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MULTIPLEX AMPLIFICATION OF DNA MICROSATELLITE MARKERS TO FINGERPRINT OLIVE OILS FROM SINGLE CULTIVARS

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Up to now, various categories of DNA-based markers have been employed in *Olea europaea* L. with cultivar identification purposes but none has been effectively used to analyse the olive oils. Since generally microsatellites have short sizes, this enables expecting good possibilities of detection also in the case of degraded DNA such as that recovered from olive oil. In this work, we aimed to apply the analysis of these markers to virgin oils obtained from five different olive cultivars, with fingerprinting purposes. Certified plant material was used and the oil was extracted from drupes by means of a laboratory-scale plant. Then, after oil centrifugation and DNA extraction from the remaining cellular residue, the amplification reactions were carried out and ten primers were tested. Out of them, four primers were chosen to achieve fingerprinting of the five types of oil and a multiplex DNA amplification was set up, basing on two of them, to obtain more information on a unique PCR reaction.

INTRODUCTION

Olive (*Olea europaea* L.) is a cultivated species for the production of high quality oil and fruits for human consumption. The crop, distributed throughout the Mediterranean basin, presents a very high number of cultivars difficult to recognise on the basis of morpho-physiological traits, so that some cases of homonymy and synonymy still exist [Fontanazza, 1993].

Molecular markers are independent by environmental conditions and are characterised by higher polymorphism than morphological and biochemical markers. Some kinds of molecular markers, such as RAPDs [Fabbri *et al*., 1995; Wiesman *et al*., 1998; Gemas *et al*., 2000], AFLPs [Angiolillo *et al*., 1999], ISSRs [Pasqualone *et al*., 2001], and recently microsatellites [Sefc *et al*., 2000; Carriero *et al*., 2002; Cipriani *et al*., 2002], have been proposed and used to achieve a better distinction of olive cultivars. However, these markers have not been effectively used to analyse the olive oils.

Microsatellites [Tautz, 1989] are simple sequence repeats, with dimensions generally comprised between 180 and 350 bp, ubiquitously interspersed in eukaryotic genomes. They show a high degree of polymorphism, due to variations in the number of repeats, that is easily detectable *via* PCR using specific primers designed on their flanking regions. Microsatellite markers are easy to handle, highly informative and repeatable and they have already been employed successfully for cultivar identification purposes in many species, including *O. europaea*.

A reliable method of cultivar fingerprinting, possibly by simultaneous amplifying multiple loci (multiplex DNA amplification), is essential to detect the presence of not declared cultivars in oils when a preserved origin denomination is awarded to them. Since the short dimension of microsatellites allows expecting good possibilities of detection also in the case of degraded DNA such as that recovered from olive oil [Pasqualone & Caponio, 2000], in this work we aimed to apply the analysis of microsatellite markers – by means of multiplex DNA amplifications – to virgin oils obtained from five different olive cultivars, with fingerprinting purposes.

MATERIALS AND METHODS

Samples. An amount of 3 kg of olives from the following Italian *Olea europaea* L. cultivars: Cima di Melfi, Nocellara del Belice, Pendolino, Picholine, and Toscanina, was collected by hand at the right stage of ripening. Certified plant material was used and the oil was extracted from drupes by means of a laboratory-scale plant available at the Section of Agro-Food Industries of Pro.Ge.S.A. Dept., University of Bari, Italy, consisting of a SK1 hammer-crusher (Retsch, Haan, Germany), whose crushing device and rotational speed adequately approximated those of the same type of machines used in the oil-milling industries, and a subsequent kneader (Agrimec Valpesana, Florence, Italy). The olive paste recovered from the crusher was put into the kneader bowl and malaxed for 30 min. The oils were finally recovered from the olive paste by means of a basket centrifuge. The plant was accurately cleaned after each batch of olives so as to avoid contamination among different cultivars.

DNA extraction. An amount of 250 mL of each oil was centrifuged at 10 000 rpm for 5 min and the DNA extraction from the resulting residue was carried out by Gene Elute Plant

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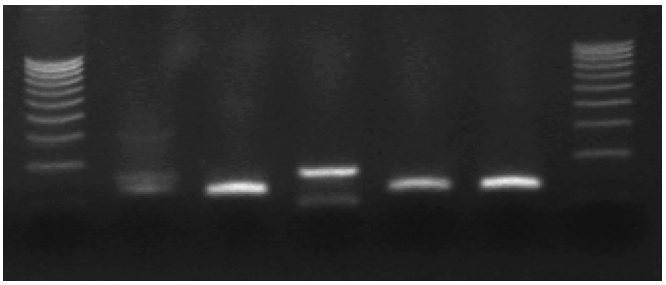

FIGURE 1. Agarose gel showing the electrophoretic patterns of the examined *O. europaea* cultivars with primer UDO99-019. The order of the cultivars is the following: Cima di Melfi, Nocellara del Belice, Pendolino, Picholine, Toscanina. On each side the marker 100 bp (Sigma) was loaded.

Kit (Sigma, St. Louis, MO, USA) following the manufacturer's indications. The concentration of the extracted DNA was determined on 8 g/kg agarose gel by comparison with λ DNA solutions at known concentration. DNA extraction from leaves was carried out with the same kit starting from 25 mg of lyophilised tissue.

Amplification and detection of microsatellite markers. Ten primer pairs of microsatellite markers, reported in Table 1, were used [Sefc *et al.*, 2000; Carriero *et al.*, 2002; Cipriani *et al.*, 2002]. Primers were synthesised by Sigma Genosys (St. Louis, MO, USA). Amplification reactions were performed in a I-Cycler programmable thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a reaction mix with the following composition: 30 ng di DNA, 1× PCR buffer, dNTP 0.25 mM, primer *forward* e *reverse* 2.5 μM each, RED*Taq* DNA polymerase (Sigma, St. Louis, MO, USA) 1 U, in a volume of 25 μL. For multiplex amplification the amount of *Taq* DNA polymerase was increased to 2 U. The amplification conditions were: 5 min at 95°C; 35 cycles at 95°C for 20 sec, at the appropriate annealing temperature for 30 sec, at 72°C for 30 sec; final elongation at 72°C for 7 min. The amplification products were separated by electrophoresis on 14 g/kg agarose gels in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA), stained by ethidium bromide and visualised under UV light. Fragment dimensions were attributed by comparison to AmpliSize and 100 base pairs (100 bp) molecular-size markers (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS AND DISCUSSION

In order to evaluate the efficiency of microsatellite markers for virgin olive oil varietal identification purposes, ten primers of microsatellite sequences were tested on the DNA extracted from the remaining cellular residue after centrifugation of the five virgin olive oils examined. Leaf DNA was initially used to assess optimal PCR conditions as well as to screen the effectiveness of the primers. Subsequently, DNA extraction from drupes and monovarietal oils, rich in PCR interfering substances such as phenolic compounds and lipids [Rossen *et al.*, 1992; Wilson, 1997], was set up.

Figure 1 shows the electrophoretic patterns obtained by amplifying the DNA of the examined oils with the primer UDO99-019. In spite of the fact that the template DNA was highly degraded (data not shown), all the samples were successfully amplified.

Table 1 reports, for each primer of microsatellite sequence, the minimum and maximum dimensions of the amplified fragments and the total number of detected

TABLE 1. Characteristics of the microsatellites analysed in the investigation and electrophoretic data of the patterns detected in the five examined oils.

Microsatellite	Primer sequence (forward and reverse, 5' to 3')	Annealing temperature (°C)	Maximum number of bands	Band sizes (min, max)	Total detected patterns
UDO99-001	AAAAATCACTTCTATTTTTGTTAG GCTCTCACGAGGAAGACTAT	50	1	120*	1
UDO99-007	TGTGTTCTTTATTTGAAGGAATCTT TCGCTTTTGTGTTACATATTCG	45	2	90, 110	2
UDO99-009	TTGATTTCACATTGCTGACCA CATAGGGAAGAGCTGCAAGG	45	3	90, 110	3
UDO99-019	TCCCTTGTAGCCTCGTCTTG GGCCTGATCATCGATACCTC	55	3	100, 290	3
GAPU59	CCCTGCTTTGGTCTTGCTAA CAAAGGTGCACTTTCTCTCG	55	1	220*	1
GAPU72	GAGGCTTTTTAATCCGAGCA AAAAAGAGGGGAGGAGAGAG	50	2	200, 210	3
GAPU101	CATGAAAGGAGG GGG ACA TA GGCACTTGTTGTGCAGATTG	55	1	195, 205	3
DCA9	AATCAAAGTCTTCCTTCTCATTTCG GATCCTTCCAAAAGTATAACCTCTC	50	2	180, 200	3
DCA11	GATCAAACTACTGCACGAGAGAG TTGTCTCAGTGAACCCTTAAACC	45	2	170, 180	2
DCA15	GATCTTGTCTGTATATCCACAC TATACCTTTTCCATCTTGACGC	55	1	260, 280	3

*a single band was obtained (monomorphic primer pair)

patterns. The patterns were composed of 1-3 fragments whose length was found ranging from the minimum of 90 base pairs to the maximum of 290. Of particular interest were the microsatellites GAPU101 and DCA15 because of their easy pattern composed by a single very polymorphic amplified fragment, able to detect three different patterns. No other microsatellite, except the useless monomorphic UDO99-001 and GAPU59, gave a single fragment, and this ensures good conditions to perform multiplex amplifications.

Out of the ten microsatellites tested, four of them were chosen to achieve fingerprinting of the five types of oil by combining their amplification patterns. The obtained fingerprinting is schematised in Figure 2 and it enabled distinguishing the five examined oils. Indeed, also combinations of a number of microsatellites lower than four, for example the two-microsatellite combinations DCA15 + GAPU101 and UDO99-007 + UDO99-019, were able to distinguish the oils from all the examined cultivars. A higher number of microsatellites was preferred in order to achieve a more complete pattern, with an adequate number of bands. Besides, the choice

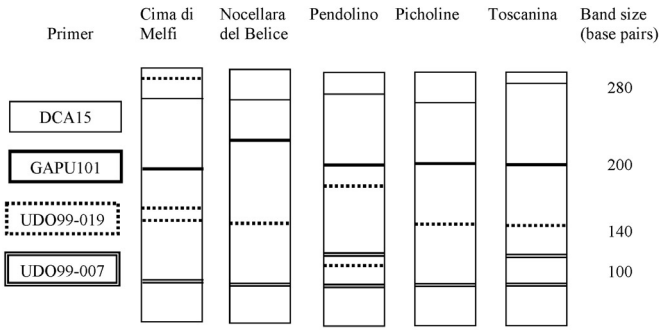

FIGURE 2. Schematised fingerprint of the examined *O. europaea* cultivars based on microsatellites DCA15, GAPU101, UDO99-007 and UDO99-019. On the right side of each amplified fragment are reported the dimensions in base pairs.

was based on a comparison of the patterns from the different microsatellites, in order, as much as possible, to avoid their overlapping.

Finally, multiplex DNA amplifications were carried out in order to achieve more information on a unique PCR reaction. To perform a multiplex amplification, it is essential for the selected primers to produce robust PCR products and amplify a single locus each [Tang *et al.*, 2003]. This was the case of the two primers DCA15 and GAPU101. Besides, since when more than one primer pair is present they will compete to anneal to the template, this has to be considered in regulating the amounts of the various primers in the reaction mixture.

Figure 3 reports the electrophoretic pattern obtained by using the primers DCA15 and GAPU101 both alone (Figures 3A and 3B, respectively) and together, in multiplex amplification. The initial trials (Figure 3C), carried out by using equal amounts of both primer pairs in the mixture of reaction, lead to a pattern essentially composed by that of DCA15, due to the higher affinity of this primer to the template with respect to GAPU101. By increasing the amount of GAPU101 primer in the amplification mixture (ratio GAPU101:DCA15 = 65:35), a pattern consistent to the sum of those obtainable by the two primers alone was achieved (Figure 3D).

CONCLUSIONS

In conclusion, it was demonstrated the possibility to apply the analysis of microsatellite markers, also by means of multiplex DNA amplifications, to virgin oils obtained from different olive cultivars, with fingerprinting purposes. This kind of analysis might be useful to recognise the presence of not declared cultivars in oils when a preserved origin denomination is awarded to them. The investigation will be extended by applying the method to the most diffused olive cultivars present in the Mediterranean basin and, especially, in Italy where a wide number of cultivars is present.

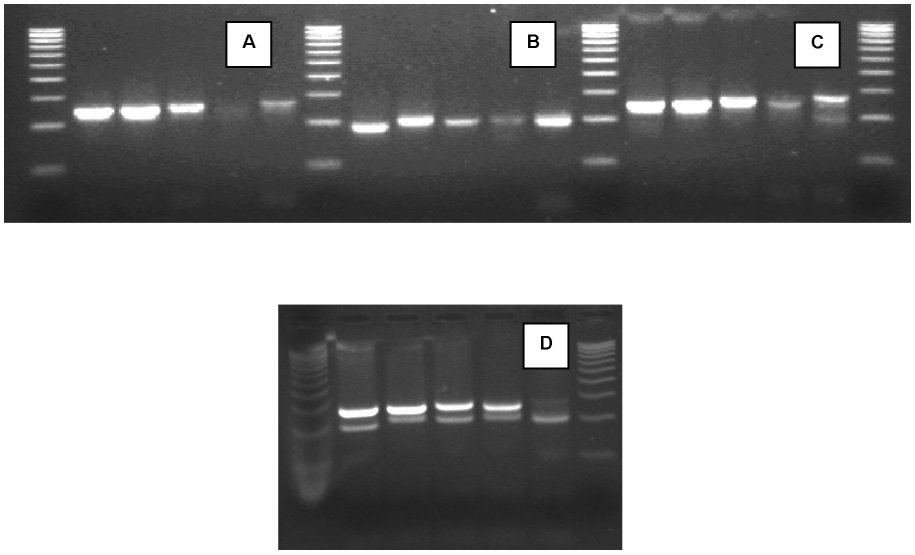

FIGURE 3. Agarose gel showing the electrophoretic patterns of the examined *O. europaea* cultivars amplified with primers DCA15 (A), GAPU101 (B) and with both these primers in equal amounts (C) or in optimised amounts (D). The order of the cultivars is the following: Cima di Melfi, Nocellara del Belice, Pendolino, Picholine, Toscanina. On each side the marker 100 bp (Sigma) was loaded.

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