JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Non-concordance between Genetic Profiles of Olive Oil and Fruit: a Cautionary Note to the Use of DNA Markers for Provenance Testing

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To investigate the contribution of paternal alleles to the DNA content of olive oil, genetic analyses of olive DNA samples from fruits, leaves, and oil derived from the same tree (cv. Leccino) were carried out. DNA extracted from maternal tissues—leaves and flesh—from different fruits showed identical genetic profiles using a set of DNA markers. Additional simple sequence repeat (SSR) alleles, not found in the maternal samples, were amplified in the embryos (stone), and they were also detected in DNA extracted from the paste obtained by crushing whole fruits and from the oil pressed from this material. These results demonstrate that the DNA profile obtained from olive oil is likely to represent a composite profile of the maternal alleles juxtaposed with alleles contributed by various pollen donors. Therefore, care needs to be taken in the interpretation of DNA profiles obtained from DNA extracted from oil for resolving provenance and authenticity issues.

KEYWORDS: Olive oil; food traceability; DNA markers

INTRODUCTION

Olive (*Olea europaea* ssp. *europaea* L.) is a woody species, one of the most ancient crops characteristic of the Mediterranean region (*1*). The fruits of the trees are harvested and consumed as table olives or milled to produce olive oil. The olive tree is traditionally grown mainly in the Mediterranean area, although recognition of the health benefits of olive oil has fuelled its spread throughout the world, mainly the United States, Canada, Australia, and Japan.

Cultivated olive is diploid ($2n = 2x = 46$) (*2*, *3*); most cultivars are self-incompatible, and the flowers are wind pollinated. Olive flowers are generally hermaphrodites, containing both male and female parts, but some cultivars are male-sterile (*4*) and others have only staminate flowers. Although a small percentage of progeny is believed to arise from selfing, even when a cultivar is considered to be self-incompatible, genotyping of seeds showed that they were products of cross-pollination in almost all cases (*5*).

Oil accumulates in the thick and fleshy mesocarp and, to a small extent, in the seed (*6*). Olive oil is extracted from the drupes in an amount that rarely exceeds 25% of fresh weight. During the milling process the whole fruits (including stones) are milled and ground into a homogeneous pulp. The pulp is then shaken during the so-called malaxation step, and the oil is recovered by either pressing or centrifugation.

There are many categories of olive oil: "virgin", "refined", "pomace", and "olive pomace" are terms used to convey quality, reflecting the extraction procedures. Protected designation of origin (PDO), protected geographical indication (PGI), and traditional speciality guaranteed (TSG) are important awards recognized by the European Union referring to the quality of the olive oils. Before such awards are given, regulations imply detailed rules on the varieties to use, the geographical area of production, and the methods of oil extraction.

As these labels reflect quality and products awarded these labels command price premiums, it is important that procedures are available to verify those labels and to recognize adulteration to protect the consumer from fraud. Because chemical analyses per se are not sufficient to verify olive oil authenticity, except in the cases of adulteration using other vegetable oils (*7*, *8*), DNA markers, which have already been used to identify olive cultivars, are increasingly being applied to solve traceability and provenance issues (*9*−*15*). However, because whole fruits are crushed in the milling process and the embryo within each olive fruit has been fertilized by pollen from another cultivar, questions about the effect of paternal DNA on the genetic profiles need to be addressed before DNA markers can be used with confidence. In this study, the presence and impact of paternal DNA are investigated throughout the oil production process using simple sequence repeat (SSR), sequence characterized amplified region (SCAR), and single nucleotide polymorphism (SNP) markers.

MATERIALS AND METHODS

Plant Material. Six young leaves were collected from spatially well-separated branches of an olive tree (cv. Leccino). Three kilograms of ripe olive fruits was collected from the same tree, and olive oil was recovered separately from 1 kg of pitted fruits and 2 kg of whole fruits. The fruits were then milled in a Tecator 1094 homogenizer, and the

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10.1021/jf061564a CCC: $33.50
Published on Web 11/03/2006

Table 1. SSR Markers Used for DNA Amplification

code	locus	primer sequence (5′ → 3′)	repeat motif	T_a[a]
DCA1	ssrOeUA-DCA1 AJ279853	GCATGCGTACGAATTCTCCTCTGAAAATCTACACTCACATCC ATGAACAGAAAGAAGTGAACAATGC	$(GA)_{22}$	63–58
DCA3	ssrOeUA-DCA3 AJ279854	GCATGCGTACGAATTCTCCCAAGCGGAGGTGTATATTGTTAC TGCTTTTGTCGTGTTTGAGATGTTG	$(GA)_{19}$	68–63
DCA4	ssrOeUA-DCA4 AJ279855	GCATGCGTACGAATTCTCTTAACTTTGTGCTTCTCCATATCC AGTGACAAAAGCAAAAGACTAAAGC	$(GA)_{16}$	63–58
DCA5	ssrOeUA-DCA5 AJ279856	GCATGCGTACGAATTCTAACAAATCCCATACGAACTGCC CGTGTTGCTGTGAAGAAAATCG	$(GA)_{15}$	68–63
DCA7	ssrOeUA-DCA7 AJ279857	GCATGCGTACGAATTCTGGACATAAAACATAGAGTGCTGGGG AGGGTAGTCCAACTGCTAATAGACG	$(GA)_{19}$	63–58
DCA8	ssrOeUA-DCA8 AJ279858	GCATGCGTACGAATTCTACAATTCAACCTCACCCCATACCC TCACGTCAACTGTGCCACTGAACTG	$(GA)_{18}$	68–63
DCA9	ssrOeUA-DCA9 AJ279859	GCATGCGTACGAATTCTAATCAAAGTCTTCCTTCTCATTTCG AATCAAAGTCTTCCTTCTCATTTCG	$(GA)_{23}$	63–58

[a] Touchdown annealing temperature (T_a) for PCR amplification.

paste was slowly shaken for 1−2 h to allow oil drop formation (malaxation) and then centrifuged for 10 min at 5000*g* at 20 °C to recover the oil phase.

DNA Extraction. DNA was extracted separately from leaves, the flesh of 3 fruits and 11 embryos, and the respective pastes obtained after malaxation and centrifugation (pomace) steps during the oil production.

DNA was extracted from 100 mg of tissue (leaf or flesh) or one embryo with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. The DNA quantity and quality were checked using agarose gel electrophoresis along with known amounts of λ DNA.

DNA was extracted from oil samples using the official Swiss method for lecithin and oil DNA extraction (*16*) with minor modifications. Briefly, 2.5 mL was transferred into a 50 mL plastic tube and mixed with 15 mL of *n*-heptane. Extraction buffer (2 mL of 6 M guanidine thiocyanate, 100 mM Tris, 20 mM EDTA) was added to each tube, and the samples were mixed and then allowed to settle briefly. The aqueous phase (2 mL) was transferred into a new 2 mL tube and centrifuged for 10 min at 10000*g*. An aliquot (1 mL) of the aqueous phase was transferred into a new tube, mixed with 0.5 mL of chloroform, and then centrifuged for 10 min at 10000*g*. The aqueous phase (500 μL) was transferred to a new tube containing 5 μL of RNase A (10 mg/mL) and incubated for 10 min at room temperature. Isopropanol (0.8 volume) and 4 μL of glycogen (20 mg/mL) were added, and the mixture was gently mixed and incubated for 30 min at room temperature. Following centrifugation (10 min at 12000*g*), the pellet was washed in 500 μL of 70% ethanol and dissolved in 60 μL of 0.1× TE (1 mM Tris, 0.1 mM EDTA, pH 8).

Molecular Markers. Seven microsatellites (DCA1, DCA3, DCA4, DCA5, DCA7, DCA8, and DCA9; *17*), nine SNPs (LS2, LS5, Lup, Chs, Cycl-1, Cycl-2, Ant-1, Ant-2, and Cbp; *18*), and two SCARs (SOD and LS1; *18*) were used to confirm the identify of the Leccino tree by genotyping the six leaves against a reference Leccino DNA, obtained from the Research Institute on Oliviculture, CNR, Perugia, Italy.

The SSR markers were profiled using a LI-COR 4200 automated genotyper. The forward primer was "tailed" by the inclusion of 17 extra nucleotides at the 5′ end, which facilitated the labeling of the products using a labeled tail primer (**Table 1**). PCRs were performed in a 10 μL volume consisting of 20 ng of DNA, 0.5 unit of FastStart *Taq* DNA Polymerase (Roche), 1× buffer (Roche), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of tailed forward primer, 0.5 μM of each reverse primer, and the tail primer labeled with IRD^{700} fluorophore (MWG Biotech). The reactions were carried out in a thermocycler Perkin-Elmer 9700 (Applied Biosystems) with the following profile: 95 °C for 5 min, 6 cycles at 95 °C for 20 s, annealing temperature (**Table 1**) for 30 s decreasing 1 °C/cycle, extension temperature 72 °C for 30 s; followed by 29 cycles at 95 °C for 20 s, annealing temperature for 30 s, 72 °C for 30 s with a final extension at 72 °C for 6 min. An aliquot (1 μL) was loaded onto a 6% polyacrylamide gel (SequaGel-6, National Diagnostics) and electrophoresed at 40 W constant. Molecular size standards were loaded on the gels to assign the size to each allele. Alleles were scored manually.

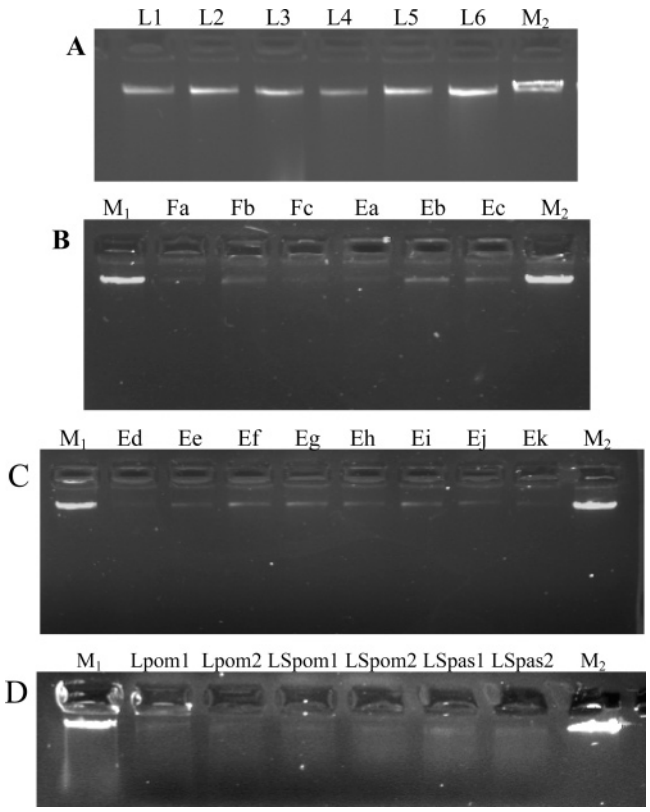

Figure 1. DNA extracted from (**A**) six young leaves of a tree of cv. Leccino (L1–L6), (**B**) three olive fruits (flesh, Fa–Fc, and respective embryos, Ea–Ec, separately), (**C**) eight more embryos, Ed–Ek, and (**D**) six paste samples obtained after the centrifugation step during the oil production process from only flesh (Lpom1 and Lpom2) and whole fruits (LSpom1 and LSpom2) and after malaxation (LSpas1 and LSpas2). M_1 and M_2 represent 25 and 50 ng of lambda DNA, respectively.

The SNP and SCAR markers were genotyped using the ABI Prism SNaPshot Multiplex System kit in an ABI Prism 3100 (Applied Biosystems) as described in Reale et al. (*18*).

To investigate the consistency in genotype profile between olive DNA extracted from material generated at various points through the oil production chain, we used a subset of markers to genotype the DNA extracted from the flesh of 3 fruits, 11 embryos, the pomaces, and the

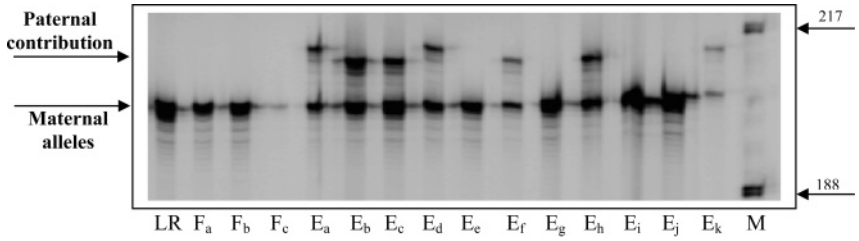

Figure 2. Microsatellite profiles of DCA1 of the reference leaf (LR), the flesh of three fruits (F_a–F_c) and 11 embryos (E_a–E_k) from the same tree. Sizes of the marker bands (M) are in nucleotides.

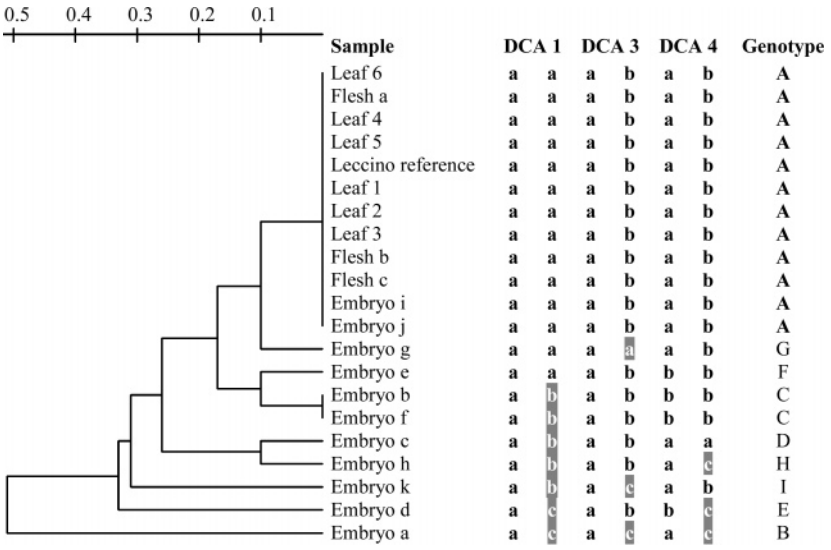

Figure 3. Dendrogram based on three SSR markers showing genetic divergence between different parts of the fruits (embryo and flesh), leaves, and reference variety by using UPGMA (*24*) cluster analysis and the Simple Matching similarity coefficient (*25*).

pastes obtained after the malaxation step during the oil production, and the oil samples as described above. Technical replicates were performed for each extraction, and the profiles presented are representative of repetitions for each genotyping assay.

RESULTS AND DISCUSSION

DNA Extraction. The amount of DNA that was extracted from leaf, flesh, embryo, and paste samples was variable: more DNA was recovered from leaves than from any other material (**Figure 1**). The quality and quantity of the DNA, as observed on agarose gels, are consistent with the finding that degradation of DNA occurs mostly in the stages of paste and pomace production (*19*). DNA extracted from olive oil samples could not be visualized on agarose gels, reflecting its low amount and/or quality.

Cultivar Identification. The identity of the olive tree was confirmed as Leccino by matching the profiles of all the SSR, SNP, and SCAR markers of the DNAs extracted from the six leaves to those of the reference Leccino DNA sample. Although intravarietal variation has been reported in some olive cultivars (*20*−*23*), no cases have been documented for Leccino.

Genotyping Pulp and Embryo of the Olive Fruit. The profiles of DNA obtained from the flesh of three fruits matched the leaf samples, whereas additional alleles were observed from the DNAs extracted from embryos (**Figure 2**). This is consistent with the flesh part of the fruit being maternal in origin, whereas the embryo also contains paternal DNA. Of the 11 embryos profiled using 3 SSRs, only 2 showed the Leccino profile. Nine embryos have non-maternal alleles that must have arisen from out-crossing (**Figure 3**). The finding of these additional alleles is consistent with Leccino being a self-incompatible cultivar. Fertilization occurs by pollen from cultivars such as Dolce Agogia, Kalamata, Aglandau, Gordal, Pendolino, Frantoio, Maremmano, Morchiaio, Gremignolo di Bolgheri, Piangente, and Trillo (*26*−*30*) depending on the varieties present in the region of cultivation, Leccino being one of the most widely diffused varieties in the world.

DNA Extraction from Different Stages in the Oil Production Chain. Paternal alleles were found in the paste and pomace of the oil-making process (**Figure 4**), albeit at lower intensities, reflecting the relative contributions of flesh and embryo tissue to the paste and pomace. DNA extracted from the paste made from pitted fruit showed only maternal alleles as expected.

There are limited reports on the extraction of DNA from olive oil (*9*−*15*). Previous works have described DNA extracted from cell residues recovered by centrifugation (*9*, *10*, *13*). However, this might not be possible when dealing with commercial olive oils because they are often filtered and thus lack sediments. Comparisons of different extraction methods have been reported (*11*, *31*). Here we use a modified method of the Swiss Official Method for extraction of DNA from lecithin and oil (*16*): this protocol allows DNA extraction starting from low volumes (ca. 2.5 mL) of oil. However, even after the products from three extractions were pooled to concentrate the template (second extraction), amplifications using 2 μL of template did not always result in visible products: only 50% of reactions were successful

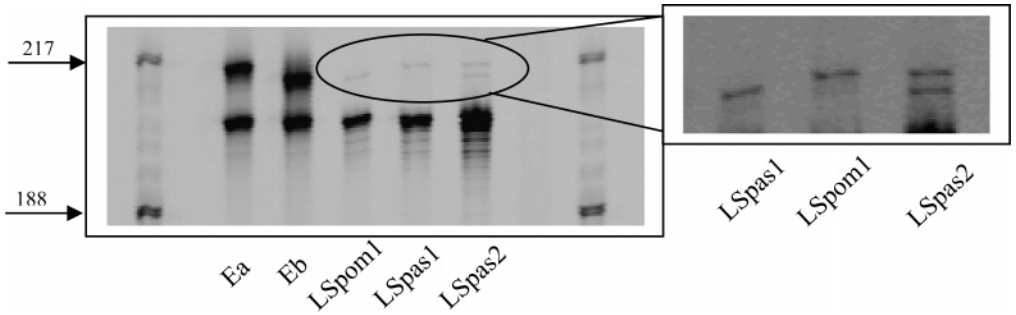

Figure 4. Amplification of SSR marker DCA1 on DNA extracted from material at different stages of the oil extraction procedure. SSR profiles of DNA extracted from pomace (pom) and paste (pas) DNA show the same-sized alleles that have been detected in the embryos (Ea and Eb from **Figure 3**). Sizes of the marker bands are given in nucleotides.

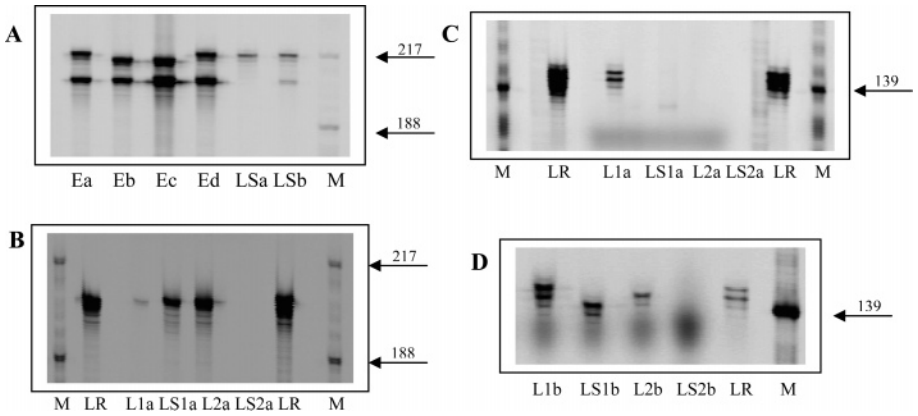

Figure 5. SSR markers DCA1 and DCA4 amplified from DNA extracted from olive oil samples: (**A**) DCA1, samples from first extraction (2.5 mL) of oil; LSa and LSb represent duplicate amplification of DNA from Leccino oil extracted from the whole fruit and Ea, Eb, and Ec represent the profiles obtained from the same embryo DNAs as shown in **Figure 2**; (**B**) DCA1 and (**C**, **D**) DCA4 samples from second extraction (2.5 mL × 3) of oil; LS2a/L2a and LS2b/L2b represent duplicate amplification (a and b) of Leccino oil extracted from the whole fruit (LS)/flesh part (L). Sizes of marker bands (M) are in nucleotides.

(**Figure 5**). Likewise, maternal alleles were amplified from oil samples with DCA1 and DCA4 (**Figure 5B**,**C**), whereas only one of the two was amplified in the DNA extracted from the destoned oil samples (**Figure 5D**). These results suggest that only small amounts of DNA can be recovered from these extractions.

In Breton et al. (*11*) 20% of olive oil samples failed to amplify. To improve the DNA yield from the oil, a higher volume of starting material could be used, because, in practical applications, oil volume would probably not be limiting. De la Torre et al. (*12*) used 500 g in total of olive oil, whereas only 200 μL was used in Testolin and Lain (*15*): in the latter case the "yield" was increased by the use of nested PCR.

The choice of markers for the amplificatioon of DNA extracted from olive oil is an important issue. Fragments longer than 300 bp could not be amplified (data not shown), which is in concordance with Pafundo et al. (*14*) and Testolin and Lain (*15*), whereas fragments of more than 1 kb were amplified by Busconi et al. (*10*). Highly informative markers such as SSRs would be prefereable due to the high heterozygosity reported in olive, especially in cases of oil mixes. Furthermore, the use of olive-specific markers would exclude amplification from contaminant DNA.

Taberlet et al. (*32*) have considered the consequences of very low DNA amounts leading to incorrect genotyping and suggested a mathematical model to reliably genotype samples when very low quantities of template DNA are used. This model takes into account the random sampling of template molecules in the extract and assumes that a single template molecule can be detected. However, non-maternal alleles were amplified from oil derived from whole fruits (**Figure 5**). These alleles can be attributed to paternal contribution (**Figure 5A**), although not all have been observed in the 11 embryos screened (**Figure 5D**). These non-maternal alleles are also found in the paste and pomace after oil extraction and within the individual embryos, indicating they are paternal in origin and not artifacts (**Figure 5A**).

A high degree of non-concordance was observed when profiles from commercial monovarietal oil were compared with leaf reference profiles (unpublished data). The interpretation of those results has been confounded by the authenticity of samples because the so-called commercial monovarietal olive oil can contain 5−10% of oil deriving from other cultivars.

This is the first study in which DNA from leaves, fruits, and oil deriving from a single olive tree is compared.

These results indicate that the profile obtained from oil is a composite of maternal and paternal alleles; therefore, cultivar identification using DNA markers is not a straightforward matter of matching the olive oil profile to a reference leaf sample or a genetic database of profiles. The number of possible allelic combinations is also exacerbated by the high levels of heterozygosity observed on olive (*18*). Notwithstanding the problems of obtaining reasonable amounts and quality of DNA from

the oil, the presence of paternal DNA from the embryos makes such analyses difficult. Paternal DNA would explain the presence of AFLP fragments in monovarietal oils not found in the olive cultivars from which the oils were derived (*14*). The presence of paternal DNA is not an insurmountable obstacle for provenance testing of oils: because the olives that make PDO, etc., oils are grown in specific regions, the pollinators are restricted and often are grown alongside the oil-producing fruit trees and may provide additional pointers to authenticity beyond the cultivar. Furthermore, the use of markers targeted to maternally inherited mitochondrial or chloroplastic DNA would solve this problem.

ABBREVIATIONS USED

PDO, protected designation of origin; PGI, protected geographical indication; TSG, traditional speciality guaranteed; SCAR, sequence-characterized amplification region; SSR, simple sequence repeat; SNP, single nucleotide polymorphism.

ACKNOWLEDGMENT

We thank Sandy Primrose for useful discussions and Andy Greenland for critical reading of the manuscript.

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Received for review June 5, 2006. Revised manuscript received September 19, 2006. Accepted September 25, 2006. This study has been carried out with financial support from the Food Standard Agency (Q01091).

JF061564A