JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 531–537 **531**

Comparative Study of Methods for DNA Preparation from Olive Oil Samples to Identify Cultivar SSR Alleles in Commercial Oil Samples: Possible Forensic Applications

CATHERINE BRETON,† DELPHINE CLAUX,† ISABELLE METTON,† GILBERT SKORSKI,† AND ANDRÉ BERVILLÉ*,‡

Phylogene, Espace Innovation 2, 110, allée C. Babbage, 30035 Nîmes Cedex, France, and INRA, UMR-DGPC, bât 33, 2 place P Viala, 34060 Montpellier Cedex 1, France

Virgin olive oil is made from diverse cultivars either mixed or single. Those ensure different tastes and typicity, and these may be also enhanced by the region of production of cultivars. The different olive oil labels correspond to their chemical composition and acidity. Labels also may correspond to a protected origin indication, and thus, such oils contain a given composition in cultivars. To verify the main cultivars used at the source of an olive oil sample, our method is based on DNA technology. DNA is present in all olive oil samples and even in refined oil, but the quantity may depend on the oil processing technology and oil conservation conditions. Thus, several supports were used to retain DNA checking different techniques (silica extraction, hydroxyapatite, magnetic beads, and spun column) to prepare DNA from variable amounts of oil. At this stage, it was usable for amplification through PCR technology and especially with the magnetic beads, and further purification processes were checked. Finally, the final method used magnetic beads. DNA is released from beads in a buffer. Once purified, we showed that it did not contain compounds inhibiting PCR amplification using SSR primers. Aliquot dilution fractions of this solution were successfully routinely used through PCR with different SSR primer sets. This enables confident detection of eventual alien alleles in oil samples. First applied to virgin oil samples of known composition, either single cultivars or mixtures of them, the method was verified working on commercial virgin oil samples using bottles bought in supermarkets. Last, we defined a protocol starting from 2 × 40 mL virgin olive oil, and DNA was prepared routinely in about 5 h. It was convenient to genotype together several loci per sample to check whether alleles were in accordance with those of expected cultivars. Thus, forensic applications of our method are expected. However, the method needs further improvement to work on all oil samples.

KEYWORDS: DNA markers; forensic applications; *Olea europaea* L.; olive; refined oil; virgin oil

INTRODUCTION

Olive (*Olea europaea* L. subsp. *europaea* var *sativa*) oil is commercialised under different grades of quality depending on acidity and on refination (**Table 1**, according to http://www.infohuiledolive.net/pdf/VERSIONI.pdf). A traditional olive oil contains water (1%) and lipids (99%). Lipids contain fatty acids: 8−25% of saturated or mono-unsaturated (mainly oleic acid; 18:1), and poly-unsaturated (3.5−21% of linoleic acid (18:2) and 0.5−1.5% of linolenic acid (18:3)). Many compounds are present: alcohols, sterols (squalen, phytosterols), flavonoids, and many other organic compounds as traces, all these compounds contributing more or less to organoleptic qualities (or taste and odor), and explain oil diversity. Specificity for olive oil is that typicity is obtained by the conjunction of a region and a cultivar or a few of cultivars. Thus, appellations in the European Union correspond to PGI (protected geographic indication) or POA (protected origin appellation). The highest oil quality is obtained by crushing fresh olives at room temperature to obtain a mixture of oil, water, and many other compounds, (**Table 2** average composition is given according to http://europa.eu.int/comm/agriculture/prom/olive).

However, for olive oil, the country origin and therefore cultivars that have produced olive are very important to know, and Besnard et al. (*1*, *2*) have previously shown that the genetic diversity of olive cultivars is strongly structured according to region and country of origins. Thus, verification of cultivars used to process an oil sample may contribute to certificate an olive oil origin and may have commercial interest and forensic applications. Generally, labels indicate the country of origin but do not give the detail of cultivars used. A controlled designation

* To whom correspondence should be addressed. Tel.: 33-4-99-61-22-33. Fax: 33-4-67-04-54-15. E-mail: berville@ensam.inra.fr.
† Phylogene.
‡ INRA.

10.1021/jf034588f CCC: $27.50
Published on Web 01/13/2004

Table 1. Olive Oil Grades and Appellations According to European Community Regulations (http://www.info-huiledolive.net/pdf/VERSIONI.pdf)

olive oil grade	acidity in oleic acid[a] content	characteristics
virgin olive oil		taste absolutely irreproachable
extra virgin olive oil	1 g per 100 g	
fine virgin olive oil	I<2 g per 100 g	
virgin olive oil	<3.3 g per 100 g	
current virgin olive oil	<3.3 g per 100 g	
lampant virgin olive oil	>3.3 g per 100 g	
refined olive oil	0.5 g per 100 g	virgin olive oil refined
olive oil	1.5 g per 100 g	mixed between refined olive oil and virgin olive oil
crude pomace olive oil		olive pomace treated with solvent
refined pomace olive oil	0.5 g per 100 g	crude olive pomace oil refined
pomace olive oil	1.5 g per 100 g	mixed olive oil between refined olive pomace oil and virgin olive oil but not lampant

[a] Acidity expressed in free oleic acid neutralized by sodium alkali.

Table 2. Average Composition of Olive Oil, Presence of Organic Compounds as Traces[a]

compounds	fatty acids	quantity
lipids (900 calories per 100 g)		99%
vitamin E, tocopherols,		150 mg/kg
saturated fatty acids		8–25%
monounsaturated fatty acids	oleic acid	55–80%
polyunsaturated fatty acids	linoleic acid	3.5–21%
	linolenic acid	0.5–1.5%
phenolic compounds		20–40 mg/kg
vitamin A, sterols, alcohol		traces
flavonoids		traces
phytosterols		traces

[a] Data compiled from http://europa.eu.int/comm/agriculture/prom/olive.

of origin (DOP, PGI, and COA) has to respect the composition in cultivars according to the registration of the denomination. Thus, here current biochemical controls are lacking to point out a mixture of cultivars, and consequently, this enables adulterated products. Variation in the oil prices seems not always justified by label information, which in many case remains insufficient. Moreover, the olive oil market is adulterated by several less quality olive oil, due to the use of anonymous cultivars.

Oil can be adulterated by other species oil such as sunflower and hazelnut, which are less expensive, thus the fraud may represent 10% of the market (*3*). However, techniques based on fatty acid composition and on identification of secondary metabolites statistically enable such an identification of the region of origin for the main cultivar(s) (*4*), but they require a series of analyses and are therefore time-consuming and expensive.

Along the oil purification performed by centrifugation and other separation processes (filtration, decantation), which broadly consist to separate oil from water to prevent oil to be cloudy, it is likely that different amounts of DNA remain both with oil and within water traces. Depending on the freshness of olive, the spectra in different compounds such as free fatty acid (responsible of acidity) may vary (Table 2), but whatever the techniques used to crush olive, the mixture will contain DNA. DNA traces stay in the oil even when it has been refined, and those traces have been used to detect a transgene in genetically modified rapeseed (*5*). Different oil bottles from different trades show different amount of residues in bottles after long shelf storage. The yield in DNA from commercialised bottles is therefore expected to be highly variable. Another consideration deals with the average size of the DNA fragments. Using refined oil, Hellebrand et al. (*5*) have shown that using PCR amplification primers they have been more successful with expected short fragments (350 bp) than with long fragments (1000 bp).

Table 3. List of Cultivars Identified by SSR Alleles According to Breton Et Al. (unpublished)

cultivar	country	cultivar	country
Aglandau	France	Leccino	Italy
Amellau	France	Lucques	France
Arbequina	Spain	Moraiolo	Italy
Aubenc	France	Négrette	France
Belgentier	France	Noirette	France
Berdanel	France	Ogliarolia	Italy
Bouteillan	France	Olearolia	Italy
Broutignan	France	Olivière	France
Cailletier	France	Pendolino	Italy
Canivano Bianca	Italy	Picholine	France
Carotina	Italy	Pigale	France
Cayet rouge	France	Poulo	France
Cayon	France	Poumal	France
Clermontaise	France	Rascasset	France
Corniale	France	Razzola	Spain
Coucourelle	France	Rougette	France
Curnet	France	Salonenque	France
Dorée	France	Sigoise	Algeria
Frantoio	Italy	Tanche	France
Giarraffa	France C	Termite di Bitetto	Italy
Grapié	France	Verdale	France
Grossane	France	Verdelé	France
Kotreiki	Greece	Vermillau	France

Several sets of SSR have been proposed for olive (*6*−*9*). Theoretically, using a dataset for SSR profiles shown accurate to identify cultivars (**Table 3**), it is possible to compare those SSR alleles present in olive oil with alleles obtained from leaf DNA found in reference cultivars. This has been used for the oil sample under study to ascertain whether an alien olive cultivar carrying a different SSR spectrum. Formerly, the identification of one cultivar in a mixture of cultivars with SSRs may be ambiguous due to allele similarities at different locus. However, if specific sequences as SCARs or STSs are available, they may enable cultivar identification.

Because we have previously shown that olive cultivars can be differentiated and identified using DNA technology and that DNA exists as traces in oil, we checked whether cultivars may be identified in a single cultivar oil using olive oil as the DNA source. We first assayed different methods to prepare DNA. We defined a running protocol for most of commercial oils and we showed that it is even working on refined commercial oil samples. We used SSRs markers by amplifying short (130, up to 250 bp) fragments that are in average sized in agreement with (*5*). Our method is usable in routine to control virgin or crude oil samples and may be used for refined oil. The potential uses of such a method in forensic application are discussed.

MATERIALS AND METHODS

Reference: Trees and Oil Samples. Leaves were harvested on reference trees (**Table 3**) either in commercial or in collection orchards (*2*, *10*). Oil samples were purchased either directly in olive mills or in stores. Different origins (France, Italy, and Spain) for oil bottles warrant diversity of denominations (PGI, COA, DOP) and of processes to obtain oil from different cultivar origins. Today manufacturers have commercialised olive oil under the name of one cultivar: Picholine, Olivière, and Arbequina. We also used such samples to check whether the corresponding cultivars may be identified. We used specially crushed

Table 4. List of Olive Oil Samples, Brands, Region of Origins, and Cultivars Eventually Listed on Labels

oil denomination	miller or trader	country	PGI	single/mix of cultivars	cultivar indicated on label	DNA	characterization
Lucques	Moulin de l'Oulibo	France	AOC	single	Lucques	direct PCR	yes
Olivière	Moulin de l'Oulibo	France	AOC	single	Olivière	direct PCR	yes
Picholine	Moulin de Villevielle	France	AOC	single	Picholine	direct PCR	yes
Aglandau	Moulin de Villevielle	France	AOC	single	Aglandau	direct PCR	yes
Bouteillan	Moulin de Villevielle	France	AOC	single	Bouteillan	direct PCR	yes
Négrette	Moulin de Villevielle	France	AOC	single	Négrette	direct PCR	yes
Arbequina	Moulin des Costières	Spain		single	Arbequina	direct PCR	yes
Picholine	Cauvin	France		single	Picholine	direct PCR	yes
Cornicabra	Cauvin	Spain		single	Cornicabra	direct PCR	yes
Arbequina	Cauvin	Spain		single	Arbequina	direct PCR	yes
Picudo	Cauvin	Spain		single	Picudo	direct PCR	yes
Picual	Cauvin	Spain		single	Picual	direct PCR	yes
Salonenque	Moulin de Virant Lançon	France	AOC	single	Salonenque	direct PCR	yes
Aglandau	Moulin de Virant Lançon	France	AOC	single	Aglandau	direct PCR	yes
Hojiblanca	Borges	Spain		single	Hojiblanca	direct PCR	
Arbequina	Borges	Spain		single	Arbequina	direct PCR	yes
Picual	Borges	Spain		single	Picual	direct PCR	yes
Siurma	Unio	Spain	DOP	mix		direct PCR	
Arbequina	Vallasera	Spain		single	Arbequina	direct PCR	yes
Arbequina	Unio	Spain		single	Arbequina	direct PCR	yes
Huile d'olive de Nyons	Le Brin d'Olivier	France	AOC	single	Tanche	direct PCR	yes
Huile d'olive de Nyons	Nyonsolive	France	AOC	single	Tanche	direct PCR	yes
Huile d'Olive Vierge Extra	Robert	France		mix		direct PCR	
Le Macine Huile d'olive Vierge Extra	Carapelli (Firenze)	Italy		mix		direct PCR	
Huile d'Olive Vierge Extra	Casino	France		mix		direct PCR	
Huile d'olive Extra vierge	Toledo	Spain	DOP	single	Cornicabra	direct and LC PCR	yes
Huile d'olive vierge extra	Gallo	Portugal		mix		direct and LC PCR	
Olio extra vergine di oliva	Bravo bis	Italy		mix		direct and LC PCR	
Huile d'olive vierge extra	Carbonell	Italy		mix		direct and LC PCR	
Huile d'olive première pression à froid Vierge extra	Bertolli Lucca	Italy		mix		direct and LC PCR	
Huile d'olive vierge extra	Borges	Spain		mix		direct and LC PCR	

olive from single cultivars to obtain oil. This was performed by IRO (Perugia, Italy). These enabled control mixtures in various proportions of two oil samples to check the susceptibility of the detection method. All origins are listed in **Table 4**.

Preparation of DNA. DNA was extracted from leaves according to the method already described (*2*). For 40-mL, 80-mL, and 400-mL oil samples, different methods were assayed using the Wizard Magnetic DNA Purification System for Food (Promega), Silica (Sigma), and Hydroxyapatite biogel (Biorad), respectively, according to provider recommendations. These techniques enabled us to obtain a native DNA solution (NDS) in 200 μL, further named NDS. NDS samples were checked for DNA content. Using a current spectrophotometer, the absorbance was not enough for experimental quantification.

Further DNA Purification from NDS. *Precipitation.* DNA precipitation was performed with ethanol, final concentration 83%, in the presence of 0.3 M ammonium acetate and after centrifugation at 8000*g* for 15 min, the pellet was washed with 70% and then dissolved into 20 μL of water.

DNA Column Purification. Different column kits were checked: either purification through silica, Nucleospin Plant (Macherey Nagel) and Wizard SV gel or PCR cleanup system (Promega), or filtration with Vivaspin 500 (Vivascience), Millipore 2X microcon (Millipore), and Nanosep centrifugal devices (Pall Life Science), according to provider recommendations (**Table** 5). The latter was retained for routine purification of DNA. The final volume of 200 μL enabled to check enough SSR primer pairs through PCR amplification reactions to differentiate cultivars.

For each oil sample (**Table 4**), two aliquot fractions of 40 mL per bottle were separately treated for DNA preparation. Each fraction led to three DNA amplification tries. Thus, our comparisons are based on at least six repeats.

DNA Amplification. *SSR Technology.* Amplification was performed according to ref 12 for 10 primer pairs that have been shown to be diagnostic for olive cultivar identification. SSR primers used may be communicated for academic work without commercial interest.

All DNA samples from oil were compared with DNA samples from leaves of cultivars that may have been used to obtain the oil samples. DNAs from Commercial oil were used in comparison with cultivars from the region of production unless the cultivar names are indicated on the label. Amplifications were performed in a final volume of 25 μL with 5 μL of DNA source according to (*12*). For each run, a blank (5 μL water instead of NDS) was performed in parallel to detect eventual contaminant DNA in preparation.

With Native DNA Solution. A set of SSR loci developed for olive was tested on oil DNA. Seven loci were selected because the amplification products were usable to detect foreign alleles in olive oil. PCR conditions were optimized at 50 cycles for a mix containing 1.5−3 mM $MgCl_2$, 1 U per reaction of Taq DNA polymerase (Sigma), and approximately 50 ng of template DNA in a total reaction volume of 25 μL.

PCR reaction was carried out using an Eppendorf Thermocycler. After 4 min at 94 °C, 50 cycles were performed with 30 s at 94 °C, 45 s at 50−60 °C depending on the primer pair, 1 min at 72 °C, and a final extension step at 72 °C for 4 min. PCR product amplification was verified on agarose electrophoresis system and finally separated on 6% vertical acrylamide gels and visualized after silver stain using the Silver Sequence kit (Promega).

Thus, we tried 5 μL aliquot fractions (out of 200 μL) of the NDS for PCR amplification and we checked with an exogenous animal beef detection system whether these NDS samples may inhibit PCR amplification to detect eventual inhibiting compounds. We also tried dilution by 2, 4, 8, and 16 of this NDS solution.

Lightcycler Technology. A 1-μL sample of the DNA solution was used in 20 μL capillary tubes with the SYBR Green mix system (Roche) and 5 μM SSR primers. The run starts by one step at 95 °C of denaturation for 6 min, followed by 45 amplification cycles. Each cycle comprises one step at 95 °C for 15 s, followed by 72 °C for 10 s and at 50 °C for 10 s. It finishes with a fusion step at 95 °C for 1 s, 65 °C for 30 min, and 95 °C for 1 s, for releasing the fluorochrome. The final cooling is at 44 °C for 1 min. Along each cycle, DNA amplifi-

Table 5: Methods Used to Prepare DNA from Oil[a]

DNA extraction methods from oil	oil sample vol	amplification of rbcl fragment	microsatellite amplification	routine usable	type of oil used
1 protocol using added water a column XL Macherey Nagel b extraction with column L Macherey Nagel c extraction with column plant Macherey Nagel	15 mL	yes	no	no	≪Picholine≫ Oil Cauvin
2 total DNA extraction protocol (ref 15)	15 mL	no	no	no	≪Picholine≫ Oil Cauvin
3 protocol CTAB precipitation CTAB (ref 1)	15 mL	no	no	no	≪Picholine≫ Oil Cauvin
4 extraction protocol of DNA: HA/CTAB	400 mL	yes	yes	no	≪Picholine≫ Oil Cauvin ≪Cornicabra≫ Oil Cauvin
5 extraction protocol of DNA: PVPP/CTAB	400 mL	no	no	no	≪Cornicabra≫ Oil Cauvin ≪Picholine≫ Oil Cauvin
6 extraction protocol of DNA: PVPP/HA (ref 14)	400 mL	yes	yes	no	≪Picholine≫ Oil Cauvin ≪Cornicabra≫ Oil Cauvin
7 extraction protocol of DNA: HA (ref 16)	200 mL	yes	yes	no	≪Picholine≫ Oil Cauvin ≪Cornicabra≫ Oil Cauvin
8 protocol with the Wizard Food kit (Promega)	40 mL 4 × 10 mL	yes	yes	yes	≪Picholine≫ Oil Cauvin ≪Cornicabra≫ Oil Cauvin
9 protocol with silica (ref 17)	80 mL 2*40 mL	yes	yes	yes	≪Picholine≫ Oil Cauvin ≪Cornicabra≫ Oil Cauvin ≪Mixture≫ Oil Casino
10 protocol with silica (ref 18, modified by Phylogene)	40 mL	yes	yes	yes	≪Picholine≫ Oil Cauvin ≪Cornicabra≫ Oil Cauvin ≪Mixture≫ Oil Casino
11 Hellebrand et al. (ref 5) Protocol.	200 mL	yes	yes	yes	≪Mixture≫ Oil Casino
12 protocol HA/CTAB	1L	yes	yes	no	≪Mixture≫ Oil Casino

[a] Methods were used according to provider recommendation or references.

cation is visualized on the monitor by comparison with the blank samples, and SSR amplifications were separated first on Agarose gel to check amplification and then on acrylamide gels for size calibration of alleles.

Beef DNA and corresponding primer pairs were used as controls to detect eventual PCR-inhibiting compounds. Purchased refined soybean oil was used to check the efficiency of the final method using STS soybean specific primer pairs.

RESULTS AND DISCUSSION

Recapitulation of the Methods to Obtain DNA from Olive Oil. Twelve different methods were assayed on oil samples as indicated (**Table 5**). Three repetitions at least of each method were performed. Several methods enabled the recovery of DNA, and we chose the most efficient for the best result in routine.

Choice of the Best Method. **Table 4** displays the oil DNA assays for experiments performed on all oil samples with the different methods. We compared the native DNA solutions obtained through their efficiency to obtain amplification products. Four methods (8−11) led to NDS solution that provided amplification after dilution by 2 to 16 times enabling a high number of PCR reactions but the best dilution has to be determined prior running the assays. Thus, we checked further the other methods. These methods required further purification (precipitation and column kits) leading to amplifiable DNA. However, the final purified DNA solution is of 200 μL, thus the number of possible amplifications remains sufficient for cultivar characterization.

Quantification of DNA from Oil. DNA quantities obtained in NDS solution by the three methods were different, but we did not try to accurately quantify DNAs. Spectrophotometer estimation of DNA was not efficient due to interfering compounds, and we suspected that these compounds may also interfere with fluorescent probes. The highest yield was obtained with Wizard Magnetic DPSF, we showed that with Hydroxyapatite and with Silica, we lose most of the DNA along the rinsing steps (not detailed).

We wondered whether the DNA solutions may contain inhibiting PCR-compounds, so we added different quantities of beef DNA (10−0.001 ng) in oil DNA. Beef DNA assays were amplified with the same efficiency without and with oil DNA samples. This shows that compounds did not inhibit PCR amplification. Thus, quantification of oil DNA using PCR is not biased.

Recapitulation of the Method Retained. Method 8 appeared to be the shortest and most adapted routine used, thus we retained it. We started from 2 × 40 mL olive oil to run two independent DNA preparations from each oil sample using the Wizard Magnetic DPSF. From each 40-mL sample were prepared four 10-mL aliquot fractions, to which were added 2 mL of lysis buffer A and 1 mL of lysis buffer B plus 25 μL (U) of RNAse A. The tubes were vigorously shaken for one min after each adjunction and stay at room temperature for 10 min. A 3-mL sample of precipitation buffer G (green color) were added, vigorously shaken, and then spun at 4000*g* for 20 min. Oil (upper phase) was eliminated, and the 4 tubes (blue color) were gathered together (about 20 mL of aqueous phase). DNA was precipitated with 0.9 V 2-propanol and 150 μL of Wizard Magnetic DPSF, and the solution was slowly stirred at room temperature for 1 h. The beads were maintained in the tube with a magnet, whereas the solution is discarded. The beads were rinsed three times with 70% ethanol. Beads were dried at 65 °C for 10 min. Beads were then suspended in 200 μL of UP water. This releases DNA, and beads were eliminated from the DNA solution with the magnet as previously. The DNA solution was further purified on the Nanosep column. Aliquot samples of this NDS are diluted 2, 4, 8, and

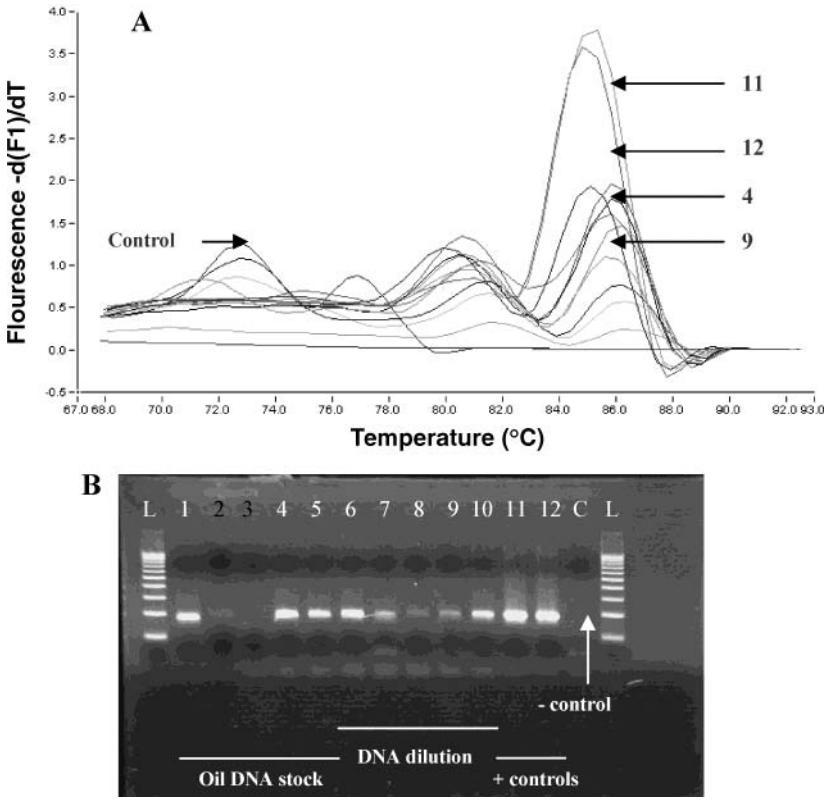

Figure 1. (A) Lightcycler records using the SYBR green kit using DNA from leaf and oil samples. (B) PCR amplification products obtained with primers amplifying rbcL sequence using DNA from oil samples and controls. rbcl primers amplified a conserved fragment in plants, sequences maybe communicated for academic work without commercial interest. L = 1 kb Ladder. 1–5, oil sample DNAs obtained with methods 4, 9, 10, 8, and 8, respectively; 6–10, same order diluted by half. Frantoio leaf DNA samples, 11, 12 (diluted 1/2). C, control without olive DNA.

16 times, to determine which is the best dilution for amplification. Eventually, further purification was performed with the Nanosep Centrifugation Devices.

SSR alleles were amplified, enabling the comparison of those obtained from the leaf DNA and those from the oil samples, according to denomination or labels. **Figure 1** shows such determination. Thus, the method provided enough DNA from 40 mL of oil to examine several loci constituting the allele patterns. We verified the SSR pattern for DNA from artificial oil mixtures or artificially mixed DNA from two cultivars. We found that we can determine all alleles of the two cultivars in the 1:1 DNA mixture (Not shown). However, with a low proportion of one cultivar (5−20%) all SSR loci did not respond equally (i.e., only one out of twelve responds for 5% of Moraiolo into 95% Frantoio DNA from oil). However, even when the composition in different cultivars is known, we detected only the main alleles, which means we detected only the main cultivar used for oil samples. Now, with this method, secondary cultivars in oil samples cannot be revealed.

Lightcycler Technology. Amplification was performed on NDS, obtained with the Wizard Magnetic DPSF. The experiments showed that beef DNA was amplified earlier and faster than olive DNA due to the quantity of the former (Not shown). At last, olive amplification products are clearly seen (**Figure 2**) once deduced the blank control. However, we observed that some loci were equally amplified in every sample using direct PCR or Lightcycler, whereas other loci were better amplified with Lightcycler than with direct PCR. Some olive oil samples did not respond at all in comparison with the blank. On average, this occurred for about 20% of the samples. However, the whole method was successfully applied to refined soybean commercial oil (not shown).

A previous method described recently in the literature was not running on commercial olive oil (*13*). DNA in wine (*14*) is probably more degraded than that in oil. The different methods that were used have led from various olive oil volumes to prepare DNA usable for PCR amplification after more or less treatments for purification. We considered different criteria for choosing the routine method. These criteria deal with the volume of oil, the time to obtain the DNA, and a simple purification step. At last, we retained the method based upon the Wizard Magnetic DPSF that enables the performance of several preparations together. Two variants may be performed, either to use the diluted NDS fraction for direct amplification without further purification or to use the DNA after purification onto a Nanosep column. Depending on the DNA quantity available from oil and the goal of the analysis, the choice of the best variant may be made.

When olive oil is due to a single cultivar, the method enabled to look for alien alleles at several loci. Cultivar olive diversity therefore allows the revellation whether foreign alleles may be due to usurpation of cultivar name. In the case of controlled mixtures of cultivars and oil samples with several cultivars, only alleles from some major cultivars were revealed. So, this is insufficient to warrant an indisputable assay using DNA technology. Consequently, our method needs further improvement to be applied on all olive oil samples. We did not check which factors may be responsible for defect of amplification, we do not think that is due to DNA quantity but rather to still organic compounds bound to DNA. However, we know that these compounds do not inhibit PCR amplification efficiency.

Different organic compounds are also used to check whether an olive oil grade announces on a label is in accordance to the content: sterol and fatty acid composition enable such a

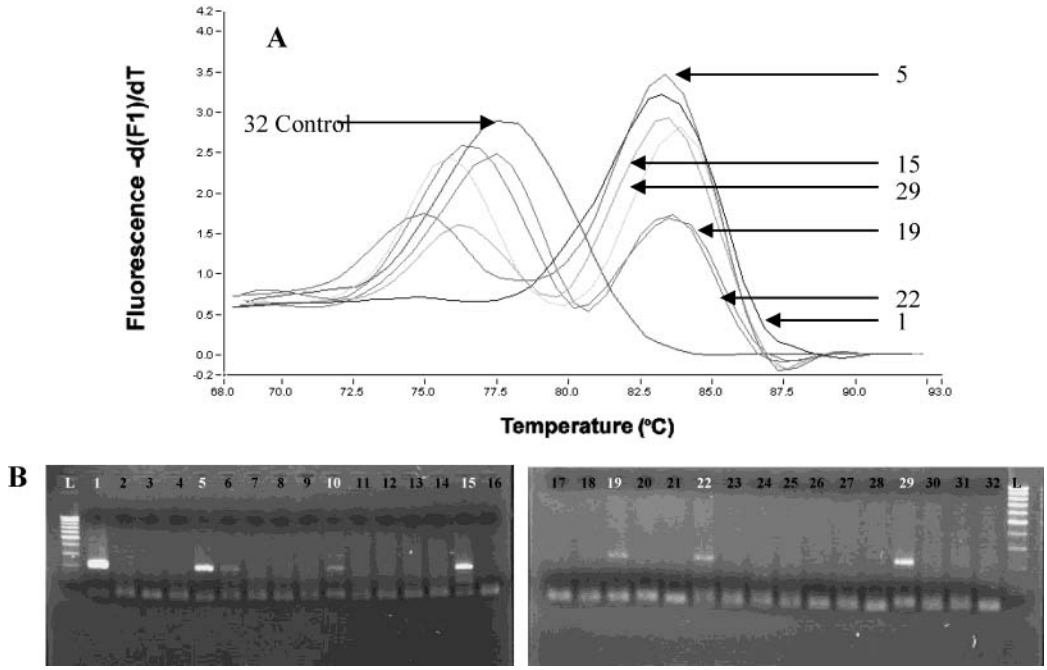

Figure 2. (A) Lightcycler records using the SYBR green kit using DNA from leaf and oil samples. (B) PCR amplification products obtained with SSR primers with DNA from oil samples and controls. SSR primers maybe communicated for academic work without commercial interest. L = 1 kb ladder. 1 = leaf reference. 5, 10, 15, 19, 22, and 29, oil samples. 32, control without olive DNA.

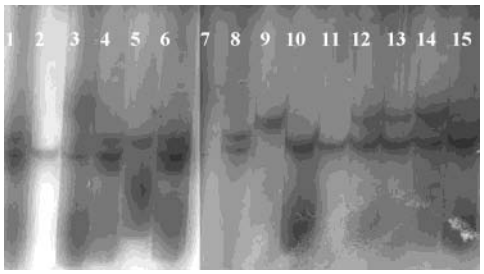

Figure 3. Electrophoregram of silver stain gel from amplified fragments with DNA from oil as template obtained with the "Wizard Magnetic DPSF" using an SSR marker. Lanes 1–6 represent leaf DNA amplification as references. 1, Cornicabra; 2, Frantoio; 3, Galega; 4, Leccino; 5, Moraiolo; 6, Picual. Lanes 7 to 15 represent amplified fragments with DNA from oil as templates using an SSR marker. 7, Frantoio; 8, Leccino; 9, Montes de Toledo; 10, Gallo; 11, Bravo; Bis 12, Carbonell; 13, Bertolli Lucca; 14, Borges; 15, Borges. SSR primers maybe communicated for academic work without commercial interest.

determination (*4*). It is clear that the knowledge of cultivars used to obtain an oil sample may help consumers to make their choice among the available oil brands and oil quality range. In most of the cases, this may eliminate oils from false origins and those that have been adulterated with such oils. However, DNA information cannot reveal whether oil processes are in agreement with labels, but many other compounds enable such controls. Consequently, DNA assays provide traceability of cultivars used as sources of oil samples. Thus, the information brought by DNA is complementary of the information brought by chemical analyses.

Our method may bring some novel features on olive oil controls for forensic applications. Our database on olive cultivars already contains several tens of entries corresponding to the main French varieties and the main oil cultivars from EU countries. For olive oil certification from a single cultivar detection of an alien allele is likely due to the high heterozygosity of olive (76% or higher). Alien alleles should indicate willful or unintentional adulteration not in agreement with labels. In many PGI not agreed cultivars are still present. This is also probably valuable for olive oils certificate from a PGI or DOP. In that last case, a series of alleles at each locus is expected and some diagnostic loci better than others have to be retained for routine applications.

Adjunction blending of chip oil from other oil crop (sunflower, hazelnut) may also be detected by secondary metabolites. They should be preferred because such oils contain specific organic compounds such as trilinoleine-TAG for sunflower that have been heated and have modified several molecules because trans-fatty acid proportion is enhanced. However, DNA traces from other species may enable to identify the source species used for adulteration as with sunflower oils.

In conclusion, we present the first protocol to obtain enough DNA from a relative low amount of virgin olive oil enabling practical detection of alien alleles in a single cultivar oil sample. Moreover, we documented the assays. The method requires further improvement for running on olive oil samples.

ABBREVIATIONS USED

CDO, controlled designation of origin; COA, controlled origin appellation; DOP, delimited origin protected; GOP, geographic origin protected; NDS, native DNA solution. PCR, polymerase chain reaction; PGI, protected geographic indication; POA, protected origin appellation; SCAR, sequence characterized amplification region; SSR, simple sequence repeat; STS, site tagged sequence; TAG, triacyl glycerol; Wizard Magnetic DPSF, Wizard Magnetic DNA preparation system for food

ACKNOWLEDGMENT

We thank our providers of olive oil samples and G. Besnard for helpful comments on the manuscript. We also thank Dr. L. Baldoni (Perugia), Mr. and Mrs. Pagès (Clermont-l'Hérault), and Mr. and Mrs. Cheylan (Lançon de Provence), for providing diverse mono-cultivar oil preparations.

LITERATURE CITED

(1) Besnard, G.; Baradat, P.; Breton, C.; Khadari, B.; Bervillé, A. Olive domestication from structure of oleasters and cultivars using nuclear RAPDs and mitochondrial RFLPs. *Genet., Sel., Evol*. **2001a**, *33 sup 1*, 251−268.

(2) Besnard, G.; Breton, C.; Baradat, P.; Khadari, B.; Bervillé, A. Cultivar identification in olive based on RAPD markers. *J. Am. Hortic. Sci.* **2001b**, *126*, 668−675.

(3) Vergniot, O. Le trafic d'or liquide. In *Pays de Provence Côte d'Azur*; Milan Presse: Toulouse, France, 2003; pp 39−41.

(4) Ollivier, D.; Souillol, S.; Noyer, C.; Guérère, M.; Pinatel, C.; Durbec, J.-P.; Artaud J. Contribution à l'étude de la traçabilité d'huiles d'olive vierges françaises: approche chimique. *Le Nouvel Olivier* **2003**, *31*, 3−7.

(5) Hellebrand, M.; Nagy, M.; Mörsel, J. T. Determination of DNA traces in rapeseed oil. *Z. Lebensm. Unters. Forsch. A.* **1998**, *206*, 237−242.

(6) Sefc, K. M.; Lopes, M. S.; Mendoça, D.; Rodrigues Dos Santos, M.; Laimer Da Câmara Machado, L. M.; Da Camara Mâchado A. Identification of microsatellites loci in olive (*Olea europaea* L.) and their characterization in Italian and Iberian trees. *Mol. Ecol.* **2000**, *9*, 1171−1173.

(7) Rallo, P.; Dorado, G.; Martin, A. Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). *Theor. Appl. Genet.* **2000**, *101*, 984−989.

(8) Carriero, F.; Fontanazza, G.; Cellini, F.; Gioro, G. Identification of imple sequence repeats (SSRs) in olive (*Olea europaea* L.). *Theor. Appl. Genet.* **2002**, *104*, 301−307.

(9) Cipriani, G.; Marrazzo, M. T.; Marconi, R.; Cimato, A.; Testolin, R. Microsatellite markers isolated in olive are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars (*Olea europaea* L.). *Theor. Appl. Genet.* **2002**, *104*, 223−228.

(10) Khadari, B.; Breton, C.; Moutier, N.; Roger, J. P.; Besnard, G.; Bervillé, A.; Dosba, F. Using molecular markers for olive germplasm management in one collection. *Theor. Appl. Genet.* **2003**, *106*, 521−529.

(11) Besnard, G.; Khadari, B.; Villemur, P.; Bervillé, A. Cytoplasmic male sterility in the olive (*Olea europaea* L.). *Theor. Appl. Genet.* **2000**, *100*, 1018−1024.

(12) de la Rosa, R.; Angiolillo, A.; Rallo, L.; Guerrero, C.; Besnard, G.; Bervillé, A.; Martin, A.; Baldoni, L. A first genetic linkage map of olive (*Olea europaea* L.) cultivars using RAPD and AFLP markers. *Theor. Appl. Genet.* **2003**, *106*, 1273−1282.

(13) Muzzalupo, I.; Perri, E. Recovery and characterisation of DNA from virgin olive oil. *Eur. Food Res. Technol.* **2002**, *214*, 528−531.

(14) Siret, R.; Gigaud, O.; Rosec, J. P.; This, P. Analysis of grape *Vitis vinifera* L. DNA in must mixtures and experimental mixed wine using microsatellite markers. *J. Agric. Food Chem.* **2002**, *50*, 3822−3827.

(15) Saghai-Maroof, M. A.; Soliman, K. M.; Jorgensen, R. A.; Allard R. W. Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 8014−8018.

(16) Siret, R. étude du polymorphisme génétique de la vigne cultivée (*Vitis vinifera* L.) à l'aide de marqueurs microsatellites: applications à la caractérisation de cépages dans les vins. Thèse de doctorat en sciences agronomiques ENSA, Montpellier, Fr.

(17) Myakishev, M. V.; Kapanadze, G. I.; Shaikhayev, G. O.; Georgiev, G. P.; Beritashvili, D.R. Institute of Gene Biology, 34/5 Vavilov St., Moscow by silica 117334, Russia, http://www.ras.ru/biogen.

(18) Boom, R.; Sol, C. J. A.; Salimans, M. M. M.; Jansen, C. L.; Wertheim-van Dillen, P. M. E.; van der Noordaa, J. Rapid and simple methods for purification of nucleic acids. *J. Clin. Microbiol.* **1990**, *28*, 495−503.11

Received for review June 4, 2003. Revised manuscript received November 12, 2003. Accepted November 13, 2003. This work was supported by the Région Languedoc Roussillon through a TRIAL Cost joint program (2001-2002) between Phylogene and INRA and partly supported in 2003 by the "Oliv-Track" program from EU QLK1-CT-2002-02386.

JF034588F