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Identification of Virgin Olive Oil from Different Cultivars by Analysis of DNA Microsatellites

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DNA analysis enables genome fingerprinting with consequent identification of different individuals. In the agro-food industry, this can have interesting applications for the identification of species and cultivars of both raw materials and processed food. In this investigation, the efficiency of DNA microsatellite analysis in identifying virgin olive oils from different cultivars was evaluated. Ten virgin oils were obtained in the laboratory from olives of 10 different cultivars and the DNA extracted from the cell residues, recovered by oil centrifugation, was used as a template with seven different primer pairs of microsatellite sequences. The electrophoretic patterns showed an adequate level of amplification and were identical to those obtained from leaves and drupes of the same cultivar. By analyzing all the patterns obtained, the smallest number of microsatellites able to distinguish the examined oils was established and an identification key for the different oils was developed.

KEYWORDS: Virgin olive oil; DNA microsatellites; cultivar identification; polymerase chain reaction

INTRODUCTION

DNA analysis enables genome fingerprinting with consequent identification of different individuals. In the agro-food industry, such a procedure can have interesting applications for the identification of species (*1*, *2*) and cultivars (*3*, *4*) of both raw materials and processed food. This can be of particular importance in cases in which protected designation of origin (PDO) has been applied for, since certain cultivars have to be present for such recognition to be awarded.

In the case of olive oil, numerous investigations have regarded the determination of quality and genuineness, but some difficulties have been encountered in distinguishing oils obtained from different cultivars due to the strong influence of environmental conditions on the characteristics examined (*5*). Moreover, *Olea europaea* L. has a considerably great number of cultivars and often cases of homonymy and synonymy occur among them (*6*).

For these reasons, DNA markers have been used for cultivar identification of olive tree due to their independence from environmental fluctuations and to their high degree of polymorphism, able to effectively distinguish even very similar cultivars and to solve homonymy cases. Many kinds of markers, such as random amplified polymorphic DNA (RAPDs) (*7*−*10*), amplified fragment length polymorphism (AFLPs) (*11*) and microsatellites, recently developed for *O. europaea* (*12*−*14*), have been used successfully. However, these markers have not been applied to olive oil DNA.

Indeed, DNA from foodstuffs can be too highly degraded or rich in DNA polymerase inhibitors to be effectively analyzed. In a previous paper (*4*), the authors used inter-simple sequence repeat (ISSR) markers to identify the varietal origin of *O. europaea* drupe stocks, and attempts made to apply the same markers to the DNA obtained from the cell residual of the oil gave inconsistent results because of its high level of degradation.

A better possibility may be offered by the analysis of microsatellite markers (*15*) due to their small size (200−300 bp), since the amplification of short fragments can usually be performed also in the presence of highly degraded DNA (*1*, *16*).

The aim of this investigation was thus to evaluate the possibility of identifying virgin olive oils from different cultivars by the analysis of DNA microsatellites. To this purpose, 10 oils were prepared in the laboratory from 10 different very common Italian *O. europaea* cultivars and were then analyzed using seven microsatellite primer pairs.

MATERIALS AND METHODS

Sample Preparation. A total of 3 kg of olives from the following Italian *O. europaea* L. cultivars: Cellina di Nardò, Cima di Melfi, Coratina, Frantoio, Leccino, Nocellara del Belice, Ogliarola, Pendolino, Picholine, and Toscanina, was hand-picked at the right stage of ripening.

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The plant material was certified and belonged to the collection of "Vivai Giannoccaro" (Bari, Italy). After the olives were washed and leaves removed, virgin olive oil was obtained from each cultivar utilizing an laboratory-scale pilot plant 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 kneader (Agrimec Valpesana, Florence, Italy). In the hammer-crusher, a vertically positioned slightly half-cone-shaped crusher chamber received a constant inflow of olives from a hopper. Inside the chamber three hammers rotated versus 30 counterbeaters embedded in the side of the chamber to break the olives. The lower part of the chamber was covered by a grid with holes of 5-mm diameter through which the olive paste passed into a collecting basket. More detailed crusher characteristics are reported in Caponio and Catalano (*17*). The olive paste recovered from the crusher was put into the kneader bowl and malaxed for 30 min. In the kneader, three blades, fixed onto the shaft of the motor, rotated so as to mix the paste. The oils were finally recovered from the olive paste by means of a basket centrifuge. The plant was accurately cleaned before changing cultivar to avoid cross contamination.

DNA Extraction. A total of 250 mL of each oil was centrifuged at 10 000 rpm for 5 min in a centrifuge (model J2-21, Beckman Instruments, Palo Alto, CA), and DNA was extracted from the resulting residue with the Gene Elute Plant kit (Sigma, St. Louis, MO) following the manufacturer's instructions. The concentration of the extracted DNA was determined on 0.8% agarose gel by comparison with λDNA solutions at a known concentration. DNA extraction from leaves and drupes was carried out with the same kit starting from 25 mg of lyophilized tissue.

Amplification and Detection of Microsatellite Markers. Seven primer pairs of microsatellite markers were used: DCA4, DCA15, DCA17 (*12*); GAPU71, GAPU89, GAPU101 (*13*); UDO99-003 (*14*), sequences are reported in the corresponding papers (*12*−*14*). Primers were synthesized by Sigma Genosys (St. Louis, MO). Amplification reactions were performed in a I-Cycler programmable thermal cycler (Bio-Rad Laboratories, Hercules, CA) in a reaction mix with the following composition: 30 ng of di DNA, 1 × PCR buffer, dNTP 0.25 mM, primer *forward* and *reverse* 2.5 μM each, RED*Taq* DNA polymerase (Sigma, St. Louis, MO) 1 U, in a volume of 25 μL. The amplification conditions were 5 min at 95 °C; 35 cycles composed of 20 s at 95 °C, 30 s at the appropriate annealing temperature (*12*−*14*) and 30 s at 72 °C; final elongation at 72 °C for 7 min. The amplification products were separated by electrophoresis on 1.4% agarose gels in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA), stained by ethidium bromide, and visualized under UV light. Fragment sizes were attributed by comparison to 100-base pair molecular-size marker (Bio-Rad Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Figure 1 shows the DNA extracted from oils compared to that extracted from drupes and leaves. As already observed in a previous paper (*4*), due to the mechanical stress connected with the olive processing, the DNA extracted from the scarce cell residue sediment recovered by centrifugation of virgin olive oils appeared to be highly degraded while the DNA obtained from leaves and drupes showed a high relative molecular mass. Similar results were found by other authors in other vegetable oils such as soybean and rapeseed oil before refining (*18*, *19*), while after refining no DNA was recovered (*18*−*21*).

The DNA extracted from oil, albeit degraded, was then used as a template for microsatellite amplifications, as was the DNA from the leaves and drupes. Acceptable levels of amplification were obtained from oil DNA with electrophoretic patterns identical to those obtained analyzing the DNA from leaves and drupes of the same cultivar (**Figure 2**). Repeated reactions proved to give reproducible patterns. This represents an advancement of previous investigations by the authors (*4*), which had relied on ISSR markers and, especially with the amplified fragments having a high molecular mass, had not obtained reproducible patterns for olive oil. Microsatellite amplification, indeed, was facilitated by the fact that in this case the amplified fragments had small sizes and were shorter than the shortest degraded fragment of DNA from oil.

Of the seven primers used, six proved to be polymorphic in that they yielded amplified fragments of different lengths for each oil type considered (**Table 1**). **Figure 3** shows the electrophoretic pattern of primers DCA4 and DCA15 in the 10 oils examined. As may be seen, the polymorphism of the microsatellites consisted of the different lengths of the amplified fragment from one sample to another due to the different number of repetitions of the basic unit. The same figure shows that the microsatellites led to a simple amplification pattern usually composed of 1−2 fragments. Since this pattern is much easier

Figure 1. Agarose gel showing the genomic DNA extracted from Toscanina drupes, Toscanina leaves, Toscanina oil, and Picholine oil, respectively.

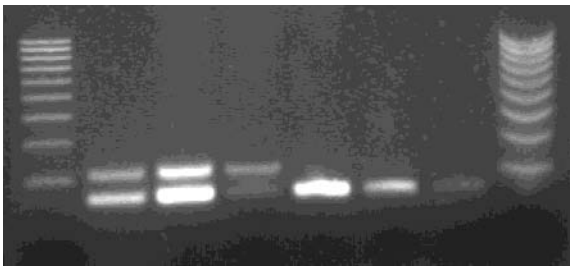

Figure 2. Amplification profile of DNA extracted from Toscanina drupes, Toscanina leaves, Toscanina oil, Cellina leaves, Cellina drupes, Cellina oil, with primer GAPU89.

Table 1. Size of the Amplified Fragments and Polymorphism Data, in the Examined Cultivars, of the Microsatellites Used in the Investigation

microsatellite	size of the amplified fragments (base pairs)	no. of distinguishable patterns	maximum number of amplified fragments per sample	discriminating power
GAPU71B	125, 150	3	2	0.46
GAPU89	185, 195, 205	3	2	0.62
GAPU101	195, 200, 205	3	1	0.46
DCA4	180, 190, 200	6	3	0.76
DCA15	260, 270	2	1	0.48
DCA17	140, 160, 170	5	2	0.72
UDO99-003	180	1	1	0
mean value		3.3	1.6	0.50

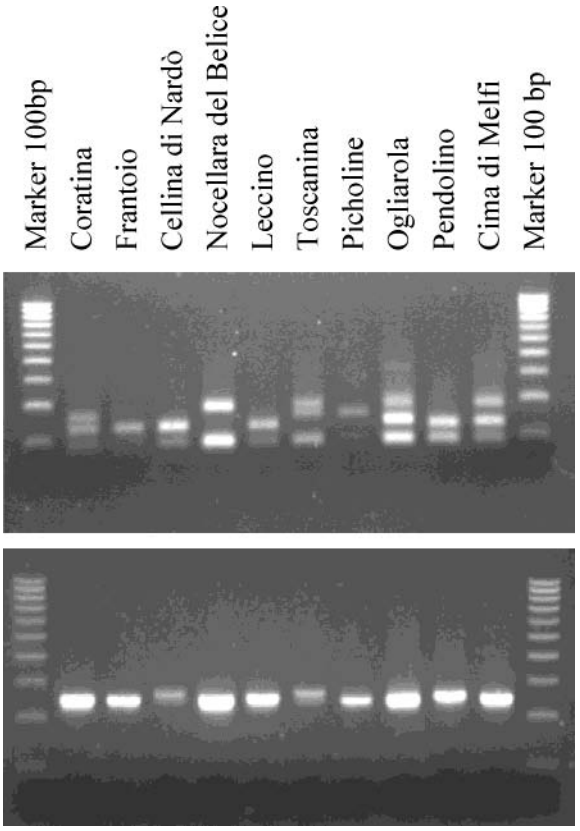

Figure 3. Agarose gel showing the electrophoretic patterns of all the examined *O. europaea* cultivars with primers DCA4 (upper) and DCA15 (lower). Cultivar names and molecular weight markers are indicated on each lane.

to screen than the pattern yielded by the ISSR, RAPD, or AFLP techniques, microsatellite analyses might be also applicable to mixtures of 3−4 cultivars, such as those usually adopted in PDO oils. On the contrary, the other markers can be applied only to single cultivars since the overlapping of complex profiles, each corresponding to a different cultivar, would render the whole pattern too complicated and thus useless.

With the aim of identifying the best primer to discriminate the cultivars examined, the degree of polymorphism detected by each primer was calculated (**Table 1**) as a discriminating power (DP) (*22*), with $DP = 1 - \Sigma p_i^2$, where p_i is the frequency of the *i*th pattern. DP expresses the ability of primers to distinguish between cultivars; it is equal to 0 for monomorphic markers and to 1 when all samples are distinguished. Apart from the monomorphic microsatellite UDO99-003, the DP values ranged from 0.46 to 0.76 for the other examined microsatellites, with the highest value for primer DCA4. This index is correlated to the number of distinguishable patterns, and, in fact, among the polymorphic markers the number of distinguishable patterns ranged from 2 to 6 with the highest number registered for microsatellite DCA4.

Since the highest number of different patterns detected by the examined microsatellites was six over 10 samples, a combination of a greater number of microsatellites should be used to distinguish more cultivars. To achieve the result of identifying all the different oils examined with the lowest number of analyses, the microsatellites having the greater discriminating power were used. A graphic identification key (**Figure 4**) was built to illustrate the way they were combined in following steps. In **Figure 4** it can be observed that the three microsatellites DCA4, DCA17, and GAPU89, used in this order, led to distinguishing the whole set of oils examined. The DCA4 primer divided the 10 samples into six groups, corresponding to six different electrophoretic patterns, and immediately distinguished oils from the Coratina, Nocellara del Belice, Picholine, and Toscanina cultivars. The oils obtained from the remaining cultivars had to undergo subsequent analyses with the other two microsatellites to be discriminated.

In conclusion, DNA microsatellites were found able to distinguish virgin olive oils from different cultivars. A good discriminating ability of these markers was observed and the identification method set up on them enabled a varietal check of the oils.

The study also demonstrated the possibility to amplify DNA extracted from cell residues present in virgin olive oils (obtained by mechanical treatment without refining).

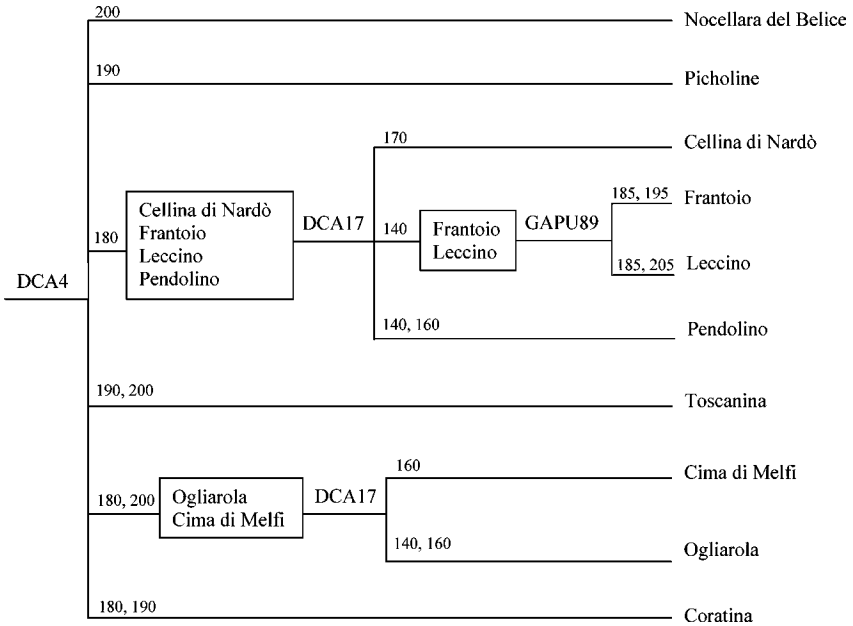

Figure 4. Identification key of the examined *O. europaea* cultivars based on primers DCA4, DCA17, and GAPU89. Each branch corresponds to a different pattern, whose size in base pair number is reported. Only polymorphic fragments have been considered.

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