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Applicability of SCAR Markers to Food Genomics: Olive Oil Traceability

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DNA analysis with molecular markers has opened a shortcut toward a genomic comprehension of complex organisms. The availability of micro-DNA extraction methods, coupled with selective amplification of the smallest extracted fragments with molecular markers, could equally bring a breakthrough in food genomics: the identification of original components in food. Amplified fragment length polymorphisms (AFLPs) have been instrumental in plant genomics because they may allow rapid and reliable analysis of multiple and potentially polymorphic sites. Nevertheless, their direct application to the analysis of DNA extracted from food matrixes is complicated by the low quality of DNA extracted: its high degradation and the presence of inhibitors of enzymatic reactions. The conversion of an AFLP fragment to a robust and specific single-locus PCR-based marker, therefore, could extend the use of molecular markers to large-scale analysis of complex agro-food matrixes. In the present study is reported the development of sequence characterized amplified regions (SCARs) starting from AFLP profiles of monovarietal olive oils analyzed on agarose gel; one of these was used to identify differences among 56 olive cultivars. All the developed markers were purposefully amplified in olive oils to apply them to olive oil traceability.

KEYWORDS: Food genomics; AFLPs; SCAR markers; olive oil traceability

INTRODUCTION

DNA analysis with molecular markers has offered a shortcut to a fast genomic description of complex organisms. A similar approach has been successfully applied to the genomic identity of "ancient DNA" and DNA traces in forensic analyses (*1*, *2*). The availability of micromethods for DNA extraction (*3*) and of molecular markers targeting sequences, which can be unequivocally attributed to a given species, could be instrumental to a scientific description of food composition. Food DNA analysis may represent an attractive and alternative choice to the more classical analytical methods, because DNA, rather than the macromolecules and metabolites, is less influenced by environmental and processing conditions (*4*).

Olive oil is a liquid food whose nutritional and health values are continuously discovered and emphasized, and the European Union is worldwide the first olive oil producer, marketing about 80% of world oil. To protect the geographical origin and the quality of olive oil, the European Union has promulgated various regulations concerning the classification of olive oils (Reg. EC n. 1989/03) (*5*), its commercialization and labeling (1019/2002) (*6*), and the definition of geographical origin (2081/92) (*7*). The latter has prompted the appearance of certificated products, referred to as protected designation of origin (PDO) and protected geographical indication (PGI). As for many high value products, PDO and PGI oils are subject to fraudulent practices, such as by admixture with other plant oils, such as cheaper olive oils or olive pomace oil, or by the use of less intensive production methods (*4*). Because genotype is such an important determinant in PDO and PGI, labeled methods to identify or confirm their plant composition (the varieties present in a batch of olive oil) are critical for the validation of product conformity.

Since the discovery of amplifiable DNA from olive oil (*3*, *8*−*11*), different molecular markers were used to target DNA in the attempt to recognize the cultivar employed for the production (*3*, *8*, *9*, *12*). In general, the approach utilized was that of transferring molecular marker information gained on olive trees to olive oil. However, DNA extracted from olive oil is highly degraded and contaminated with inhibitors of PCR reactions, which may limit the applicability of molecular markers to internal traceability. An alternative approach could be that of isolating molecular markers directly within the DNA of a monovarietal oil that can be used to construct a molecular fingerprint comparable with that of its cultivar of origin. This approach offers the advantage of finding sequences which are easier to amplify by PCR in DNA of oil than sequences taken from DNA of leaves, which are good for leaves but gave poor results with olive oil.

Pafundo et al. (*8*) were able to obtain an AFLP (amplified fragment length polymorphism) profile of olive oil with a

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similarity of about 70% with the AFLP profile of leaves of its respective cultivar. It was also shown that the extraction of DNA from olive oil is the limiting step in this approach. In fact, some differences were found in fingerprints of DNA extracted from the same oil in different times, even though some AFLP fragments were highly reproducible.

Because AFLP technology is a difficult application for degraded and contaminated DNA, the development of sequence characterized amplified region (SCAR) markers (*13*−*15*) derived from AFLP profile of olive oil can be instrumental to simplify the determination of varietal composition of an oil sample. The development of SCAR markers and their use for the characterization of olive germplasm were previously reported by Hernández et al. (*16*), showing how they can be visualized through ethidium bromide staining and can be applied to multiplexed PCR and microarray platforms.

The present study reports the development of SCAR markers directly derived from AFLP profiles of olive oil. To avoid the usual procedure for SCAR isolation from polyacrylamide gel electrophoresis, expensive, time-consuming, and requiring the use of radioactive isotopes, a procedure to visualize AFLPs of oil in agarose gel was developed. Moreover, also the use of silver staining, for polyacrylamide gel electrophoresis, limits the possibility to sequence the recovered fragments. From the AFLP sequences, SCAR markers were obtained, and fragments of the expected length were amplified from DNA extracted both from plant material and oils. These SCAR markers could be used to produce a PCR platform to identify the cultivars contributing to an olive oil. This same approach could be applied to traceability and labeling of other complex food matrixes.

MATERIALS AND METHODS

Figure 1 is a scheme of the entire procedure used to obtain SCAR markers from AFLP profiles of monovarietal oil.

Plant Material. Leaves and drupes of *Olea europaea* cultivars from different countries (**Table 1**) were collected in the year 2002 from single plants and were used for DNA extraction and for oil production. The extravirgin monovarietal oils were produced in the country of origin, following the standard methods employed in oil factories. Leaves were stored at −20 °C, while oils were maintained at room temperature in the dark until DNA extraction.

DNA Extraction from Leaves and Monovarietal Olive Oils. Genomic DNA was extracted from fresh leaves by using the method previously established for olive (*17*). DNA from olive oils was extracted with the method developed by Palmieri (*18*) with the following modifications: the incubation at 48 °C was performed for 2 h and the incubation at −80 °C was performed overnight.

AFLP Analysis. The AFLP analysis was carried out as described previously (*8*), using the primer combinations *Eco*RI-AAA/*Mse*I-CAA and *Eco*RI-AAA/*Mse*I-CAC. *Eco*RI primer was labeled with the fluorescent dye Cy5.5. Amplified products were loaded on the automatic sequencer CEQ 2000 XL (Beckman-Coulter, Fullerton, CA), using the following parameters: capillary temperature 50 °C, denaturation temperature 90 °C for 120 s, injection voltage 2 kV for 30 s, and separation voltage 6 kV for 50 min. The electropherograms were analyzed with Fragment Analysis of CEQ 2000 DNA Analysis System Software and with Genographer Software (*19*).

Gel Electrophoresis and Fragment Recovery from AFLP. The amplification products were separated by electrophoresis on 4% (w/v) high-resolution and low-melting NuSieve GTG agarose (Cambrex, East Rutherford, NJ) in 1× TAE buffer (40 mM Tris-Acetate, 1 mM EDTA), stained with ethidium bromide (EtBr) (0.004% w/v). The electrophoresis was carried out at 50 V for 30 min and then at 90 V for 30 min in the dark. The gel was then stained with EtBr. Fragments were visualized under UV light, and their sizes were calculated by comparison to a 100 base pair molecular-size marker (Amersham Biosciences, Piscataway, NJ), using Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). Finally, chosen AFLP bands (**Table 2**) were purified with the GFX PCR DNA and band purification kit (Amersham Biosciences).

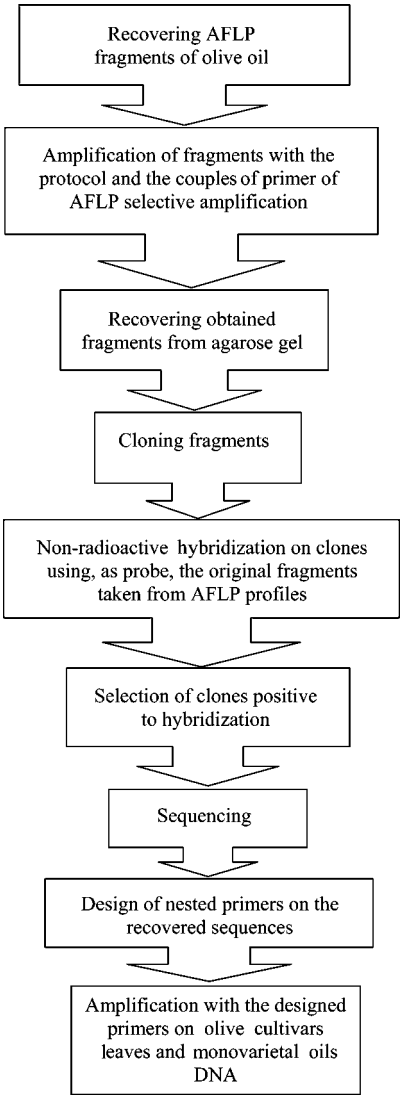

Figure 1. Flowchart of the procedure used to develop SCAR markers from AFLP profiles of monovarietal olive oils.

Cloning and Sequencing of AFLP Fragments. Purified DNA fragments were initially amplified with the same pairs of primers used in the selective amplification for AFLP. Using the pGEM-T Easy Vector System (Promega, Madison, WI), the reamplified fragments were bound into the pGEM vector, and the ligation products were used to transform the JM109 high-efficiency competent cells. To confirm that cloned bands were those taken in origin from AFLP profiles, nonradioactive colony hybridization was carried out using as probe the original fragment purified from AFLPs. Hybridization was done by using the ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences). The colonies that gave a positive signal were chosen, and the cloned fragment was sequenced. To do this, plasmid DNA was extracted from the transformed cells with the Wizard Plus SV Mini-Preps DNA Purification Kit (Promega) and then was digested with Eco*RI* to verify the presence of the fragment and to check for its length. Three positive clones for each fragment were chosen and sequenced, with the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman-Coulter), using M13 universal primers. The three sequences of each fragment were aligned and compared using the program CLUSTAL-W (www.embl-ebi.com). Nucleotide BLAST searches were carried out for similarities between fragment sequences and those present in databases, using the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). BLAST parameters were set to default values.

Table 1. Panel of Olive Cultivars and Relative Oils Considered in This Work[a]

cultivar	country of origin	test with CP-rpl16T and G219/172H primers	test with G230/162T primers	available oils
Adramytini	Greece	yes		
Aglandau	France	yes		
Arbequina	Spain	yes	yes	yes
Azeiteira	Portugal	yes		
Biancolilla	Italy	yes		
Blanqueta	Spain	yes		
Blanqueta	Portugal	yes		
Bosana	Italy	yes		
Bouteillan	France	yes		
Cailletier	France	yes		
Canino	Italy	yes		
Carolea	Italy	yes		yes
Carrasquenha	Portugal	yes		
Cellina di Nardò	Italy	yes	yes	yes
Changlot Real	Spain	yes		
Chemlali	Tunisia	yes		
Cobrançosa	Portugal	yes		
Coratina	Italy	yes	yes	yes
Cordovil de Serpa	Portugal	yes		
Cornezuolo	Spain	yes		
Dolce Agogia	Italy	yes	yes	
Dritta	Italy	yes	yes	
Farga	Italy	yes		
Frantoio	Italy	yes	yes	yes
Galega	Portugal	yes		
Gentile di Chieti	Italy	yes	yes	yes
Ghjermana	France	yes		
Hojiblanca	Spain	yes	yes	yes
Koroneiki	Greece	yes		
Leccino	Italy	yes	yes	yes
Lechin de Granada	Spain	yes		
Madural	Portugal	yes		
Manzanila Cacerena	Spain	yes		yes
Manzanila de Jean	Spain	yes		
Memecik	Turkey	yes		
Moraiolo	Italy	yes		yes
Nocellara del Belice	Italy	yes	yes	yes
Ogliarola Leccese	Italy	yes		yes
Ottobratica	Italy	yes	yes	yes
Petit Ribier	France	yes		yes
Picholine	France	yes		yes
Picholine Morocaine	Morocco	yes		
Picual	Spain	yes		yes
Picudo	Spain	yes		
Redondal	Portugal	yes		
Redondil	Portugal	yes		
Sabina	France	yes		
Salonenque	France	yes	yes	yes
Sigoise	Algeria	yes		
Sinopolese	Italy	yes		
Tanche	France	yes	yes	yes
Tonda Iblea	Italy	yes	yes	yes
Verdeal	Portugal	yes		
Verdial de Huevar	Spain	yes		
Verdale de l'Hérault	France	yes		
Zinzala	France	yes		

[a] Reported are the cultivars and oils tested with the primers designed on AFLP recovered fragments.

The nucleotide sequence data reported in this paper appeared in the DDBJ/EMBL/GenBank nucleotide sequence databases with the following accession numbers: AB286868 for the sequence "AFLP-2OilT"; AB286869 for the sequence "AFLP-CVH"; AB286870 for the sequence "AFLP-333OilT"; and AB286871 for the sequence "AFLP-1OilT".

Nested PCR in Olive Leaves and Oils. On the basis of sequences obtained, specific primers were designed for nested PCR in DNA from olive leaves, using the Primer Express v.2.0 software (Applied Biosystem division of Perkin-Elmer Corp., Foster City, CA). The primer sequences are listed in **Table 3**.

Table 2. Characteristics of AFLP Fragments Chosen to Develop Scar Markers

fragment name	length	AFLP selective primers combination	origin
AFLP-CVH	253 bp	*Eco*RI-AAA/*Mse*I-CAA	Hojiblanca leaves
AFLP-333OilT	333 bp	*Eco*RI-AAA/*Mse*I-CAC	Tanche oil
AFLP-1OilT	378 bp	*Eco*RI-AAA/*Mse*I-CAA	Tanche oil
AFLP-2OilT	253 bp	*Eco*RI-AAA/*Mse*I-CAA	Tanche oil
AFLP-3OilT	139 bp	*Eco*RI-AAA/*Mse*I-CAA	Tanche oil
AFLP-5OilS	333 bp	*Eco*RI-AAA/*Mse*I-CAA	Salonenque oil
AFLP-6OilS	253 bp	*Eco*RI-AAA/*Mse*I-CAA	Salonenque oil
AFLP-7OilH	329 bp	*Eco*RI-AAA/*Mse*I-CAA	Hojiblanca oil
AFLP-8OilH	253 bp	*Eco*RI-AAA/*Mse*I-CAA	Hojiblanca oil

Table 3. Characteristics of Primers for Nested PCR

name of original AFLP fragments	length of AFLP fragments	name of primers for nested PCR	primer sequence (5–3); F: forward, R: reverse	expected length of amplicon
AFLP-CVH	253 bp	G219/172H	F: GTCAATGTGGCATTTCGTCG R: AATTTCGAGCTTCATTTT-ACCGTT	172 bp
AFLP-333OilT	333 bp	Cp-rpl16T	F: CCCAAAAGAACCAGATTC R: TCGCGAGAGCCTTTACCTGA	262 bp
AFLP-2OilT	230 bp	G230/162T	F: AATCACTTGAATGCCCACGTG R: CAAGGGTGTGAGCGACTGTTC	162 bp

The amplifications were carried out in a final volume of 20 μL starting from 20 ng of DNA in a Robocycler thermal cycler (Stratagene, La Jolla, CA). For each olive and leaf sample, three replicates of the amplification were done.

Amplifications with the primers CP-rpl16T were carried out in the presence of 1× PCR buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$ pH 8.3, 20 mM $MgCl_2$), 0.1 μM of forward and reverse primers, 0.3 mM dNTPs, and 1U JumpStart RED AccuTaq La DNA polymerase (Sigma, St. Louis, MO) at the following conditions: 95 °C for 5 min; 40 cycles at 95 °C for 50 s, 58 °C for 50 s, 68 °C for 1 min; and 68 °C for 15 min.

The amplifications with the primers G219/172H and G230/162T were carried out in the presence of 1× PCR buffer (500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$ pH 8.3, 20 mM $MgCl_2$) (Sigma), 1 mM $MgCl_2$ (Sigma), 0.1 μM of primers, (0.4 μM for G230/162T), 0.5 mM dNTPs, and 1.2U JumpStart RED AccuTaq La DNA Polymerase (Sigma) at the following conditions: 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 59 °C for 45 s, 68 °C for 1 min; and 68 °C for 20 min.

The amplification products were analyzed by capillary and gel electrophoresis and were recovered and purified from the agarose gel as described above. They were sequenced using CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman-Coulter), following the protocol for sequencing PCR products. The sequences were aligned and compared with the specific original AFLP derived fragment, using the program CLUSTAL-W.

RESULTS

Comparison between Olive Oil AFLP Profiles Visualized through Capillary Electrophoresis and Agarose Gel. AFLP profiles of four monovarietal oils (obtained from cultivars Tanche, Salonenque, Hojiblanca, and Arbequina) and of leaves of cultivar Hojiblanca were obtained with a protocol described by Pafundo et al. (*8*). Cultivar specific DNA extracted from leaves was utilized as reference material to value the reliability of the approach throughout. After the fingerprints were visualized on automatic sequencer, AFLP fragments were separated using a high-resolution/low-melting agarose gel that finely resolves PCR products with sizes ranging from 10 to 1000 bp. **Figure 2** reports the comparison among three different analytical methods applied to AFLPs in monovarietal oil Tanche: (1)

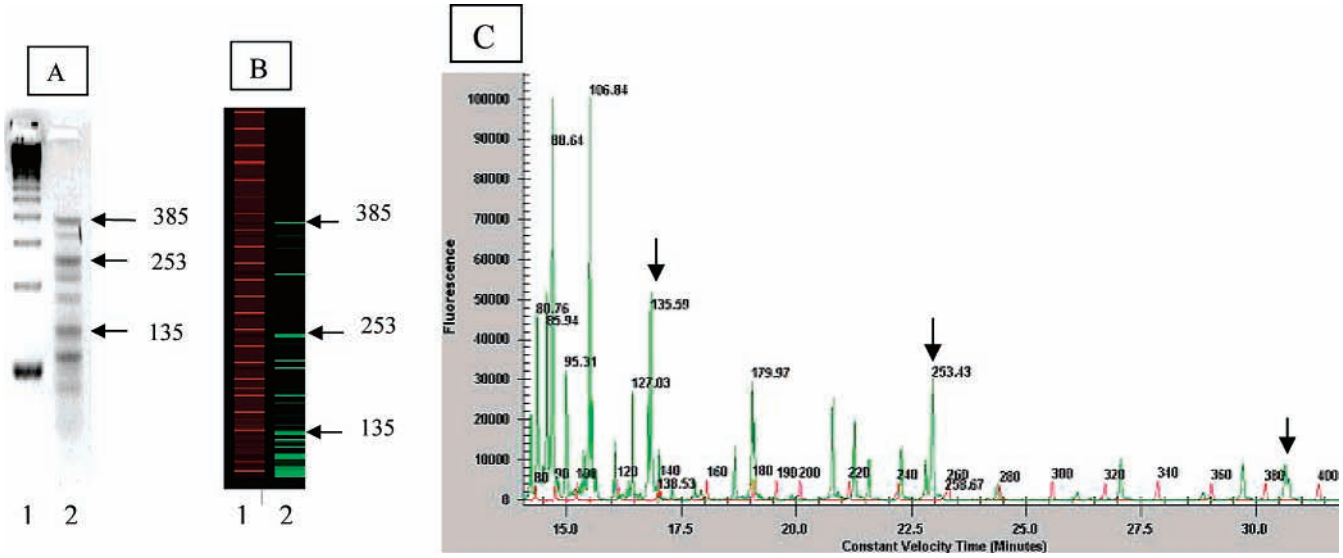

Figure 2. Comparison of detection methods for AFLP profile of Tanche oil obtained with primer selective combination *Eco*RI-AAA/*Mse*I-CAA. (**A**) Agarose gel, lane 1: 100 base pair ladder; lane 2: Tanche oil. (**B**) Virtual gel obtained with the software Genographer, lane 1: Check 600 bp ladder; lane 2: Tanche oil. (**C**) Capillary electrophoresis, check 600 bp ladder (basal line). In all figures, the arrows mark the fragments of 135, 253, and 385 bp starting from the bottom in **A** and **B** and from the left in **C**.

electropherogram resulting from capillary electrophoresis; (2) virtual gel obtained by processing electropherogram with Genographer software; and (3) electrophoresis on agarose gel. A good correspondence of the profiles obtained with the different analytical methods was found both for the molecular weights and for the intensity of bands and peaks. In fact, some of the most intense bands corresponded to the highest peaks of the capillary electropherogram. A good fragment separation was obtained both with capillary electrophoresis and agarose gel, both with a low background signal. Similar results were obtained with other oils (data not shown). Fingerprints obtained from leaf DNA presented a smear on the gel, because they possessed many bands with fragments of similar lengths, and so it became very difficult to separate them in agarose gel. Therefore, only few bands characterized by a high fluorescent signal intensity were resolved and recovered. As reported previously (*8*), both common bands to all the oils analyzed and specific ones were found in AFLP profiles.

Cloning and Sequencing of AFLP Bands from Olive Oils and Leaves. Hellebrand et al. (*20*) reported for rapeseed oil that fragments longer than 300 bp were hardly amplifiable. A similar result was found by Pafundo et al. (*8*) who reported that, in AFLP profiles of olive oil, fragments longer than 350 bp did not show up. For this reason, specific primers for olive oil DNA were designed on internal sequences of selected fragments to produce shorter amplicons that can be more easily amplified. These primers were further used to amplify DNA extracted from leaves of a broad set of olive cultivars to determine any possible polymorphism in the fragments isolated.

Eight distinguishable bands taken from the oil profiles and one taken from the profiles of Hojiblanca leaves were chosen to develop the food genomic molecular marker approach. The criteria used for this selection were (1) intensity of bands in the gel, because it can be related to their abundance in the sample and to difficulty in recovering the fragments; (2) their length, because longer fragments can be more informative than the shorter ones; (3) their reproducibility in DNA extractions from the same oil at different times; and (4) the absence of bands near those of interest that can lead to copurification of other fragments. In this way, we found three bands specific for Tanche oil (AFLP-1OilT, AFLP-3OilT, and 333OilT), one specific for Hojiblanca oil (AFLP-7OilH), and one specific for Salonenque oil (AFLP-5OilS). Other bands (AFLP-333OilT, AFLP-5OilS, AFLP-2OilT, AFLP-6OilS, AFLP-8OilH) showed the same size in all oils analyzed; however, they were equally chosen because fragments of the same size did not have necessarily the same sequence. A distinct band was selected from the complex AFLP profile of the Hojiblanca leaves (AFLP-CVH) (**Table 2**). **Figure 3** reports an example of AFLP profiles of monovarietal oils on agarose gel and some interesting fragments taken for further analyses.

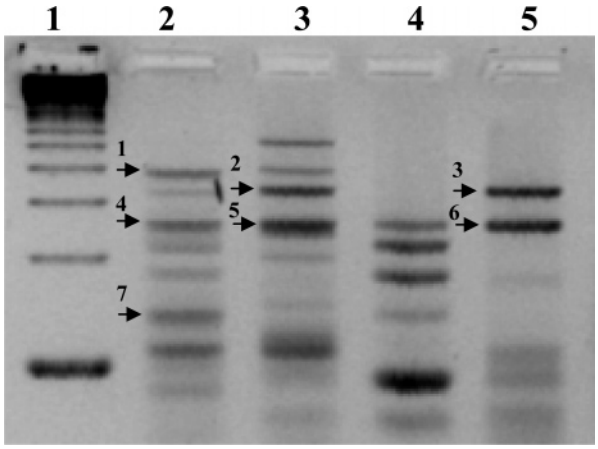

Figure 3. AFLPs obtained with primer selective combination *Eco*RI-AAA/*Mse*I-CAA of oil DNA in agarose gel. Lane 1: 100 base pair ladder; lane 2: Tanche oil; lane 3: Salonenque oil; lane 4: Arbequina oil; lane 5: Hojiblanca oil. The arrows indicate the size of some bands considered to develop SCAR markers (1: 378 bp; 2: 333 bp; 3: 329 bp; 4: 253 bp; 5: 253 bp; 6: 253 bp; 7: 139 bp).

All these fragments, recovered from agarose gel, were amplified with the same fluorescent pairs of primers used in AFLP selective amplification, and the amplicons were loaded on high-resolution/low-melting agarose gel. In this way, the verified conditions were that (1) the original fragment was reamplifiable; (2) it had exactly the length of the original fragment; and (3) it was unique, because no other fragment with very similar molecular weight could be copurified. After these

screenings, several fragments (AFLP-5OilS, AFLP-3OilT, AFLP-6OilS, AFLP-7OilH, AFLP-8OilH) were excluded from further analyses because they were not reamplified or they gave either fragments of different length with respect to the original or multiple bands.

Therefore, AFLP-CVH, AFLP-333OilT, AFLP-1OilT, and AFLP-2OilT were considered in developing the new markers. To confirm that their sequences corresponded with the fragments taken from fingerprints, the fragments were cloned, and a nonradioactive hybridization test was performed, using as probe the same AFLP fragment. Sequencing all the cloned PCR products obtained from reamplification showed that they were identical to the original AFLP fragments and that these fragments were unique. The program BLAST-N showed that AFLP-333OilT had a high homology (93%) with a region across the intron and exon 2 of the chloroplastic ribosomal gene L16 of *Nicotiana tabacum* and *Atropa belladonna* (*21*), AFLP-CVH and AFLP-2OilT were partially homologous to plant genomic regions, whereas AFLP-1OilT was homologous to a gene of the fungus *Gibberella zeae*, and so it was excluded from successive analyses.

Sequences have been submitted to the database DDBJ (http://www.ddbj.nig.ac.jp/).

Development of SCAR Markers in Olive Oils. Specific primers for selected fragments were used to amplify their internal regions. The name assigned to these primers and the length of the expected amplicons are reported in **Table 3**. All primers were first tested on DNA extracted from a few samples of leaves and oils to verify that amplification worked in both conditions. All amplifications yielded fragments of the expected length (data not shown). Amplifications were then extended to 56 cultivars of leaf DNA with primers G219/172H and CP-rpl16T and to 14 cultivars of leaf DNA with primers G230/162T. The results obtained in leaves were compared with those of monovarietal oils, in particular, with 19 monovarietal oils with primers G219/172H and CP-rpl16T and with 14 monovarietal oils with primers G230/162T (**Table 1**). The amplicons were analyzed on capillary electrophoresis to evidence small differences in size and because of the small quantity of some amplified products, which could not be visible on traditional agarose electrophoresis.

G219/172H primers gave a unique fragment with the expected length of 172 bp, monomorphic in all the cultivar leaves (data not shown).

CP-rpl16T primers produced polymorphic fragments able to recognize four groups of cultivars: 1, with the expected fragment of 262 bp; 2, with a smaller fragment of 109 bp; 3, with both fragments; 4, with no fragment (**Table 4** and **Figure 4A**,**C**,**D**). The height of the peaks corresponding to these fragments in the electropherograms varied; however, the intensity of the peak of 109 bp was generally lower than that of 262 bp (**Figure 4C**,**D**). Therefore, primer pair CP-rpl16T was able to discriminate some groups of cultivars. The fragment of 109 bp was sequenced, and it did not have significant homologies with the fragment of 262 bp.

With primers G230/162T, the expected fragment with length of 162 bp was found in all the cultivars analyzed, except Ottobratica and Gentile di Chieti (**Figure 4E**,**G**). Some cultivars presented other relevant peaks with lengths ranging from 300 to 500 bp (**Figure 4E**). Some of these fragments were sequenced and resulted differently from each other. Probably, these primers annealed to different regions on olive genome, so they can produce multiple amplifications. However, these fragments could also be used to develop SCAR markers using internal specific primers eventually, but they were not considered in this work.

Table 4. Classification of Cultivars on the Basis of Amplification Products Obtained with CP-rpl16T Primers

group 1 fragment of 262 bp	group 2 fragment of 109 bp	group 3 fragments of 109 bp and 262 bp	group 4 no fragments
Adramytini	Canino	Carrasquenha	Aglandau
Blanqueta	Changlot Real	Dolce Agogia	Arbequina
Bosana	Chemlali	Frantoio	Azeiteira
Bouteillan	Dritta	Leccino	Biancolilla
Cailletier	Galega	Sinopolese	Blanqueta (Portugal)
Carolea	Hojiblanca	Tonda Iblea	Cellina di Nardò
Coratina	Picholine	Verdial de Huevar	Cobrançosa
Farga			Cordovil de Serpa
Gentile di Chieti			Cornezuolo
Ghjermana			Koroneiki
Lechin de Granada			Redondal
Madural			Redondil
Manzanila Cacerena			Sabina
Moraiolo			Manzanila de Jean
Nocellara del Belice			Memecik
Ogliarola Leccese			
Ottobratica			
Petit Ribier			
Picholine Morocaine			
Picual			
Picudo			
Salonenque			
Sigoise			
Tanche			
Verdeal (Portugal)			
Verdale de l'Hérault (France)			
Zinzala			

All the monovarietal oils analyzed with G219/172H presented the expected monomorphic fragment of 172 bp (data not shown).

In the case of CP-rpl16T, a correspondence between monovarietal oils and leaves of the same cultivar was observed, for cultivars of group 1, with the only fragment of 262 bp (**Figure 4B**), and for cultivars of group 4, which did not give any amplification products (data not shown). The fragment of 109 bp found in the DNA from leaves was not amplified in the oils so far tested: in fact, in DNA from leaves, its signal was always lower than that of the 262 bp fragment. The low amount of DNA extracted from oil could contribute to making this fragment hardly detectable.

G230/162T primers amplified in monovarietal oils only the fragment with length of 162 bp, and the longer fragments were not amplified (**Figure 4F**,**H**). Oils from cultivars not showing this fragment did not have any amplification. The longer fragments observed in DNA from leaves were not detected in the derived oils, probably because of the degradation of DNA extracted from oil. Therefore, fragments longer than 300 bp could hardly be of use in olive oil.

Fragments of expected length obtained from amplification of leaf and oil DNA with the primers were all sequenced to confirm their identity. Comparison of leaves, oils, and original AFLP fragments revealed that they had the same sequences.

DISCUSSION

Tracking the original components into a complex food matrix (traceability) conceptually and practically resembles the tracing of DNA fingerprints in archaeological samples or in biological residues left on the crime scene (*1*, *22*). All these have the following in common: (1) the presence of a microamount of DNA; (2) the high degradation of the same DNA; and (3) the presence of many interfering substances, which make each

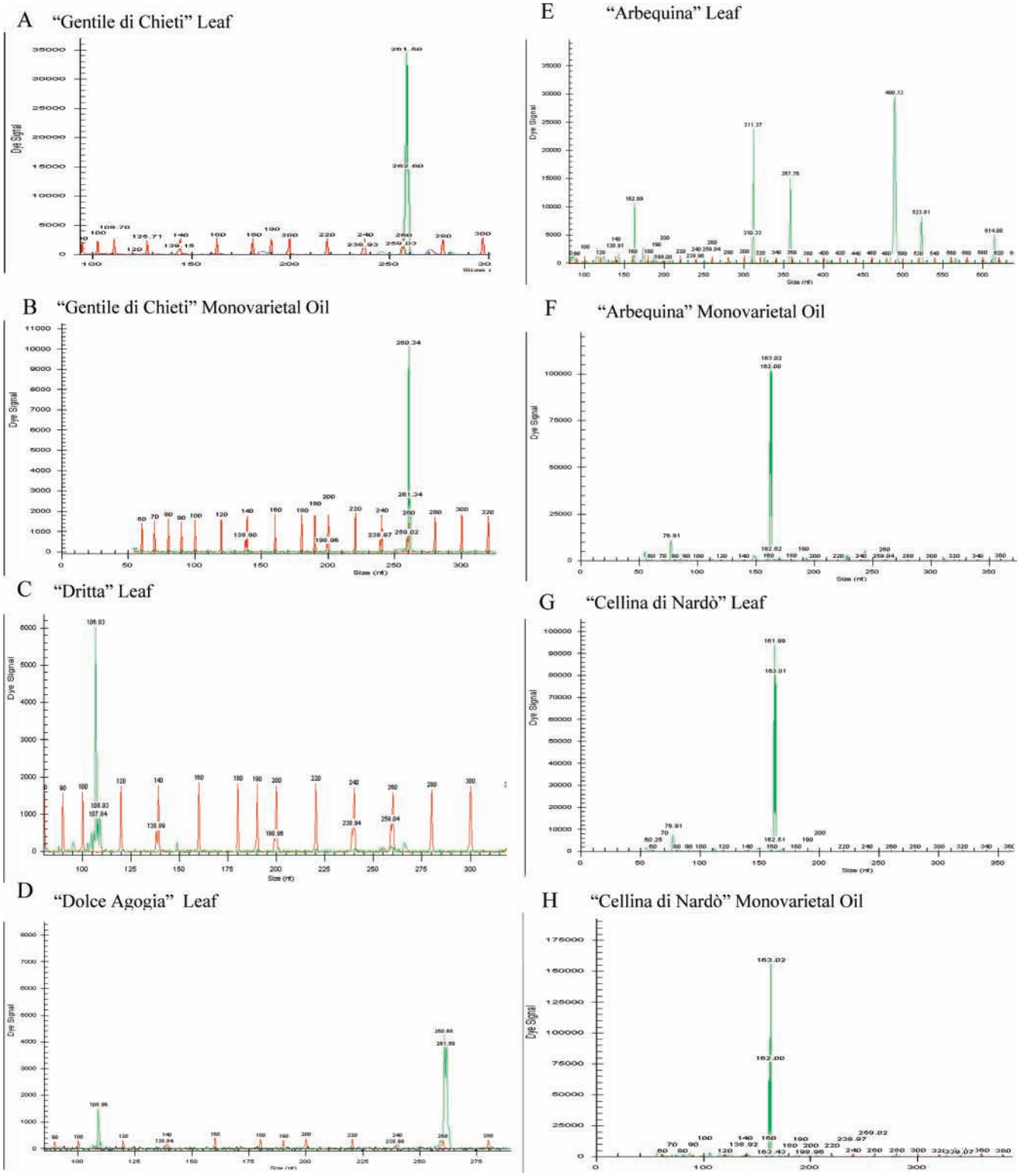

Figure 4. Amplification pattern of cultivars and oils with CP-rpl16T and G230/162T. Electropherograms obtained in capillary electrophoresis with the primer labeled with the fluorescent dye Cy5.5. The basal peaks represent 600 bp ladder. Amplification with CP-rpl16T primers: (**A**) Gentile di Chieti leaf; (**B**) Gentile di Chieti monovarietal oil; (**C**) Dritta leaf; (**D**) Dolce Agogia leaf. Amplifications with G230/162T primers: (**E**) Arbequina leaf; (**F**) Arbequina monovarietal oil; (**G**) Cellina di Nardò leaf; (**H**) Cellina di Nardò monovarietal oil.

analysis at risk of significance. To build a bridge between the organism's genomics (in many cases well developed) and trace genomics (in most cases still poor), informative molecular markers have been introduced, coupled to high-throughput genotyping platforms like multiplex real-time PCR and microarray (*23*). The genomic analysis of olive oil (*Olea europaea* L.) could benefit from the olive genomics which produced many markers of genotypic variability that can be used for identity screening. Several works describe the application of molecular markers to genetic recognition of the cultivar composition of a monovarietal PDO or PGI oils. However, these works describe the application of multilocus markers, such as RAPDs or AFLPs (*9*, *10*) or microsatellites (*3*, *12*, *24*). RAPDs and AFLPs give complex profiles that can be applicable to monovarietal oils

but not to mixtures of three to four cultivars, such as those usually adopted in PDO oils. Single-locus microsatellites are more effective at this aim, but they are not applicable to high-throughput screening such as microarray. Moreover, not all molecular markers identified in olive trees (usually from leaves) are equally transferable to oil: some sequences are under-represented in this matrix or are too long to be amplified (*8*). Following all these considerations, we attempted the direct identification of molecular markers from the oils to find directly the sequences most suitable for PCR amplification.

Performing the electrophoresis for AFLPs of olive oil on high-resolution agarose gel, for instance, allowed separation of fragments with similar lengths, overcoming all problems of acrylamide electrophoresis. This aspect and the fact that AFLP fingerprints of olive oil DNA had fewer bands than those of leaf DNA allowed to recover in a simple way fragments from the gel, greatly limiting problems of the coextraction of multiple fragments (*25*). The high correspondence between the profiles obtained with agarose and capillary electrophoresis was an index of the reliability of the method used.

The AFLP profiles of the monovarietal olive oils analyzed presented fragments with different intensity of the fluorescence signal, probably related to their abundance in the sample. The abundance of a fragment after PCR is related to its possibility of being extracted from a complex matrix and also being amplified in the presence of inhibitors (*8*, *11*). For this reason, those amplicons directly derived from oil DNA which are visible on agarose gel may be more amenable for application than "cultivar amplicons". Many oil amplicons were reproducible in PCR replicates of the same oil (*8*): some were common to many oils, while others were specific. Both common and specific oil amplicons were analyzed in our study, because the common fragments can have differences in their sequences which were not detectable on the agarose gel. The sequences found by analyzing four of these AFLP fragments had partial or complete homology with plant genes. We did not find any specific correspondence with olive genes because of the little sequence information on this plant present in databases.

The junction region between the intron and second exon of the chloroplastic L16 ribosomal gene, first found in *Spirodela oligorhiza* (*26*), corresponded to the AFLP-333T fragment. This is an interesting result because the junction regions are supposedly sites where the evolution piled up mutations (*27*), and therefore, they can be a source of markers (*28*). Moreover, previous studies reported that chloroplast DNA variations can allow to distinguish different chlorotypes in the genus *Olea* L. and in *Olea europaea* (*29*, *30*), and this information can be used to interpret DNA fingerprinting in olive oil. Because small differences in the size of amplicons can hardly be detected on agarose gel, fluorescent capillary electrophoresis was chosen to analyze the PCR products. This technique also offers the possibility of finding low abundant amplicons, such as those derived from oil DNA, that cannot be revealed with traditional ethidium bromide staining.

A nested PCR conducted on some cultivars and monovarietal oils showed that DNA of leaves was easily amplified with the primers used for oil AFLPs. Moreover, the identity of sequences of oil and leaf amplicons excluded that fragments isolated in oils can derive from sources other than olive.

A screening conducted on a larger number of cultivars showed that one fragment (G219/172H) was monomorphic, whereas two others (CP-rpl16T and G230/162T) showed differences both in size and number in the different samples, and in particular CP-rpl16T was considered as SCAR marker. The amplification of oil DNA showed that the monomorphic fragment was detectable in all samples analyzed, with a signal intensity comparable with that of the leaf DNA. This result confirmed once more that oil DNA amplifications with some specific marker can give results comparable with the respective cultivars.

Amplifications of the chloroplast fragment CP-rp116T separated the cultivars in four groups. The two fragments of 262 bp and 109 bp were not homologous; this means that they were amplified by common primers but belong to different loci. However, since the goal of our work is to find reliable markers to trace olive oil composition, fragments also derived from different loci can be useful.

The fragments of 262 bp and 109 bp obtained with CP-rpl16T primers could be used in a quantitative PCR (*31*, *32*) or microarray (*33*) assay, designing specific primers on their sequences. Also, the fragment of 162 bp and the longer ones, obtained with G230/162T, resulted in polymorphism and are useful for traceability, providing that highly specific primers are designed to avoid multiple amplifications. In general, when the fragments were small or well amplified in leaf DNA, they could be retrieved in oil DNA; however, it was difficult to amplify fragments longer than 300 bp or scarcely represented in the olive genome. This drawback could be overcome by improving the DNA extraction procedure or by reducing the size of the amplicons.

Future research will concern the application of these markers to develop a compositional test able to identify a cultivar in a monovarietal olive oil. These markers will be applied also in a high-throughput platform to assess and quantify the contribution of a single cultivar in commercial multivarietal oils. For this purpose, CP-rpl16T was tested in real-time PCR assay on some monovarietal oils with encouraging results (data not shown). The fragment with similarity to a part of a gene of strain *PH-1 Gibberella zeae* is being used to develop a marker specific test to identify fungal contaminations in plants and oils.

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