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Traceability of Plant Contribution in Olive Oil by Amplified Fragment Length Polymorphisms

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Application of DNA molecular markers to traceability of foods is thought to bring new benefit to consumer's protection. Even in a complex matrix such as olive oil, DNA could be traced with PCR markers such as the amplified fragment length polymorphisms (AFLPs). In this work, fluorescent AFLPs were optimized for the characterization of olive oil DNA, to obtain highly reproducible, high-quality fingerprints, testing different parameters: the concentrations of dNTPs and labeled primer, the kind of *Taq* DNA polymerase and thermal cycler, and the quantity of DNA employed. It was found that correspondence of fingerprinting by comparing results in oils and in plants was close to 70% and that the DNA extraction from olive oil was the limiting step for the reliability of AFLP profiles, due to the complex matrix analyzed.

KEYWORDS: Food traceability; olive oil DNA; olive cultivars identification; improved AFLPs; reproducibility

INTRODUCTION

Olive (*Olea europaea* L.) cultivation and olive oil production started in the Mediterranean basin in the third millennium B.C. (*1*); even then they represented a relevant aspect for all Mediterranean cultures. Because of its importance in the diet and its beneficial effect on health (*2*), the consumption of olive oil is increasing throughout the entire world. In recent years, the request for higher food safety has raised a greater interest in the determination of product origin and authenticity. Olive oil traceability becomes instrumental to ensure the consumer's protection (*3*), particularly for extra virgin olive oil, the quality of which is highly related to the cultivars employed and to the environmental conditions of growth. Investigations concerning the origin and authenticity of olive oil through the chemical analysis of different oil's components (*4*) have showed that these can indeed be affected by the environment of the growing areas and by seasons (*5*). Conversely, plant DNA sequence in olive oil should be independent from the environment, and it might be used to trace specific plant genotypes in this complex food matrix. For the same reason, DNA analysis is gaining great attention in food forensics (*3*), for the recognition of raw materials in processed food or to discover misdescriptions and frauds (*6*).

Different studies (*7*−*9*) have reported that it is possible to obtain DNA from a small sample of olive oil, and it can be amplified by PCR. DNA markers have been developed to characterize plant genome, such as random amplified polymorphic DNA (RAPDs) (*10*), simple sequence repeats (SSRs) (*11*), restriction fragment length polymorphisms (RFLPs) (*12*, *13*), and, in particular, amplified fragment length polymorphisms (AFLPs) (*14*), which may allow the simultaneous screening of a large number of loci, without any need of preliminary sequence knowledge. For these advantages, and for their high reliability, AFLPs have been widely used for genotyping in a large number of crops and wild species, including olive (*15*, *16*). Busconi et al. (*9*) previously reported an AFLP fingerprinting of olive oil partially superimposable and agreeable with the cultivar from which the oil was made. Actually, DNA extraction from olive oil is characterized by a very low yield, by the extent of degradation, and by richness in phenolic compounds and polysaccharides, which can inhibit the activity of both restriction enzymes and DNA polymerases, giving rise to AFLP profiles that are not completely reliable.

To improve the applicability of AFLPs to traceability of DNA extracted from olive oil, we performed a detailed evaluation of the entire process, focusing on the evaluation of the reproducibility of profiles (i) in different DNA extractions, (ii) in different DNA restrictions, (iii) in the preselective amplifications, and (iv) in the selective amplifications and comparing AFLP fingerprints obtained from oil with AFLPs from the corresponding plant material. These parameters were evaluated on DNA from olive oil extracted with a new implemented method (*17*; Palmieri and Donini, submitted for publication).

The AFLP fingerprinting procedure, reported here, was tested on monovarietal olive oils obtained from four cultivars, Salonenque, Tanche, Arbequina, and Hojiblanca, but the method

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could be applied to traceability of the origin and authenticity of olive oil containing or made from other popular cultivars.

MATERIALS AND METHODS

Plant Material. Leaves and olives of the two French *Olea europaea* cultivars Salonenque and Tanche, and of the two Spanish *Olea europaea* cultivars Arbequina and Hojiblanca were collected in 2002 from a single plant for each cultivar, and used for DNA extraction and oil production. The procedure for extra virgin monovarietal oil production followed the standard methods employed in oil factories, with particular care to avoid any contamination with other oils. Leaves were stored at −20 °C, whereas oils were maintained in the dark at room temperature until DNA extraction.

DNA Extracted from Leaves. Genomic DNA was extracted from fresh leaves of the four cultivars by using a CTAB method (*16*).

DNA Extracted from Olive Oil. DNA was extracted from olive oil according to three different methods: (i) a method developed by Palmieri and Donini (*17*; submitted for publication), based on DNA extraction from paraffin with modifications, starting from 2 mL of olive oil; (ii) the method described by Busconi et al. (*9*); and (iii) the same CTAB method used for olive leaves. The quantification of the DNA extracted from oil samples was made with the spectrophotometer Biophotometer (Eppendorf, Hamburg, Germany).

AFLP Analysis. AFLP analysis was performed with a capillary electrophoresis system. The AFLP technique was carried out as described by Vos et al. (*14*), with minor modifications. Different quantities of oil DNA (50, 100, 200, and 250 ng) were double-digested with 5 units of *Eco*RI and *Tru*1I or *Tru*9I (isoschizomers of *Mse*I) and 1× restriction buffer in 40 μL at 37 °C for 3 h. Different restriction buffers were tested: buffer R+ (Fermentas, Vilnius, Lithuania), 10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mg/mL BSA; buffer OPA (Amersham Biosciences, Piscataway, NJ), 100 mM Tris acetate, pH 7.5, 100 mM magnesium acetate, and 500 mM potassium acetate; buffer B (Promega, Madison, WI), 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT; buffer MultiCore (Promega), 25 mM Tris acetate, pH 7.5, 100 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT; buffer Y+/Tango (Fermentas), 33 mM Tris acetate, 10 mM magnesium acetate, 66 mM potassium acetate, and 0.1 mg/mL BSA, pH 7.9; buffer A (Roche Applied Science, Mannheim, Germany), 33 mM Tris acetate, 10 mM magnesium acetate, 66 mM potassium acetate, and 0.5 mM DTT. Each buffer was used with the restriction enzymes provided by the same manufacturers. Buffers OPA, B, MultiCore, and A were supplemented with acetylated BSA, of the same manufacturer, at a final concentration of 0.1 μg/μL. The digestion was followed by the addition of 1 unit of T_4 ligase (Fermentas), 1× of the same buffer used in the digestion, 5 pmol of adapters for *Eco*RI, and 50 pmol of adapters for *Mse*I, 1.5 mM ATP, and acetylated BSA at 0.1 μg/μL in a final volume of 10 μL. The ligation was performed at 37 °C overnight.

Different dilutions of restricted/ligated products were tested, as 1:1 and 1:5 from restriction/ligation reaction to preselective PCR, and as 1:1, 1:10, and 1:20 from preselective to selective amplification.

PCR amplification conditions were optimized by varying the following parameters: (i) dNTP and primer concentrations; (ii) *Taq* DNA polymerases with different characteristics; and (iii) PCR in standard and real-time thermal cyclers. The dNTP concentrations tested in preselective amplification were 0.4 and 0.8 mM and in selective PCR were 0.3, 0.4, 0.5, and 0.8 mM. The *Eco*RI primer concentrations in the selective amplification were 0.1, 0.2, and 0.3 μM. The *Taq* DNA polymerases tested were Taq DNA polymerase (Amersham Biosciences), FastStart Taq DNA polymerase (Roche), and JumpStart RED AccuTaq La DNA polymerase (Sigma, St. Louis, MO). The thermal cyclers utilized were a Genius (Techne Cambridge Ltd., Duxford, Cambridge, U.K.) and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA).

On the basis of these preliminary experiments, the AFLP procedure was carried out in the following conditions: 8 μL of ligated DNA was preamplified in a final volume of 50 μL, with 1× PCR buffer [500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, pH 8.3, 20 mM $MgCl_2$], 0.3 μM of each preselective primer (*Eco*RI + A and *Mse*I + C), 0.4 mM dNTPs, and 1.5 units of FastStart Taq DNA polymerase by Roche. Preselective amplifications were performed in an ABI Prism 7000 thermal cycler, at the following conditions: 95 °C for 5 min; 25 cycles at 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min; 72 °C for 10 min. Aliquots of 5 μL from each reaction were selectively amplified in a final volume of 20 μL, with 1× PCR buffer [500 mM Tris-HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, pH 8.3, 20 mM $MgCl_2$], 1 mM $MgCl_2$, 0.2 μM *Eco*RI + AXX selective primer, 0.3 μM *Mse*I + CXX selective primer, 0.4 mM dNTPs, and 2 units of FastStart Taq DNA polymerase by Roche. Reactions were performed in an ABI Prism 7000 thermal cycler at the following conditions: 95 °C for 5 min; 13 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min and 30 s, with the decrease of 0.7 °C in the annealing temperature at each cycle; 22 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and 30 s; 72 °C for 10 min. The *Eco*RI + AXX selective primers were labeled with the fluorescent dye Cy5.5. The following primer combinations were checked for selective amplification: *Eco*RI + AAG/*Mse*I + CTG; *Eco*RI + ATT/*Mse*I + CAA; *Eco*RI + AAG/*Mse*I + CAA; *Eco*RI + ATT/*Mse*I + CAC; *Eco*RI + AAA/*Mse*I + CTG; *Eco*RI + AAA/*Mse*I + CAA.

Genomic DNA (250 ng) of different olive cultivars was used to obtain the AFLP profile of the plant; the protocol for AFLP analysis was the same as described for olive oil.

Amplified products from selective amplification were loaded and run 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 V for 30 s; separation voltage, 6 V for 50 min. Results were analyzed with Fragment Analysis of CEQ 2000 DNA Analysis System software.

Statistical Analyses. A nonparametric statistical method reported by Friedman (*18*)

$$\mathrm{Fr} = \sum_{i=1}^{k}\left(Ti - \frac{N(k+1)}{2}\right)^2$$

where *Ti* is the ranks sum for each group or condition, *N* is the number of samples considered for each group, and *k* is the number of groups or conditions under analysis, was applied to test for reproducibility.

A nonparametric statistical test proposed by Kruskal and Wallis (*19*) and by Kruskal (*20*)

$$g = \left(\frac{12}{N(N+1)}\sum_{i=1}^{k}\frac{R_i^2}{n_i}\right) - 3(N+1)$$

where *R* is the ranks sum for each group, n_i is the number of observations for each group, *N* is the total number of observations, and *k* is the number of groups under analysis, was used to compare AFLP profiles in cultivars and oils.

RESULTS

Comparison of AFLP Profiles Obtained with Different DNA Extraction Methods from Monovarietal Olive Oil. Three different methods of DNA extraction from olive oil were compared. In these first experiments the standard AFLP protocol (*15*) was used, and for DNA extraction the evaluation was done not only to obtain a good profile but also with the potential applications of the method to analyses in an industrial context considered. The profile obtained with the CTAB method presented few peaks (data not shown). The method developed by Busconi et al. (*9*) started from at least 40 mL of olive oil, whereas the Palmieri and Donini method began with only from 2 mL of oil; for this reason, the latter was chosen.

Experimental Conditions of AFLP Analysis in Monovarietal Olive Oil. In the AFLP protocol developed were varied

(i) the restriction buffer, (ii) the dNTP and (iii) the fluorescent primer concentrations, (iv) the dilution of restriction/ligation and preamplified products, (v) the *Taq* DNA polymerase, (vi) the thermal cycler, (vii) the amount of oil DNA, and (viii) the selective primer combination. All of these experiments were made in duplicates for each monovarietal oil used. At the beginning was used the restriction OPA ("one for all") buffer, 0.3 mM dNTPs, 0.1 μM fluorescent primer, no dilution of restricted/ligated and amplified products, a standard *Taq* DNA polymerase and a standard thermal cycler, 250 ng of DNA from oil, which is the same amount utilized for DNA extracted from olive leaves (*18*), and the selective primer combination *Eco*RI + AAA/*Mse*I + CAA. These experiments were conducted on two monovarietal oils to compare the conditions in different samples and to verify that the results were reproducible. Results obtained from oil, after capillary electrophoresis, were visualized as electropherograms, where peaks of fluorescence corresponded to fragments of particular length and the height of each peak was related with the number of copies of that amplified fragment. The parameters used to assess the efficiency of the protocol were (i) the maximum number of peaks, (ii) the average intensity of the signal, (iii) the intensity of lower peaks, because the presence of peaks below a certain threshold, set at 8000 relative fluorescence units (RFU), can indicate a background of unspecific amplification, and (iv) the intensity of the highest peak, because it showed the maximum reading of fluorescence. This last observation is not always confirmed, because the high fluorescence intensity of some peaks could be due to the preferential amplification of certain fragments; nevertheless, in general it is recognized as a sign of a good amplification. In each experiment, not all of these parameters were considered simultaneously, because they were chosen for their relevance in the different tests.

The first attempt to improve AFLP analysis in olive oil was a new setup of the restriction conditions. For this purpose, different restriction buffers were tested for their capacity to increase the signal intensity in the AFLP profile: buffer A by Roche gave the highest average signal in both oils (data not shown), and therefore it was utilized for further trials.

At this point, the efforts were directed to assess the optimal conditions for preselective and selective amplifications. In general, in AFLP protocol dilutions of restricted/ligated and preamplified products are done before the further steps. Therefore, in our work we performed experiments on diluted samples, but the best results were obtained without any dilution. In fact, the use of diluted samples led to a drastic decrease in fluorescence intensity (data not shown). The second parameter considered was the dNTP concentration. For this purpose, concentrations of dNTPs varying from 0.3 to 0.8 mM were tested in preselective and selective amplification conditions. As shown in **Figure 1A**, the efficacy of amplifications, evaluated as the mean fluorescence intensity, increased until 0.4 mM and then slightly decreased. Therefore, 0.4 mM dNTPs was considered to be the optimal concentration for further analyses. Because signal intensity was a consequence of the fluorescent labeling of the *Eco*RI selective primer, three concentrations of the dye were tested in the selective amplification: 0.1, 0.2, or 0.3 μM. As shown in **Figure 1B**, the highest intensity of signal was obtained with a primer concentration of 0.2 μM. With a primer concentration of 0.1 μM, fewer peaks were above the threshold of fluorescence set to 8000 RFU. The highest point of the curve of **Figure 1B** was reached at 0.2 μM primer concentration, and then the fluorescence signal decreased.

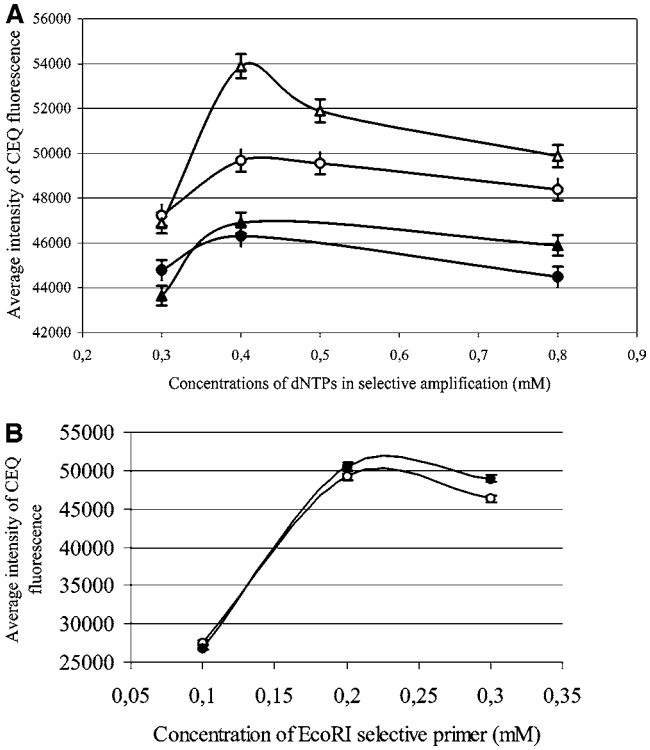

Figure 1. Improvement of amplification conditions for AFLP analysis in monovarietal olive oils. (**A**) Effect of different concentrations of dNTPs in monovarietal oil from Salonenque (circles) and Tanche (triangles) in preselective and selective amplifications. Open symbols indicate 0.4 mM dNTPs in Salonenque (○) and in Tanche (△), and solid symbols indicate 0.8 mM dNTPs in Salonenque (●) and in Tanche (▲) in preselective amplification conditions. (**B**) Variations of signal intensity with different concentrations of Cy5.5-*Eco*RI selective primer in monovarietal oil from Tanche (open symbols) and Salonenque (solid symbols). Bars represent the error interval calculated as 1% on the total value.

Modifications of these parameters did not significantly affect the number of peaks, and therefore signal intensity was considered for evaluating the process.

With standard *Taq* DNA polymerase a background of peaks of low intensity (<8000 RFU) was observed, and then we tested whether the utilization of more specific *Taq* polymerases improved the quality of profiles. For this purpose, two Hot Start enzymes, FastStart Taq DNA polymerase by Roche and JumpStart RED AccuTaq La DNA polymerase by Sigma, which are "high fidelity" and able to amplify very small amounts of DNA, were tested. Evaluation was made considering not only the average fluorescence intensity produced with these DNA polymerases but also the number of peaks observed after capillary electrophoresis, with the intensity of the lowest peak as well. The utilization of different *Taq* polymerases affected all parameters considered: as shown in **Figure 2A**, *Taq* DNA polymerase by Amersham Biosciences gave the highest number of peaks, but the mean and the minimal intensity were lower. These values increased with *Taq* polymerase by Sigma and with enzymes purchased by Roche that gave an electropherogram with peaks of the highest intensity. On the basis of these considerations, Taq DNA polymerase by Roche was considered to be the most efficient and utilized in further trials.

Two different thermal cyclers were tested: (i) Genius, a standard thermal cycler; and (ii) ABI Prism 7000, a rapid cycle thermal cycler for real-time PCR, used in this case to perform standard PCR. For all of the parameters analyzed, the number

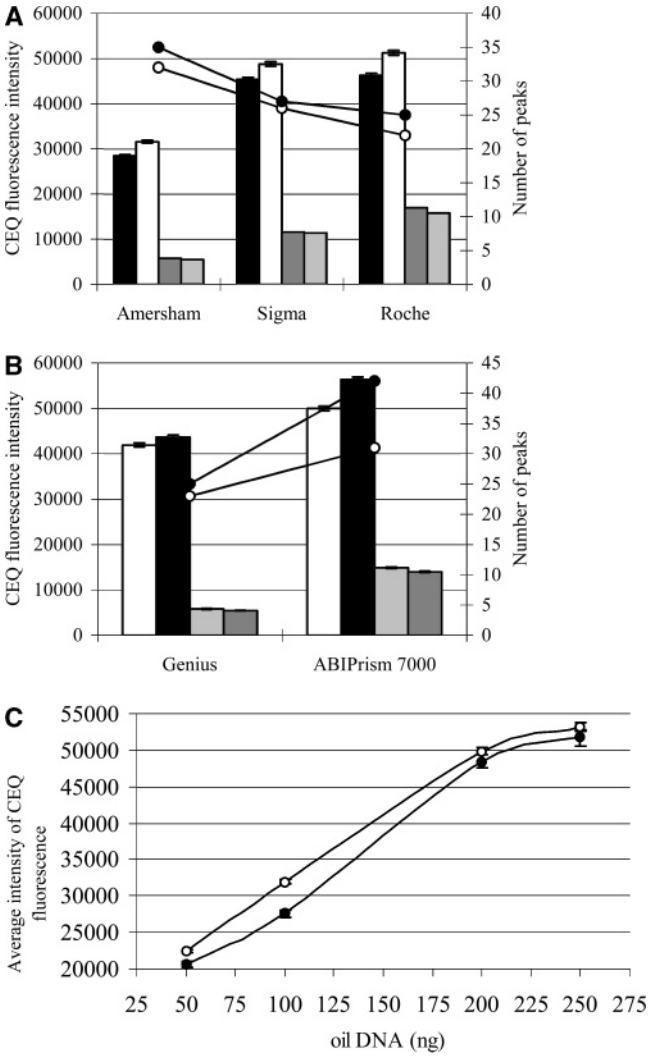

Figure 2. Effect of varying the amplification conditions on AFLPs in monovarietal olive oils. (**A**) Histograms represent the effects of different *Taq* DNA polymerases with different characteristics on average signal intensity in monovarietal oil from Tanche (black bars) and Hojiblanca (open bars), on the signal intensity of the lowest peak in monovarietal oil from Tanche (dark gray bars) and Hojiblanca (light gray bars); number of peaks in monovarietal oil from Tanche (open symbols) and Hojiblanca (solid symbols). (**B**) Effect of different thermal cyclers on average signal intensity in monovarietal oil from Tanche (open bars) and Arbequina (closed bars); signal intensity of the lowest peak in monovarietal oil from Tanche (light gray bars) and Arbequina (dark gray bars); and number of peaks in monovarietal oil from Tanche (open symbols) and Arbequina (closed symbols). (**C**) Variations of signal intensity with different concentrations of DNA in monovarietal oil from Arbequina (solid symbols) and Hojiblanca (open symbols). Bars represent the error interval calculated as 1% on the total value.

of peaks obtained and the average and minimal intensities, the ABI Prism 7000 gave the best results (**Figure 2B**).

Considering the low amount of DNA extracted from oil, usually no more than 10 ng/mL of oil, it was important to determine the lowest amount of DNA required to obtain a good AFLP profile, by reducing the quantities of DNA, with respect to those generally suggested in the standard AFLP protocol. As shown in **Figure 2C**, the efficiency increased almost linearly with the DNA concentration, from 50 to 200 ng, whereas in the range between 200 and 250 ng of DNA the signal intensity did not increase proportionally; therefore, an amount of 200 ng of DNA for each reaction was considered to be optimal to perform AFLPs on DNA extracted from olive oil.

Finally, six different primer combinations were used on Tanche oil DNA, to evaluate the effect of three different nucleotides in 3′ ends of the selective primers on the number of peaks and on the intensity of the lower and the higher peaks. The combination *Eco*RI + ATT/*Mse*I + CAA gave the better results (data not shown).

Reproducibility of AFLP Profiles on DNA from Monovarietal Olive Oil. On the basis of the previous experiments, reproducibility tests were applied on DNA extracted from olive oil.

Reproducibility tests were performed by comparing the AFLP profile of DNA extracted from four olive oils (Arbequina, Hojiblanca, Salonenque, and Tanche). The trials were made for all four oils using two selective primer combinations, *Eco*RI + AAA/*Mse*I + CAA and *Eco*RI + ATT/*Mse*I + CAC. The results were treated as percentage of the common fragments present in the two/four samples on the total fragments obtained in the trial, using the two selective primer combinations. We considered only the peaks detected by the instrument with fluorescence >2000 RFU, because fragments with lower signal intensity may derive from DNA degradation.

In **Figure 3A** the distribution of the obtained results is shown, considering the reproducibility of AFLPs between profiles obtained (i) by four DNA extractions performed at intervals of weeks, (ii) by restriction/ligation products from two digestions carried out at different times, (iii) by two amplified products of the same preselective PCR, and (iv) by two replicates of the identical selective amplification. The reproducibility in AFLP profiles was close to 60% using DNA extracted at different times, whereas it was close to 86% using different restriction/ligation products. In the case of two replicates of the preselective amplification, the value was near 96% and it increased to 99% when the products of the same selective amplification were considered. In **Figure 3B** testing of the reproducibility by using different oils and in the same steps of the entire AFLP procedure is shown. The results were comparable in all of the monovarietal oils. The application of Friedman's nonparametric statistical method (*18*) showed that reproducibility values in each step were significantly different (Fr 80, $p \leq 0.001$). No significant difference was found in reproducibility when different monovarietal oils were analyzed.

To evaluate the correspondence between cultivar and oil, six extraction replicates of the same oil, extracted at intervals of weeks, were used. This parameter was evaluated for three conditions (**Table 1**): (i) using for the comparison bands common to all six extracted samples; (ii) using bands common to 50% of the extracted samples; and (iii) considering bands in all of the extracted samples (common and not common). Also in this case, only the fragments with intensity in the electropherogram up to 2000 RFU were considered. The same two selective primer combinations were utilized. The correspondence values found in these three conditions changed significantly, as confirmed by the nonparametric statistical method proposed by Kruskal and Wallis (*19*, *20*) ($g = 9.846$, $p \leq 0.025$). The maximal correspondence reached 70.10% when all of the peaks were taken into consideration.

DISCUSSION

A first analysis of olive oil DNA with the standard AFLP protocol produced similar profiles by using either the method

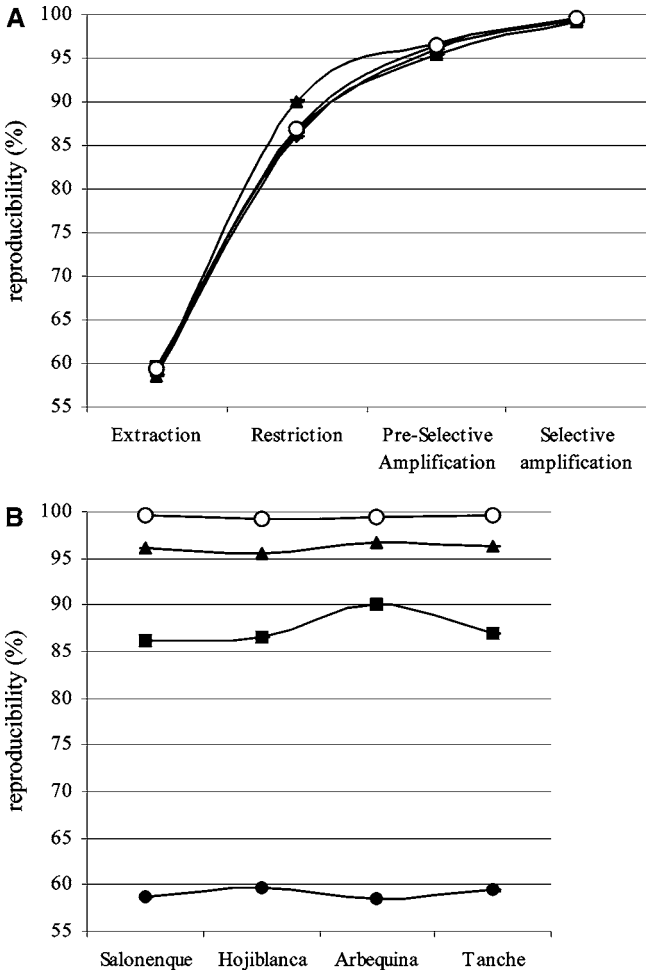

Figure 3. Results of reproducibility tests. (**A**) Importance of the different steps of AFLP methodology, extraction, restriction, preselective amplification, and selective amplification, on the reproducibility of AFLPs in four monovarietal oils: Salonenque (◆); Tanche (○); Hojiblanca (■); Arbequina (▲). (**B**) Influence of the use of different oils, Salonenque, Tanche, Hojiblanca, and Arbequina, on the reproducibility of AFLP profiles, analyzed in different steps of the method: extraction (●); restriction (■); preselective amplification (▲); selective amplification (○). The results obtained are reported as percentages. Data are the average between the results obtained with the two primer combinations, rounded off excess.

Table 1. Correspondence (as Percentages) between Cultivar and Oil AFLP Profiles[a]

cultivar	bands common to all extracted samples	bands common to 50% of the extracted samples	bands (common and not common) in all extracted samples
Salonenque	36.10	48.40	68.80
Arbequina	33.90	48.10	70.10
Tanche	35.40	48.30	69.50
Hojiblanca	34.30	47.50	69.20

[a] Data are the average between the results obtained with the two primer combinations, rounded off excess.

developed by Busconi et al. (*9*) or the method developed by Palmieri et al. (*17*) (data not shown). Therefore, application of a molecular marker technology to the analysis of a complex food matrix, such as olive oil, required a series of adjustments. First, optimization of restriction conditions was considered, because the double digestion was an important and delicate step in AFLP analysis (*21*). The presence of inhibitors and the relevant degradation of DNA extracted from olive oil (*8*) can modify the capacity of the restriction enzyme to cut into the proper restriction site. Therefore, an appropriate restriction buffer was chosen, in which the presence of different reagents, such as DTT or different concentrations of the basic components, could improve the performance of restriction enzymes.

The optimization of the AFLP protocol required also the evaluation of the dilution of the double restriction/ligation and of the preselective products. It was observed that a dilution of the sample may have a negative effect, different from the results previously reported by Busconi et al. (*9*). The different effects of dilution can be related to the different extraction methods utilized and to the different qualities of DNA obtained. Other authors (*22*, *23*) have reported that increasing the dNTP and labeled primer concentrations above the standard values, 0.2 mM and 0.1 μM, respectively, can positively affect the AFLP experiments. This seems to be consistent with our results: in fact, the number of fragments obtained increased with the concentration of labeled primers. However, for dNTP concentration >0.5 mM and for *Eco*RI + AXX, labeled with Cy5.5, >0.25 μM, these increments may have negative effects on AFLP resolution. Probably, the performance of the DNA polymerase could be hampered by the excess of some substrates.

A good AFLP profile should contain a high number of peaks and a low background with peaks of low intensity; these are generally products of unspecific amplifications, due to the low quality of DNA extracted from oil, and they can bias the interpretation of results. We tried to improve the AFLP methodology to have a higher fluorescence intensity of the AFLP but with also a reduction in the background, utilizing standard *Taq* DNA polymerase and a conventional thermal cycler or Hot Start *Taq* polymerase and a real time thermal cycler. Due to their sensitivity and high fidelity, the use of a Hot Start *Taq* and a real time cycler allowed good AFLP profiles, with peaks easily recognizable, to be obtained. Therefore, this aspect proved to be important in assessing the reliability of the method, in particular, to distinguish between profiles obtained from different monovarietal oils.

Another critical step was optimizing the quantity of DNA to be used. As reported previously by Sauzo and Hall (*22*), increasing the DNA concentration may improve the signal intensity of AFLP profiles. However, because the amount of DNA extracted from olive oil was low, but rich in inhibitory compounds, it might be desirable to reduce its amount as much as possible to avoid effects on the profile's quality. Our results suggest that increasing the DNA amount above 200 ng does not improve significantly the quality of AFLPs. However, below this concentration of DNA, the quality of AFLP profiles was reduced.

We have found in performing AFLP fingerprinting on DNA from olive oil that profiles (**Figure 4**) included 20−30 peaks corresponding to fragments with molecular weights from 60 to 350 bp; the average fluorescence signal was 35000 RFU. These values varied depending on selective primer combinations, because of the capacity of the selective primer to amplify a specific part of the restriction products, due to the particular nucleotides in the 3′ end of its sequence.

Although reliability of AFLP profiles for genotyping was already assessed in different studies (*24*), fingerprinting DNA extracted from food matrix and in particular from olive oil represented a new and formidable challenge. Olive oil, in our

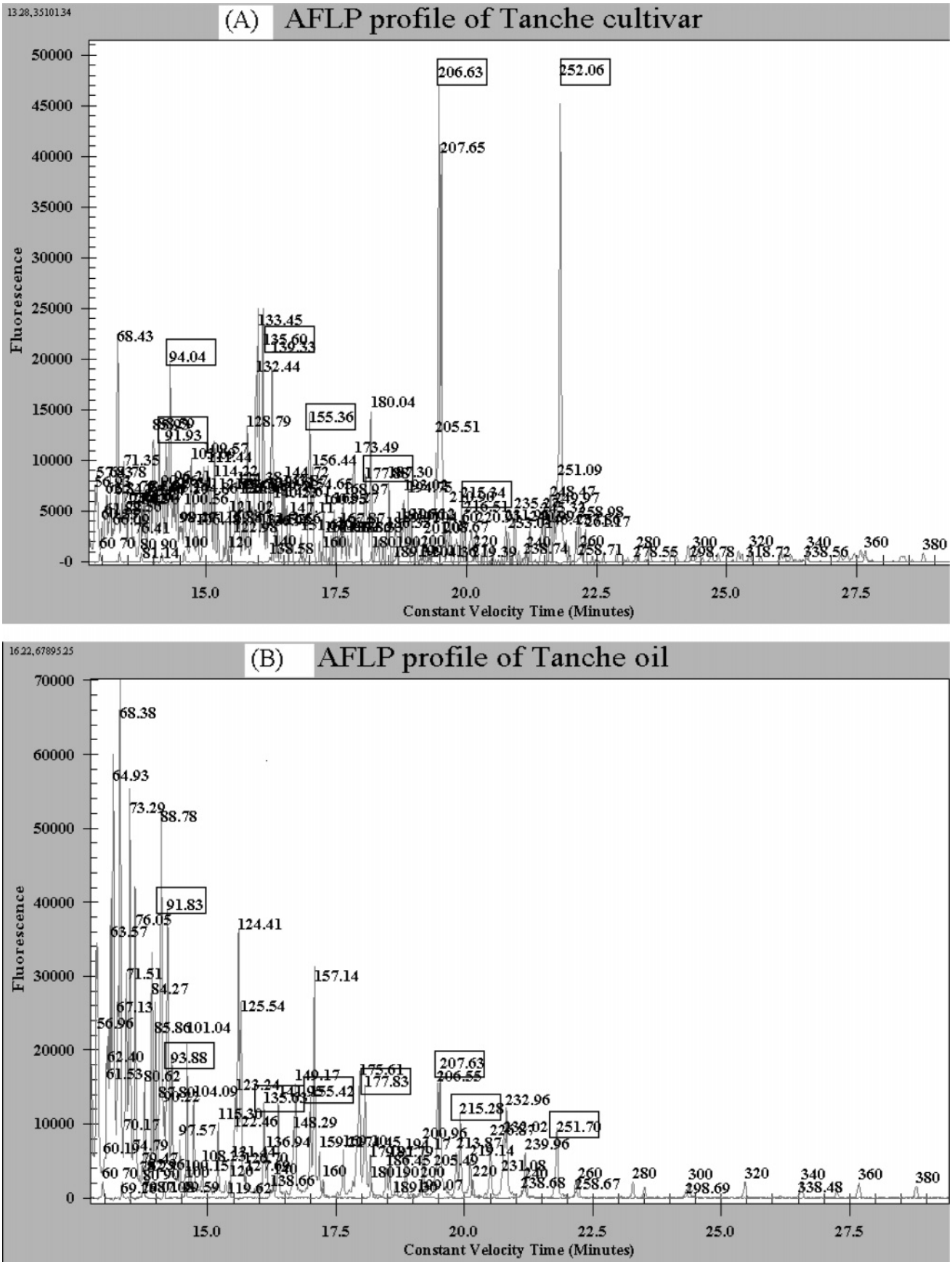

Figure 4. AFLP profiles of Tanche cultivar and its corresponding monovarietal oil. The electropherograms were obtained through capillary electrophoresis with *Eco*RI+AAA primer, labeled with the fluorescent dye Cy5.5: (**A**) fingerprinting of Tanche cultivar; (**B**) fingerprinting of Tanche monovarietal oil. Basal line: CEQ DNA Size Standard Kit 600. Rectangles indicate peaks corresponding between cultivar and oil.

case, contained a large number of inhibitors of enzyme activity potentially leading to artifactual and nonreliable profiles. Therefore, the reproducibility along the entire AFLP process, from the selective amplifications to the DNA extractions, to find and tag critical steps, was tested. Reproducibility within the same DNA extraction, in all steps considered to perform AFLPs, was high, when peaks above a certain threshold only were considered for evaluation. Reproducibility along the entire AFLP procedure was represented in a curve, which started from the lowest value (reproducibility for DNA extraction) and reached the highest value of 99% for selective amplification. Our conclusion is that DNA extraction from olive oil was the most critical step in the whole AFLP procedure. Matrix composition may have a great effect on AFLP reproducibility related to DNA extraction. DNA purity can vary in different preparations, affecting the activity of enzymes used for AFLP procedure. Moreover, degradation of DNA can affect the size and number of fragments recovered in AFLPs: if a fragment that possesses a restriction site is broken down or not cut down, it will not be detected in the AFLP profile. A positive note is that several fragments, with high intensity and very reproducible among different oil samples, were found.

Correspondence between AFLP profiles in cultivars and oils reached a maximum at 70.10% when bands present in all replicates were considered (**Table 1**). This value decreased to 33.90% when bands common to all samples only were considered. Although some bands were not always reproducible in the oil replicates, they were always present in the cultivar but can be amplified or not in the oil depending on the DNA quality. By adding all fragments identified in different extractions, a "virtual oil AFLP profile" can be generated, which matches with the "real cultivar AFLP profile". Consequently, reading and reproducibility of olive oil AFLP profiling rest on the analysis of independent DNA extractions for the same food source.

Moreover, in AFLP profiles of DNA from plants there were a lot of longer fragments, with different fluorescence intensities, but the coincidence with the oils' AFLPs was restricted to the shorter fragments, below 250 bp. Moreover, the oil DNA gave some fragments that were not detectable in DNA from plants.

To improve the traceability of olive oil, also in terms of costs and efficiency, the conversion of fragments of AFLP patterns to a simple, codominant PCR-based marker could be advisable, to obtain a sequence-characterized amplified region (SCAR), as those derived from RAPD markers developed from olive, which were previously reported (*9*, *25*). Development of SCAR markers derived from reproducible fragments obtained through AFLP fingerprinting (*26*−*30*) of monovarietal oil will be even more useful. Some of these SCAR markers have already been designed, and they will be tested in different olive cultivars to find polymorphisms, and in the corresponding oils for the same purpose. The same SCAR markers, due to their single-locus specific nature, can help in distinguishing cultivar contribution to a given olive oil sample.

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