDO products should be protected and a solution could be the use of DNA fingerprinting. In this work, the efficiency of a panel of SSR molecular markers for olive oil varietal identification and their possible use in certification purposes are evaluated.

MATERIALS AND METHODS

Plant and oil sample preparations: Young leaves of olive cultivars Carolea, Coratina, Frantoio, Leccino, and Nocellara were collected from the National Repositories in Follonica (GR, Germplasm Repository) Italy. Cold pressed unfiltered virgin olive oil of Carolea and Frantoio were also obtained from industrial oil mills and stored at 4°C until required. After harvesting of plant tissues, they were frozen in liquid nitrogen and stored at -80°C for later processing.

DNA extraction, quantification and qualification: Commercial kits (LB Link-Biotech ExtMan (Link-Biotech, Bio-technology and Research Company), Promega Wizard (Southampton, England), Qiagen QIAamp DNA stool and Qiagen Plant Mini kit (both from QIAGEN, Hilden, Germany) that are currently used for DNA extraction from food to analyze genetically modified organisms (GMO) were also tested and adapted to the oil samples.

A Hoefer DyNA Quant 200 Fluorometer (Pharmacia Biotech, USA), was used for the accurate quantification of DNA at low concentrations using bisbenzimide dye (Hoechst 33258, Sigma, USA). To test the quality of DNA extracted from oil samples, several microsatellite markers selected from literature were tested by means of PCR amplification and electrophoretic separation. This method of evaluating the quality of DNA was adopted because very small amounts of DNA were recovered from oil samples and it was impossible to estimate the quality of DNA by means of a simple electrophoresis of the samples as they were.

The choice of SSR markers: There are a substantial number of SSRs isolated from olive available in the literature (Carriero *et al*., 2002; Cipriani *et al*., 2002; De La Rosa *et al*., 2002; Rallo *et al*., 2000; Sefc *et al*., 2000) with corresponding demonstrations of their high capacity for discriminating olive cultivars (Bandelj *et al*., 2002; Belaj *et al*., 2004). Five microsatellites of the series ssrOeUA-DCA (ssrOeUA-DCA3, ssrOeUA-DCA4, ssrOeUA-DCA9, ssrOeUA-DCA16 and ssrOeUA-DCA17) isolated by the Wien group (Sefc *et al*., 2000) and two of the GAPU series (GAPU59, and GAPU101) isolated by Agrobios (Carriero *et al*., 2002), were selected for their high polymorphism in olive species, their easily scored patterns and their small-scale stuttering (Table 1).

Table 1. List of seven microsatellites used for olive cultivar identification.

Literature reference	Locus	Motif	Forward 5´ → 3´	Reverse 5´ → 3´	T anneal (°C)
Sefc *et al*., 2000	ssrOeUA-DCA3	$(GA)_{19}$	CCCAAGCGGAGGTGTATATTGTTAC	TGCTTTTGTCGTGTTTGAGATGTTG	50
	ssrOeUA-DCA4	$(GA)_{16}$	CTTAACTTTGTGCTTCTCCATATCC	AGTGACAAAAGCAAAAGACTAAAGC	55
	ssrOeUA-DCA9	$(GA)_{23}$	AATCAAAGTCTTCCTTCTCATTTCG	GATCCTTCCAAAAGTATAACCTCTC	55
	ssrOeUA-DCA16	$(GT)_{13}(GA)_{29}$	TTAGGTGGGATTCTGTAGATGGTTG	TTTTAGGTGAGTTCATAGAATTAGC	50
	ssrOeUA-DCA17	$(GT)_9(AT)_7$ AGATA$(GA)_{38}$	GATCAAATTCTACCAAAAATATA	TAATTTTTGGCACGTAGTATTGG	50
Carriero *et al*., 2002	GAPU101	$(CT)_9$	CATGAAAGGAGGGGGACATA	GGCACTTGTTGTGCAGATTG	57
	GAPU59	$(GA)_8(G)_3(AG)_3$	CCCTGCTTTGGTCTTGCTAA	CAAAGGTGCACTTTCTCTCG	57

Polymerase chain reaction on agarose gels: Amplification of SSR markers by the polymerase chain reaction (PCR) was performed in a 25 µl reaction volume containing 20 ng of DNA, 0.5 µM forward and reverse primers (forward primer was labeled with either the FAM, 6-carboxyfluorescein, or HEX, 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein, fluorescent dye), 200 µM of each dNTPs, 0.5 U *Taq* polymerase (Amersham Biosciences, Piscataway, N.J., USA), 2.5 µl of 10x PCR buffer. Amplifications were carried out using a thermocycler machine, PTC-200 (MJ Research, USA) programmed for 2 min at 94°C, followed by 27 cycles of 45 s denaturation at 94°C, 45s at the annealing temperature reported in Table 1 for each SSR, 1 min extension at 72°C.

Touch down PCR (Don *et al*., 1991) was adopted in several cases to obtain a clearer signal. PCR products were then loaded onto 1% (w/v) agarose gels containing ethidium bromide (1:20000 v/v) or Syber Green (1:10000 v/v), electrophoresed at 1 V/cm for 50 min and photographed under a UV light.

PCRs on capillary sequencer: There are two official and lab-made methods for purification of PCR products before loading in capillary sequencer. The most suggested method of PCR product clean-up (Jakupciak *et al*., 2005) concerns the use of a special plate under vacuum filtration (Montage PCR_{96} Plates, Millipore Corp., Billerica, MA). Seventy-five µl of deionized H_2O were added to each well of the secondary amplification plate to a final volume of 100 µl/well. The contents of each well were then transferred to the Millipore filtration plate and placed on the vacuum manifold for 30 to 45 minutes or until all of the wells were dry. Once dry, 65 µl of deionized H_2O were added to each well. The plate was then placed on a shaker at high speed for 10 minutes.

The lab-made PCR purification was performed using a method based on the precipitation of DNA in ethanol to remove impurities, with a final re-suspension of DNA in sterilized water. For each 10 µl PCR product, 27.5 µl of 96% ethanol and 1 µl of ammonium acetate was added, followed by centrifugation 37000 rpm for 40 min. Samples were then centrifuged for one minute at 270 rpm, bottom-side-up, to eliminate the supernatant. The second precipitation of samples with 70% ethanol (100 µl) was followed by centrifugation at 270 rpm for 2 min. Finally, samples were recovered by re-suspension in 20 µl of sterile water after solvent residues from the samples had evaporated by leaving them for 20 minutes in open air.

One microlitre of desalted PCR product was then mixed with 2.75 µl of loading solution (70% formamide, 1 mM EDTA), 0.25 µl ET-ROX-labeled Et400-R size standard (Amersham Biosciences), and 1.0 µl deionized H_2O, followed by centrifugation at 900 rpm for 2 min, denaturation at 95°C for 4 min, cooling on ice, and analysis on a MegaBACE 500 capillary sequencer (Amersham Biosciences) using Genetic Profiler v2.0 software to estimate allele sizes.

RESULTS

Quality and quantity of DNA extracted from oil: Several standard DNA extraction procedures and commercial kits designed to amplify DNA from foodstuffs suspected of containing GMO were tested to obtain DNA from olive oil samples. Small amounts of DNA were extracted from unfiltered and cotton-filtered olive oil, though in most cases, the DNA concentration was below the sensitivity of the fluorimeter.

Table 2. Comparison between different DNA extraction procedures with regard to DNA quality as evaluated by suitability as template for 7 SSR PCR-based analyses[a].

Protocol	DCA3	DCA4	DCA9	DCA16	DCA17	GAPU-59	GAPU-101
CTAB (control)	+	++	0	0	+	++	+
LB ExtMan kit	+	0	+	+	+	+	+
Promega Wizard kit	+	0	0	+	0	+	+
Qiagen QIAamp DNA stool kit	++	++	0	++	+	++	+

[a] Cold-pressed oil from the cultivar 'Carolea', electrophoresis on 1% (w/v) agarose gel. Key: ++: PCR product of expected size and clearly visible on gel, +: PCR product of expected size, weak bands, 0: lack of amplification.

Considering the data provided in Table 2, the most consistent results were achieved using the Qiagen QIAamp DNA stool extraction kit for the isolation of DNA from olive oil. Other methods had more erratic PCR amplifications. For the isolation of DNA from plant tissue, better results were obtained with the DNeasy Plant Mini Kit (data not shown). Table 3 presents the profile of the five olive cultivars using the 7 SSRs selected from the literature. This database was created as a reference for the analysis of samples of monovarietal oils from the Carolea and Frantoio cultivars.

Identification of olive cultivars in monovarietal oils and blends: Figure 1 shows agarose gel separation of PCR products derived from DNA recovered from olive oil and leaf amplified with the microsatellite DCA9. The amplification of DNA recovered from the oils seems less consistent, with weak bands or lack of amplification indicating that the DNA recovered from the oils still contains some naturally occurring inhibitors and polyphenols. These components, even in picogram (pg) amounts, interfere with the PCR reaction by inhibiting the *Taq* activity and annealing of primer pairs and in turn the DNA chain elongation. In such conditions, either erratic amplification or stuttering may happen. This problem was overcome by improving the purification process through the use of Montage PCR_{96} Plates which eliminated the inhibitors (PCR buffer, salts, polyphenols). To remove polyphenol compounds that interfere with DNA amplification, several techniques are recommended, such as high speed centrifugation and methanol/hexane pretreatment (Rabiei, 2006), or the use of *Candida tropicalis* under metabolic induction in a biodegradation process (Ettayebi et al., 2003); it is important not to shear DNA during these supplementary processes. Optimum results were obtained by ethanol precipitation of DNA accompanied with centrifugation at 37000 rpm (153054.20 g) to remove unwanted compounds after DNA extraction. Using the refined DNA extraction process, it was thus possible to consistently reproduce the DNA profile of the two monovarietal oils (Fig. 2).

In general, most of the SSRs tested with templates obtained from leaves and the corresponding oils of the same cultivar provided at least some consistency in allelic profiles for both leaf and oil-derived DNA templates. Figure 3 presents the results obtained with the primer DCA3, which provided the best fluorescent detection of DNA extracted from oil. Additionally, the amplicons were of the same size in both leaves and oils. Also shown in Figure 3, are the allelic SSR pat-

Table 3. PCR amplification of 7 SSRs from leaf samples of five olive cultivars.

Cultivar/SSR	DCA3	DCA4	DCA9	DCA16	DCA17	GAPU59	GAPU101
Carolea	232-254	132-166	164-199	127-155	115-177	214-220	193-218
Coratina	239-243	132 [a]	182-194	150-172	117	214	199-218
Frantoio	238-244	132-134	183-207	151-157	117-143	210-214	185-199
Leccino	242-252	132-134	163-207	152-176	109-119	210-214	199-201
Nocellara	242-248	188-190	163-173	152-176	117-145	214	201-207

[a] Profiles with a single allele mean homozygosity or the presence of a null allele, which cannot be ascertained without segregation analysis.

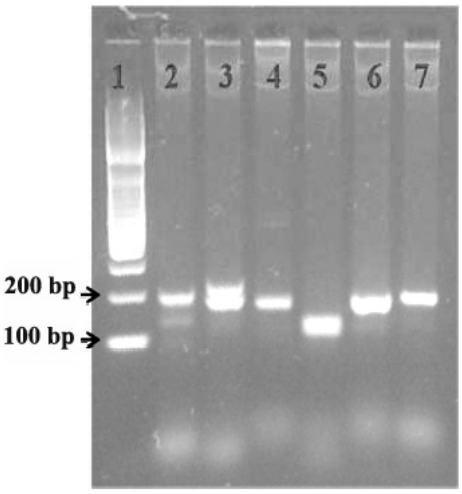

Figure 1. Agarose gel separation of PCR products derived from DNA recovered from olive oil and leaf amplified with the micresatellite DCA9. Lane 1 is 100-bp ladder DNA (bio-rad Richmond, USA); lane 2 Carolea leaf; lane 3 Frantoio leaf; lane 4 Frantoio oil; lane 5 Carolea oil; lane 6 Carolea and Frantoio oil (1:10); lane 7 Carolea and Frantoio oil (10:1).

terns resulting from oil blends made with two different cultivars at different ratios (1:1, 1:2, 1:5, 1:10). It should be noted that all SSRs studied had a high annealing temperature, 50-57°C, which helped in obtaining more specific amplifications. The technique of SSR amplification was well suited to the identification of the cultivar of origin in monovarietal virgin oils. For oils blended from different cultivars, such as with PDO products, the numbers of cultivars present in the oil and their ratios in the mixture could limit the correct interpretation of the SSR allelic patterns and the attribution of a given allele to the right variety.

DISCUSSION

The greatest challenges in using DNA technology in oil characterization is the low quality and high-level degradation of the DNA recovered from the fatty matrices, along with the impact of oil extraction processing on the size of the DNA fragments recovered. Several supports have been shown by Breton *et al*. (2004) to retain DNA from variable amounts of oil such as silica extraction, hydroxyapatite, magnetic beads, and spun column. Doveri *et al*. (2008) proposed using the official Swiss method for lecithin and oil DNA extraction, while Pafundo *et al*. (2005) tried several methods such as those proposed by Busconi *et al*. (2003) and Palmieri (2003) and cetyl-trimethyl ammonium bromide (CTAB). In all cases the quantity of extracted DNA from olive oil was too low to be visualized on the agarose gel. However, unlike some protocols, the quantity of DNA is not a crucial factor. Having quality DNA, even in small amounts, is more

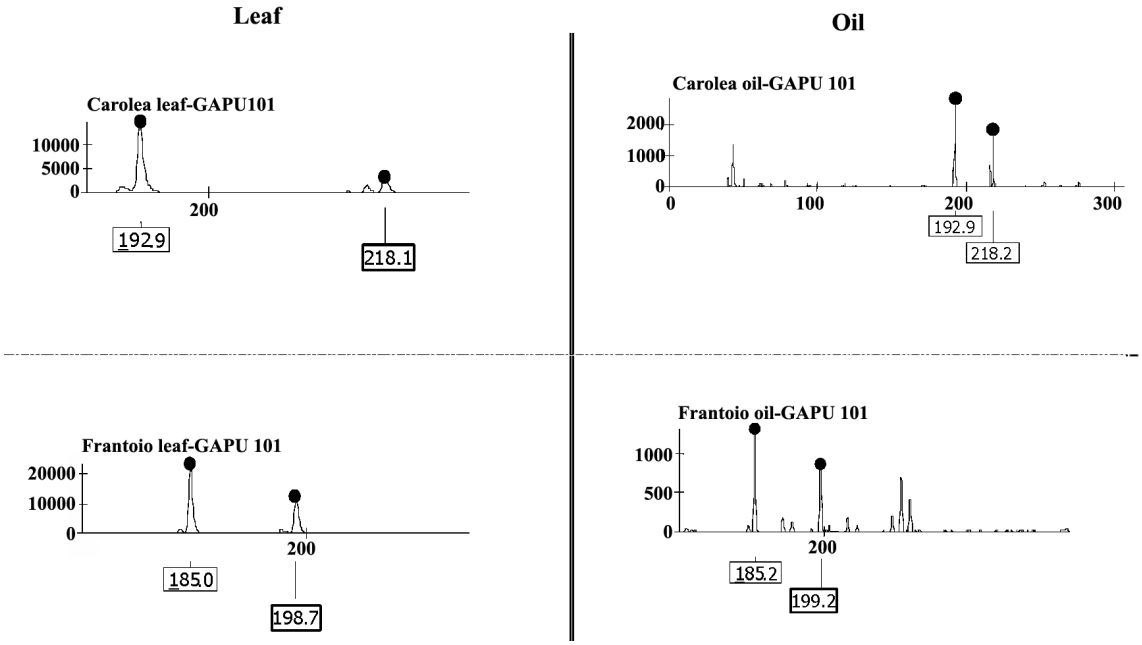

Figure 2. Electropheregram of PCR products separated by capillary electrophoresis of microsatellite locus GAPU 101, obtained from DNA extracted from Carolea and Frantoio leaves and oils. Allele sizes are below the x axis. The scores on Y-axis are the intensity of amplified allele detection.

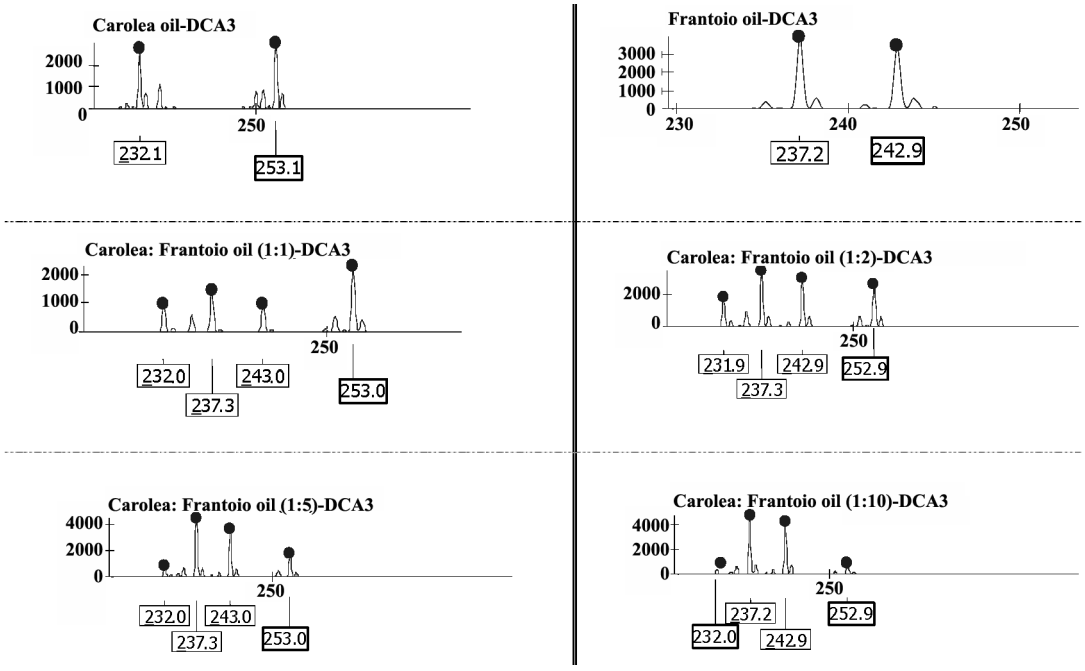

Figure 3. Electropheregram of PCR products separated by capillary electrophoresis of microsatellite locus ssrOeUA-DCA3 obtained from recovered DNA of Carolea and Frantoio oils and mixtures of the two at 1:1, 1:2, 1:5, 1:10 ratios. Allele sizes are below the x axis. The scores on Y-axis are the intensity of amplified allele detection.

beneficial than higher quantities of low grade DNA that does not amplify. Thus, the main criteria in choosing the best DNA extraction method is the ability of the method to isolate DNA free of the natural inhibitors (polyphenols, …) present in the samples. For amplification of the recovered DNA, we used SSRs mainly because they are short sequences, usually 100-200 bp long including the flanking regions (Table 1). This helps to overcome the problem of DNA degradation that occurs due to the presence of free nucleases (De la Torre *et al*., 2004; Muzzalupo and Perri 2002). Short tandem repeat markers were also selected because they are locus specific and co-dominant, allowing any peak produced by the PCR amplification to be correctly interpreted.

In examining the results, the microsatellite profiles obtained with the monovarietal oil-derived DNA were generally consistent with the cultivar used (DCA3, DCA4, DCA16, DCA17), although some ambiguities were recorded (such as GAPU59). These ambiguities are likely due to contamination of monovarietal oils by other cultivars grown in the same block or by contamination occurring at the mill (the presence of some traces of olive drupes from the former lot). It must also be considered that in some cases the lack of matching in leaf and oil profiles may have been caused by the presence of embryos in berry seeds that included the alleles of pollinators, as most cultivated olive are self-incompatible and the flowers are wind pollinated (Doveri and Lee 2007). Alba *et al*. (2009) defined the possible presence of additional alleles due to paternal contribution in oils extracted from entire drupes (as it happened in the experiments of this study).

Other cases of mis-amplification were recorded as a missing allele, due either to the preferential amplification of one of the two alleles in oil-derived DNA templates, or to excessive degradation of the DNA template of the missing allele, which would have prevented the production of a sufficient number of copies to be detected.

In testing a mixture of two monovarietal oils at different ratios (1:1, 1:2, 1:5 and 1:10 v:v), the analysis was less reliable due to several problems as described by Breton *et al*. (2004). The first problem arose from the fact that one or more peaks were missing not only at the extreme ratio of 1:10, where one cultivar was minimally represented, but also at more balanced ratios. The most likely explanation for such a phenomenon is the differential amplification of the alleles, an occurrence well known in PCR (Reysenbach *et al*.,

1992). In general, PCR preferentially amplifies shorter alleles, providing fewer copies of longer alleles and a resulting lower signal. However, this is not always true and for the latter cases, few convincing arguments are available in the literature. In this study, DCA3 and DCA17 provided a concordant pherogram of both pairs of alleles from the two varieties included in the blend, even at the 1:10 ratio. Others markers, like DCA9 and GAPU101, reproduced the allele combinations in many cases, but were limited by a low intensity of several signals. GAPU59 provided the least reliable results due to the presence of unknown picks, stuttering and a complete void of amplification in some instances (data not shown).

Overall, the technique of SSRs amplification applies rather well to identifying the cultivar of origin in monovarietal virgin oils. For blended cultivar oils such as PDO products, the number of cultivars presents in the sample and their ratio in the mixture would limit cultivar identification. Therefore, to have reproducible results, selection of a set of markers of highly robust amplification patterns is suggested. The present research offers a number of molecular tools suitable for proving olive oil authenticity. These tools provide a reliable protocol for DNA extraction from cold pressed olive oil and protocols to recognize 'the genetic background' of the oil by means of SSRs amplification.

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