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Traceability of Origin and Authenticity of *Olive Oil*

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1. Introduction

Olive (*Olea europaea* L.) is one of the oldest agricultural tree crops worldwide and is an important source of oil with beneficial properties for human health. *Olive oil* is produced solely from the fruit of the olive tree (*Olea europaea* L.) and differs from most of the other vegetable oils in the method of extraction, allowing it to be consumed in crude form, hence conserving its vitamins and other natural healthy high-value compounds.

In comparison to commonly used vegetable oils, the cost of *olive oil* is higher. As such, *olive oil* is more prone against adulteration with other cheaper oils in order to increase profits. Several grades of *olive oil* are marketed which also command different prices. There is also the possibility of the addition of cheaper grades of *olive oil* to better graded ones for the same economic reasons. The presence in highly prized *olive oil*s of lower grade material is sometimes revealed by specific analytical methods.

Mixing low-grade sunflower, canola or other oil with olive's industrial chlorophylls, and flavouring it with beta-carotene has been brought to light and sold as *olive oil*. The FDA does not have the resources to test all the imported *olive oil* for adulteration, and some products are difficult to test. One can always expect adulteration and mislabeling *olive oil* products (Mueller, 1991). Christy et al., (2004) used near-infrared (NIR) spectra in the region of 12,000–4000 cm ($^{-1}$) to detect adulteration of *olive oil* with sunflower oil, corn oil, walnut oil and hazelnut oil. It has reported that adulteration of virgin *olive oil* with hazelnut oil could be detected only at levels of 25% and higher with Fourier transform infrared spectroscopy (Kumar et al., 2011). However, Rabiei (2006) has reported the use of molecular approach in revealing hazelnut-adulterated *olive oil* at level of less than 10% of hazelnut.

Several methods have been proposed for monitoring the adulteration of virgin *olive oil*s with other edible oils. In the last 10 years, technology and knowledge have undergone a great advance in the fight against adulteration; however, in the same way, knowledge of defrauders has also been increased. This enables them to prepare more sophisticated adulterations that make useless the methodologies proposed to detect them. Those oils normally added to virgin *olive oil* can be, either *olive oil*s of lower quality (e.g. olive-pomace *olive oil* or virgin *olive oil* obtained by second centrifugation of the olives), or seed oils (e.g. corn, soybean, palm or sunflower oil, among others) (Peña et al., 2005).

It has long been known that the chemical composition of virgin *olive oil* is influenced by genetic (variety) and environmental (climatological and edaphologic conditions) factors. So the olive production area is greatly responsible for the specific characteristics of *olive oil*.

The analytical analyses have their limits. This has promoted a growing interest towards the application of DNA-based markers since it is independent from environmental conditions. Specific protocols for DNA isolation from *olive oil* have been developed (Breton et al., 2004; Busconi et al., 2003; Consolandi et al., 2008; De la Torre et al., 2004). The application of DNA-based methods requests the knowledge on nucleotide sequences of olive. This information for olive is back to 1994, when the first *Olea europaea* L. sequence has deposited in NCBI. Table 1 provides brief information on olive genomics presented on NCBI from 1994 to July 2011 consist of Nucleotides, ESTs and GSS accessions.

T*raceability* in food is a recently developed concept of control of the whole chain of food production and marketing that would trace back to each step of the process. In a narrower sense, *genetic traceability* is performed to find out the genetic identity of the plant material from which the transformed products have originated. The recognition of the genetic background underlying food products aims to prove the authenticity of valuable food and to discourage from the adulteration with extraneous material of lower cost and value. Recently, Rotondi et al., (2011) has performed *olive oil* traceability by means of a combination of the chemical and sensory analyses with SSR biomolecular profiles. Her group demonstrated that the genetic (SSR analysis) component and the selected fatty acids (eicosenoic, linoleic, oleic, stearic, palmitic and linolenic), seems to represent a possible tool for inter- and intra-varietal characterisation and for monovarietal traceability.

Year	Olea europaea accessions on NCBI database*			
	Total nucleotide sequences	**Nucleotide**	**EST****	**GSS*****
1994	3	3		
1995	1	1		
1996	1	1		
1997	13	13		
1998	1	1		
1999	11	11		
2000	39	39		
2001	57	57		
2002	57	57		
2003	88	64	24	
2004	23	23		
2005	213	213		
2006	44	44		
2007	337	335		2
2008	186	186		
2009	4891	55	4,836	
2010	1871	690	1,159	22
07/2011	33			
Total sequences	7,869	1,793	6,019	24

*http://www.ncbi.nlm.nih.gov, **EST: expressed sequence tags, ***GSS: genome survey sequences

Table 1. Olive genomics information present on NCBI database from 1994 to July 2011

2. General description of olive plant

Olive (*Olea europaea* L.) is the main cultivated species belonging to the monophyletic *Oleaceae* family that comprises 30 genera and 600 species, within the clade of Asterids, in which the majority of nuclear and organellar genomic sequences are unknown. The Olea genus includes 30 species and has spread to Europe, Asia, Oceania and Africa (Bracci et al., 2011). The wild olive or oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris*) and the cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) are two co-existing forms of the subspecies *europaea* (Green 2002). Other five subspecies constitute the *Olea europaea* complex including *laperrinei*, present in Saharan massifs; *cuspidata*, present from South Africa to southern Egypt and from Arabia to northern India and south-west China; *guanchica* present in the Canary Islands; *maroccana* present in south-western Morocco; and *cerasiformis* present in Madeira (Green 2002).

The Mediterranean form (*Olea europaea*, subspecies *europaea*) includes the wild and cultivated olives is a diploid species (2n = 2x = 46) (Kumar et al., 2011). The origin of the olive tree is lost in time, coinciding and mingling with the expansion of the Mediterranean civilisations which for centuries governed the destiny of mankind and left their imprint on Western culture.

The common olive is an evergreen tree that grows up to ~12m in height with a spread of about 8 m. However, many larger olive trees are found around the world, with huge, spreading trunks. The trees are also tenacious, easily sprouting again even when chopped to the ground. Sometimes it is difficult to recognize which is the primary trunk. The tree can be kept at a height of about 5m with regular pruning. Olives are long-lived, with a life expectancy of greater than 500 years (Kumar et al., 2011).

Most olive-growing areas lie between the latitudes 30◦ and 45◦ north and south of the equator, although in Australia some of the recently established commercial olive orchards are nearer to the equator than to the 30◦ latitude and are producing a good yield; this may be because of their altitude or for other geographic reasons.

The olive fruit is termed a *drupe* botanically, which are green in color at the beginning and generally become blackishpurple when fully ripe. A few varieties are green even when ripe, and some turn a shade of copper brown. Olive fruits consist of a carpel, and the wall of the ovary has both fleshy and dry portions. The skin (exocarp) is free of hairs and contains stomata. The flesh (mesocarp) is the tissue that is eaten, and the pit (endocarp) encloses the seed. Olive cultivars vary considerably in size, shape, oil content and flavor. Raw olive fruits contain an alkaloid that makes them bitter and unpalatable. A few varieties are sweet enough to be eaten after sun-drying (Wiesman, 2009).

Olive cultivars are basically classified into “oil olives” and “table olives,” and oil cultivars predominate. Olive cultivars are also classified according to the origin of the cultivar – for example, Spanish, Italian, Greek, Syrian, Moroccan, Israeli, etc. The most popular cultivars are: Picual, Arbequina, Cornicabra, Hojiblanca and Empeltre in Spain; Frantoio, Moraiolo, Leccino, Coratina and Pendolino in Italy; Koroneiki in Greece; Chemlali in Tunisia; Ayvalik in Turkey; Nabali, Suori and Barnea in Israel and The West Bank; Picholin in France; Mission in California; and various varieties in Australia. The table olive cultivars include Manzanilla and Gordal from Spain; “Kalamata” from Greece; “Ascolano” from Italy; and “Barouni” from Tunisia (Jacoboni & Fontanazza, 1981; Weissbein, 2006).

The large number of cultivars, added to the many cases of synonymous and homonymous name, makes particularly difficult the description and classification of olive varieties (Fabbri et al. 2009). Notice that two varieties are synonymous when they have different names but the same profile of fingerprinting, and two varieties are homonyms when they have the same name but different fingerprinting profiles.

The size of olive germplasm is controversial: about 1,250 varieties (or in some other references 1,275 cultivars, Sarri et al., 2006), cultivated in 54 countries and conserved in over 100 collections, were included in the FAO olive germplasm database (Bartolini 2008), also if this number is certainly higher because the lack of information on many local cultivars and ecotypes (Cantini et al. 1999). The most part of these cultivars comes from southern European countries such as Italy (538 varieties), Spain (183), France (88) and Greece (52) (Baldoni & Belaj 2009). Due to this richness of the germplasm, olive is an unusual case among horticultural crops and its biodiversity can represent a rich source of variability for the genetic improvement of this plant.

3. Olive oil

It has been known that climate, soil, variety of tree (cultivar) and time of harvest account for the different organoleptic properties of different *olive oil*s. Two factors are influential: where the olives are grown and which harvesting methods are implemented. Certain locations yield more bountiful harvests; consequently their oil is sold for less. Olive trees planted near the sea can produce up to 20 times more fruit than those planted inland, in hilly areas like Tuscany. It is in these land-locked areas that the olive trees' habitat is pushed to the extreme; if the conditions were just a little more severe, the trees would not survive. Extra-virgin oils produced from these trees have higher organoleptic scores.

It is extremely difficult to establish the origins of *olive oil* using DNA technologies. One approach has been **to target yeasts associated with olives and** *olive oil*. Target for characterization was the LTR retrotransposon (Ty element) (Lenoir et al., 1997, as cited in Popping, 2002) using amplified fragment length polymorphism or similar techniques. This method has been more successful for olives, where different yeast strains are associated with olives and *olive oil*. The yeast strains in *olive oil* appear to be associated with the production site (fattoria) where the *olive oil* was produced. And since the number of production sites is limited, the *olive oil* can be traced back to the fattoria.

But this technique is not yet applicable for routine analysis. For the identification of the origin of *olive oil*, a second, non-DNA-based technology has proven very useful. The technology is called **site-specific natural isotope fractionation nuclear magnetic resonance (SNIF-NMR)** (Gonzalez et al., 1999; Martin et al., 1996).

The basis of this technology is that certain elements have naturally occurring stable isotopes (^{16}O and ^{18}O, ^{1}H and ^{2}H, ^{12}C and ^{13}C). The ratios of the different stable isotopes vary from one geographic location to another. These ratios are maintained in the organic material from that region, e.g. plants, animals etc. The SNIF-NMR technology allows measuring these stable isotope ratios at individual positions in a given molecule.

With an appropriate database listing the location and typical stable-isotope distribution, the origin of *olive oil* can be identified (Popping, 2002).

Other techniques such as proton transfer reaction mass spectrometry (PTR-MS), nuclear magnetic resonance spectroscopy (NMR) or high performance liquid chromatography (HPLC) has also been addressed using different methodologies (Luykx & van Ruth, 2008). However, the chemical composition of *olive oil* may differ among seasons and growing areas. Several investigations concerning the origin and authenticity of *olive oil* have shown that chemical analyses per se are not sufficient to assure *olive oil* authenticity or to reveal *olive oil* region (Gimenez et al., 2010). Christopoulou et al., 2004 expressed that no single known parameter could detect the presence of hazelnut and almond oils in *olive oil* which have many chemical characteristics (fatty acid profile, sterol composition, …) similar to *olive oil*.

Several Protected Denomination of Origin (PDO) *olive oil* regions have been established by legislation to ensure both producer's profits and consumer's rights. In this context, it is mainly the identification of the olive cultivar used for the oil production which is of importance for authentication (Luykx & van Ruth, 2008) since the contribution of cultivars is known for each designation (Gimenez et al., 2010). Different PDO labels such as "Oliva Cilento", "Colline Salernitane", and "Penisola Sorrentina", have been granted for the extra-virgin *olive oil* produced in Campania, and some others are in the process of assignment.

4. World olive oil production

According to the report of the International Olive Council (IOC) (the International *Olive oil* Council, IOOC, until 2006), Mediterranean countries account for around 97 percent of the world's olive cultivation, estimated at about 10,000,000 hectares. There are more than 800 million olive trees currently grown throughout the world, of which greater than 90 percent are grown for oil production and the rest for table olives. It is estimated that more than 2,500,000 tons of *olive oil* are produced annually throughout the world.

Since the mid-1990s, Spain has consistently been the largest producer; in the year 2004/05 it produced 826,300 tons of *olive oil* and it is expected a sum of 2,948,000 tons for total world olive oil production in 2010/11. The main producer is still European Union (EU), with 2.1 million tons, of which 1.2 from Spain (-14% from the previous campaign), 336000 tons produced by Greece, 480000 tons by Italy, 67500 tons by Portugal, 65000 tons by Cyprus and 6000 tons by France. Out of EU, IOOC estimated a production of 193500 tons from Syria, 160000 tons from Turkey, 12000 from Tunisia, 150000 from Morocco, 48000 from Algeria, 24900 from Palestine, 19000 from Jordan, 18000 from Australia, 17500 from Argentina, and 15000 from Lybia.

The world *olive oil* consumption (2010-2011) will reach 2.98 million tons, with a 3.65 % increase from the previous campaign 2009-2010 (*IOC website*).
IOOC has estimated that the world export will increase of 5.05% and reach 707000 tons, with EU (438000 tons) as the main commercial power, followed at great distance by Morocco (40000 tons), Syria (50000 tons), Tunisia (90000 tons), and Turkey (38000 tons).

The import for the period between October 1st 2010 and September 30th 2011 is estimated at 648000 tons, with a 2.93% increase from the previous year (*IOC website*).

The pattern of production of *olive oil* during these years shows big fluctuations from one year to the next; however, Spain, Italy and Greece remain the three largest *olive oil* producing countries, dominating the world annual *olive oil* production. This signifies a high level of uncertainty regarding production levels. In the year 2004/05, Spain, Italy and

Greece produced 32, 28 and 13.5 percent of the world's *olive oil*, respectively. However, the recent expansion of the *olive oil* industry and significant contribution to the global *olive oil* market by several other countries, such as Australia and the United States, may lead to stabilization of the market in the near future.

5. *Olive oil* traceability

Food traceability implies the control of the entire chain of food production and marketing, allowing the food to be traced through every step of its production back to its origin. The verification of food traceability is necessary for the prevention of deliberate or accidental mislabeling, which is very important in the assurance of public health. Thus, several regulations provide the basis for the assurance of a high level of protection of human health and consumers' interest in relation to food.

In the case of *olive oil*s, the increase in the demand for high-quality *olive oil*s has led to the appearance in the market of *olive oil*s elaborated with specific characteristics. They include oils of certain regions possessing well-known characteristics, that is, *olive oil*s with a denomination of origin, or with specific olive variety composition, that is, coupage or monovarietal *olive oil*s. *Olive oil*s obtained from one genetic variety of olive or from several different varieties are called monovarietal or coupage, respectively. Monovarietal *olive oil*s have certain specific characteristics related to the olive variety from which they are elaborated (Montealegre et al., 2010). However, coupage *olive oil*s are obtained from several olive varieties to achieve a special flavor or aroma.

The appearance of denominations and protected indications of origin has promoted the existence of oils labeled according to these criteria. Regulation 2081/92 (2) created the systems known as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and the ''Traditional Speciality Guaranteed" (TSG) to promote and protect food products (Table 2).

General regimen	Origin	Characteristics	Restriction
Protected Designation of Origin (PDO)	In that region, specific place, or country	Quality essentially or exclusively due to a particular geographical area	Produced, processed and prepared in a given geographical area
Protected Geographical Indication (PGI)	In that region, specific place, or country	Slightly less strict; food reputation of a product from a given region is sufficient	One of the stages of production, processing, or preparation takes place in the area

*Council Regulation (EC) 510/2006, March 20, 2006.

Table 2. General regimen for food and certain other agricultural products based on Regulation 510/2006*

For example, an *olive oil* with a PDO denomination requires meeting precise definition of several parameters such as cultivar, geographical origin, agronomic practice, production

technology, and organoleptic qualities (Gimenez et al., 2010), and all of these parameters have to be investigated to study its traceability and to certify its quality. Among the above-mentioned factors, the two first are the most important (Montealegre et al., 2010).

Additionally, a Database of Origin and Registration (DOOR) was created to support these denominations (Montealegre et al., 2010). Based on the report of the *International Olive Council* (http://www.internationaloliveoil.org) gave the world production of *olive oil* in 2008/2009 as 2,669.5 million tons and it consumption for the same period as 2,831.5 million tons. It is quite clear that some of the *olive oil* sold has been mislabeled. *Olive oil* is priced from $13–105 for 500 mL where as canola oil and sunflower oils available from less than $1–10 for 500 mL.

The introduction of certifications of origin and quality for virgin *olive oil* as PDO makes necessary the implementation of traceability procedures. It seems that DNA analysis to be a promising approach to this problem, since it is less influenced by environmental and processing conditions in respect to other methods (i.e.; metabolites).

Any research dealing with *olive oil* traceability is focused on investigating the botanical or geographical origin. However, the concept of geographical traceability, in which the objective is the geographical location of the olive tree, is slightly different from the concept of botanical traceability, in which the olive used for the *olive oil* production is the aim. In both cases, the selection of the markers (compounds with discriminating power) to be studied is complicated because the composition of extra virgin *olive oil*s is the result of complex interactions among olive variety, environmental conditions, fruit ripening, and oil extraction technology (Araghipour et al., 2008).

5.1 Traceability to the botanical origin

The verification of the cultivars employed to produce an *olive oil* sample may contribute to address the oil origin. This fact may have commercial interest in the case of monovarietal *olive oil*s or *olive oil*s with PDO because these high-quality *olive oil*s may be adulterated by other oils of lower quality, using anonymous or less costly cultivars (Breton, 2004).

As the quality of an *olive oil* depends on the olive variety from which it is elaborated, the production of *olive oil*s from certain varieties has increased (Sanz-Cortes et al., 2003). The olive variety selection is based on its adaptation to different climatic conditions and soils. In addition, whereas some cultivars are characteristic of a given zone, others can be found in several countries (Japon-Lujan et al., 2006). As a consequence, one olive variety can be cultivated and nominated in a different way in distinct geographical locations, which makes the differentiation of olive varieties in *olive oil*s quite complex. (Montealegre et al., 2010)

Traditionally, differentiation among olive cultivars has been supported by numerous morphological (study of the form or shape) and pomological (the development, cultivation, and physiological studies of fruit trees) traits. Unfortunately, morphological traits have been difficult to evaluate, are affected by subjective interpretations, and are severely influenced by the environment and plant developmental stage (Sanz-Cortes et al., 2003). Nowadays, several efforts have been focused on the investigation of one or several compounds present in *olive oil*s usable to differentiate olive varieties (Montealegre et al., 2010). Compositional markers (substances that take part of the composition of the olive oils) include major and minor components. Major, (sterols, phenolic compounds, volatile compounds, pigments,

hydrocarbons, and tocopherols, fatty acids and triglycerides, components may provide basic information on olive cultivars. Minor components, can provide more useful information and have been more widely used to differentiate the botanical origin of *olive oil*s (Montealegre et al., 2010).

5.2 Compositional markers

There are several parameters, major and minor compositional markers, with varied discriminant power used for *olive oil* traceability according to the variety of olive participated in the production of the oil (Arvanitoyannis et al., 2007).

To relate the fatty acid composition of *olive oil*s with the cultivar, Mannina et al. (2003) studied *olive oil* in a well- limited geographical region, with no consideration of the pedoclimatic factor (soil characteristics such as temperature and humidity). A relationship between the fatty acid composition and some specific cultivars has been observed (Montealegre et al., 2010).

The *volatile fraction* in *olive oil*s, which represents one of the most important qualitative aspects of this oil, consists of a complex mixture of more than 100 compounds, but the most important substances useful for olive cultivar differentiation are the products of the lipoxygenase pathway (LOX). Only a subset of volatile compounds and a combination among them could provide valuable information for olive cultivar differentiation (Montealegre et al., 2010). Three volatile compounds [hexyl acetate, hexanal, and (E)-hex-2-enal] and the total concentration of ketones have nominated to distinguish the olive varieties (Tena et al., 2007) hich consequently could be used for olive oil, as well. However, it has been found that the level of (E)-hex-2-enal in the analyzed samples showed a variability that suggest an influence of genetic factors on the biosynthesis of this compound. In fact, genetic (Tura et al., 2008; Mahjoub-Haddada et al., 2007) and geographic (Mahjoub-Haddada et al., 2007) factors influence the volatile compound production of the olive fruits and affect the differentiation of *olive oil*s according to their olive variety. The volatile compound contents allowed differentiation among monovarietal *olive oil*s and even identification of the technique used for *olive oil* production (Torres Vaz-Freire et al., 2009).

The color of a virgin *olive oil* is due to the solubilization of the lipophilic chlorophyll and carotenoid pigments present in the fruit. The green-yellowish color is due to various pigments, that is, chlorophylls, pheophytins, and carotenoids (Cichelli & Pertesana, 2004). Chlorophyll a is the major chlorophyll pigment, followed by chlorophyll b. The carotenoid fraction is included lutein, violaxanthin, neoxanthin, β-carotene, β-cryptoxanthin, and luteoxanthin (Montealegre et al., 2010).

Several researchers reported the same qualitative composition in chlorophyll and carotenoid pigments, independent of the olive variety and the time of picking (Giuffrida et al., 2007 ; Roca et al., 2003).

Cerretani et al. (2006) showed that the carotenoid and chlorophyll content determination using UV-vis spectrophotometry was not useful to discriminate oils produced from different olive varieties. Lutein/β-carotene ratio has been reported as a tool to differentiate oils from a single cultivar.

Tocopherols and hydrocarbons are the compositional markers less studied to date to differentiate *olive oil*s. An important common aspect is that the content and composition of

these markers are highly affected by the environmental conditions, the fruit ripening, and the extraction technology (Montealegre et al., 2010).

6. DNA-based markers

Molecular markers are investigated as a diagnostic tool for food authenticity and traceability of variety/type composition of complex food matrices in an increasing number of projects (Palmieri et al., 2004). DNA-based methods make an important contribution to protect high-quality *olive oil*s.

Significant amounts of DNA are present in *olive oil* obtained by cold pressing (Consolandi et al., 2008). However, the filtration process lowers DNA concentrations, which tend to disappear due to nuclease degradation (De la Torre et al., 2004; Muzzalupo et al., 2002). On the other hand, the length of storage after milling of the oil can affect the use of DNA as an analyte for molecular traceability. Pafundo et al., 2010 observed a significant decrease of quality of DNA extracted from olive oil, with a consequent loss of information a month later from olive oil production.

Spaniolas et al. (2008b) has used lambda DNA as a marker to monitor the length of DNA fragments in olive oil during storage time when determined the varietal origin of olive oil. Lambda DNA is a linear molecule of approximately 50 kb, a length that probably resembles that of olive DNA present in olive oil. Based on the fact, PCR-based fingerprinting techniques, which require templates longer than 100 bp, might not be able to successfully amplify the target sequences from olive oil samples low in DNA content and stored for several months, they conduct their analyses. They have deduced the detection of polymorphic markers requiring DNA templates shorter than 100 bp might have a wider range of applications in DNA fingerprinting of olive oil.

In *olive oil*, once the barrier of DNA extraction has been overcome, several markers could be used to identify olive cultivars that made up a certain *olive oil*. (Consolandi et al., 2008)

DNA recovery methods from *olive oil* have been developed by many authors (Busconi et al. 2003; Doveri et al. 2006; Pasqualone et al. 2007; Consolandi et al. 2008). Several commercial kits, providing adapted protocols, were used in different works (Martins-Lopes et al. 2008; Spaniolas et al. 2008a; Ayed et al. 2009; Pafundo et al. 2010). All of these studies confirmed that the DNA of the cultivars is recoverable from extra virgin *olive oil*, but it has low quantity and quality. The first researches, carried out using genomic DNA extracted from drupes. That DNA had a good potential to amplify correctly using RAPDs markers (Cresti et al. 1997). By means of SCAR and AFLP markers, Busconi et al. (2003) were able to show that DNA recovered from *olive oil* had both organellar and nuclear origin. Pafundo et al. (2005) traced the cultivar composition of monovarietal *olive oil*s by AFLPs, suggesting that DNA extraction is the most critical step affecting the procedure. Pafundo et al. (2007), performed amplification of DNA isolated from *olive oil* using AFLPs. They have also developed some SCARs to amplify successfully the DNA extracted from *olive oil*. Using SSR analysis, Pasqualone et al. (2007) demonstrated that microsatellites are useful in checking the presence of a specific cultivar in a PDO oil, thus verifying the identity of the product. However, they obtained only the marker profile of the main cultivar in the oil: no signal was detected for the secondary varieties. Montemurro et al. (2008) analyzed ten virgin monovarietal *olive oil*s prepared in the laboratory by AFLP markers. They were able to

distinguish all the *olive oil*s examined, even if only a partial correspondence with the AFLP profile obtained from the leaves was obtained. Martins-Lopes et al. (2008) evaluated the efficiency of RAPD, ISSR and SSR molecular markers for *olive oil* varietal identification and their possible use in certification purposes (Bracci et al., 2011).

Consolandi et al. (2008) reported the development of a semi-automated SNP genotyping assay to verify the origin and the authenticity of extra-virgin *olive oil*s. The authors developed a Ligation Detection Reaction (LDR)/Universal Array (UA) platform by using several olive SNPs. They found that 13 accurately chosen SNPs were sufficient to unequivocally discriminate a panel of 49 different cultivars (Bracci et al., 2011).

Doveri et al. (2006) published a cautionary note on the use of DNA markers for provenance testing. Their observations were based on non-concordance between the genetic profiles of *olive oil* and fruit. They suggested that this could be due to the contribution of pollen donors in DNA extracted from the paste obtained by crushing whole fruits. They concluded that care needs to be taken in the interpretation of DNA profiles obtained from DNA extracted from oil for resolving provenance and authenticity issues (Martins-Lopes et al., 2008). It is to note that, the possible presence of additional alleles due to paternal contribution in oils extracted from entire drupes, should be taken into consideration for variety traceability purposes when comparing the amplification profiles of leaves with the corresponding oils for (Alba et al., 2009)

In a recent study, Pafundo et al. (2010) investigated the effect of the storage time on the degradation of the DNA purified from the oil; a negative correlation between storage time and quality–quantity of recovered DNA has been observed. The authors showed that 1 month after the production of the oil the degradation increases making harder traceability goals (Bracci et al., 2011). Table 3 presents a brief report on the application of DNA-based molecular markers for cultivar traceability in *olive oil*.

Molecular marker	Developers	References
RAPD (Random Amplified Polymorphic DNA)	Williams et al. (1990)	Muzzalupo and Perri (2002), Martins-Lopes et al. (2008)
AFLP (Amplified Fragment Length Polymorphism)	Vos et al. (1995)	Busconi et al. 2003, Pafundo et al. 2005
SCAR (Sequence Characterized Amplified Region)	Paran & Michelmore (1993)	De la Torre et al. (2004), Pafundo et al. (2007)
SSRs (Simple Sequence Repeats)	Morgante & Olivieri (1993)	Martins-Lopes et al. (2008), Alba et al. (2009), Rabiei et al. (2010)
ISSR (Inter Simple sequence Repeats)	Zietkiewicz et al. (1994)	Pasqualone et al. (2001), Martins-Lopes et al. (2008)
Chloroplast and mitochondrial (Direct sequencing)	Botstein et al. (1980)	Intrieri et al. (2007)

Table 3. Applications of DNA-based molecular markers for cultivar traceability in *olive oil*

6.1 Random Amplified Polymorphic DNA (RAPDs)

The applicability to the traceability of cultivars in the *olive oil* by means of RAPDs was evaluated by Muzzalupo & Perri (2002). This kind of markers was used, together with other DNA-based markers, in the construction of the first olive linkage maps (De la Rosa et al. 2004; Wu et al. 2004) as well.

6.2 Amplified Fragment Length Polymorphism (AFLPs)

AFLPs have been widely used for DNA fingerprinting of cultivars (Angiolillo et al. 1999; Owen et al. 2005), to analyse the relationships between wild and cultivated olive (Baldoni et al. 2006), for the construction of linkage maps (de la Rosa et al. 2003) and for cultivar traceability of *olive oil* (Busconi et al. 2003; Pafundo et al. 2005).

6.3 Development of sequence-characterized amplified regions (SCARs)

SCARs have been used for cultivar identification (Busconi et al. 2006) and in *olive oil* traceability (De la Torre et al. 2004; Pafundo et al. 2007). Putative associations of several SCAR markers with fruit characteristics (Mekuria et al. 2002) and resistance to pathogenic fungi (Herna´ndez et al. 2001) were found, suggesting the applicability of this type of marker for marker-assisted breeding programs (Bracci et al., 2011).

6.4 Simple Sequence Repeats (SSRs)

Many authors have reported on SSR development in olive and several of them are currently available for DNA analysis (Cipriani et al. 2002; De la Rosa et al. 2002; Rabiei & Tahmasebi Enferadi 2009; Sabino Gil et al. 2006; Sefc et al. 2000). This technique benefits from the use of microsatellites which are short stretches (1-6-bp long) of DNA, tandemly repeated several times. The number of repeats can vary from one individual to another. Besides, they are abundant in eucaryotic genomes. A combination of several SSRs loci allows virtually discrimination of individuals originated through sexual reproduction, especially in out-crossing species, where the level of heterozygosity is high.

In Olea europaea, these markers have been used for different applications such as cultivar discrimination (Sarri et al. 2006; Fendri et al. 2010), study of relationships between wild and cultivated olive tree (Belaj et al. 2007), construction of association maps (De la Rosa et al. 2003), paternity analysis (Mookerjee et al. 2005) and identification of *olive oil* varietal composition (Alba et al. 2009; Ayed et al. 2009; Rabiei et al. 2010). A list of recommended SSR markers and protocols for olive genotyping has been provided with the aim to develop a robust method to track the origin of olive cultivars (Doveri et al. 2008; Baldoni et al. 2009) (table 4).

Series	Number	Institute	References
ssrOeUA-DCA	15	Boku, Vienna, Austria	Sefc et al 2000
IAS-oli	5	CSIC, Córdoba, Spagna	Rallo et al 2000
GAPU	20	Agrobios, Matera, Italia	Carriero et al 2002
UDO	30	Università di Udine, Italia	Cipriani et al 2002
EMO	7	ETSIAM, Córdoba, Spagna	De La Rosa et al 2002
Totale	*77*		

Table 4. List of microsatellites isolated in olive which their related primers are available in the literature, most of them have been used in case of *olive oil* traceability (Bracci et al., 2011)

For studying the informative potential of the microsatellites, the observed (*Ho*) and expected (*He*) heterozygosities generally are calculated using the software POPGENE ver. 1.31 (Yeh et al., 1999, as cited in Alba et al., 2009). *He* values were estimated using the formula proposed by Nei et al. (1973):

$$H_e = 1 - \sum p_i^2 \quad (1)$$

where *pi* is the frequency of the *ith* allele. The power of discrimination (PD) [21] of microsatellite primer pairs are also calculated as reported by Cipriani et al. (2002), where the allele frequency of the *He* formula is replaced by the genotype frequency.(Alba et al., 2009)

A research carried on the use of SSRs as a tool to identify the genetic background of *olive oil* which was involved the analysis of DNA sequences using a panel of seven simple sequence repeats (SSRs) to provide genotype-specific allelic profiles (Rabiei et al., 2010). The amplified SSR fragments and the DNA profiles from the monovarietal oil corresponded to the profiles from the leaves of the same cultivar. The most reliable SSR in providing correct allele sizing in distinguishing either single cultivar *olive oil* samples or the different ratios of their blends are DCA3, DCA4, DCA16, DCA17, and GAPU101, while DCA9, GAPU59 produced less concordance against data obtained by the genetic analysis of leaf samples. Desalted PCR product has been analyzed on a MegaBACE 500 capillary sequencer (Amersham Biosciences) using Genetic Profiler v2.0 software to estimate allele sizes (figure 1).

Rabiei et al., 2010 concluded PCR product purification and selection of a set of markers with a highly robust amplification pattern is needed to have reproducible results in certify the genetic background of *olive oil*.

6.5 Inter simple sequence repeat (ISSR) polymorphisms

ISSRs are DNA fragments of about 100–3,000 bp located between adjacent, oppositely oriented microsatellite regions. These markers were used with success to distinguish 10 Italian varieties, by analysing genomic DNA extracted from the olive fruit (Pasqualone et al. 2001), and for the study of cultivar traceability in *olive oil* (Pasqualone et al. 2001, Martins-Lopes et al. 2008).

6.6 Chloroplast genome sequencing

A very important results, recently published, in *Olea europaea* L. genomic studies have been the DNA sequencing of the entire plastome of the Italian cultivar 'Frantoio' (Mariotti et al. 2010). This sequence has a length of 155,889 bp and showed an organization and gene order that is conserved among numerous Angiosperms. The olive chloroplast contains 130 genes and 644 repetitive sequences (among which 633 mono-nucleotide SSRs, 6 di-, 3 tetra- 2 penta-nucleotide SSRs were identified) (Bracci et al., 2011)

The annotated sequence was used to evaluate the content of coding genes, the extent, and distribution of repeated and long dispersed sequences and the nucleotide composition pattern. These analyses provided essential information for structural, functional and comparative genomic studies in olive plastids. Furthermore, the alignment of the olive plastome sequence to those of other varieties and species identified 30 new organellar polymorphisms within the cultivated olive. chloroplast DNA polymorphisms has been used as molecular markers to identify cultivars of *Olea europaea* L. (Intrieri et al. 2007).

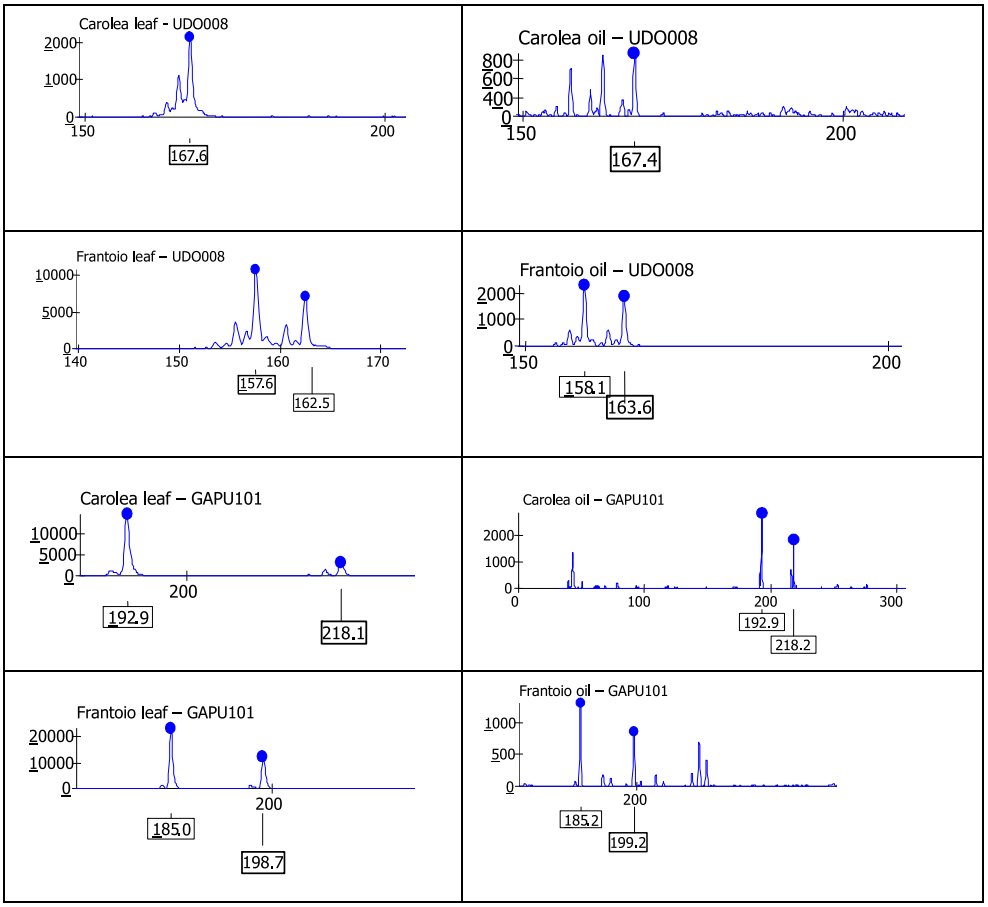

Fig. 1. Electropheregram of PCR products separated by capillary electrophoresis of microsatellite loci UDO008 and GAPU 101, obtained from DNA extracted from Carolea and Frantoio leaves and oils. Allele sizes are below the x axis. The scores on Y-axis are the intensity of amplified allele detection (Rabiei et al., 2010).

6.7 Expressed Sequence Tags (ESTs)

Understanding the function of genes and other parts of the genome is known as functional genomics. In olive, efforts to improve the identification and annotation of genes are prevalently based on EST identification, which are predominantly related to pollen allergens and characteristics of olive fruit (Bracci et al., 2011).

The first nucleotide sequences isolated in 1994 in olive coded for allergenic proteins (Villalba et al. 1994, as cited in Bracci et al., 2011) (Table 1).

6.8 Real time-PCR

The detection of frauds, either due to the mixtures with oils of other species such as hazelnut, or to the certification of PDOs would need quantitative tools. At its best, conventional PCR remains a semi-quantitative technique, and therefore, it is not optimal for authentication purposes when quantification is needed (Gimenez et al.; 2010).

The use of real-time chemistries allows for the detection of PCR amplification during the early phases of the reaction, providing a distinct advantage over detection of amplification at the final phase or end-point of the PCR reaction. qRT-PCR is a useful tool in the development of molecular markers for *olive oil* authentication since it allows inspecting the PCR efficiency. Besides qRT-PCR should be used for the optimisation of the amplicon size and the DNA isolation procedure which are critical aspects in *olive oil* authentication. The potential of cpDNA for *olive oil* authentication should be addressed in the future (Gimenez et al.; 2010).

6.9 DNA barcode

Several sequences from noncoding spacer region between psbA-trnH and partial coding region of matK of plastid genome provided a good discrimination of pure *olive oil* and its admixture by other vegetable oils such as canola and sunflower.

The plastid based molecular DNA technology has a great potential to be used for rapid detection of adulteration easily up to 5% in *olive oil* (Kumar et al., 2011).

7. Paternity analysis

Similar to other woody species, olive is characterized by a long juvenile phase that ranges between 10 and 15 years. This represents a great obstacle to breeding programs and makes the genetic improvement of olive very difficult and expensive. Although seedling-forcing growth protocols have been developed to reduce the length of this phase, the evaluation of the agronomic performance of mature olive plants still requires at least 5 years of experimentation (Santos-Antunes et al. 2005). For this reason, the application of molecular markers both to confirm the parental origins of the progeny and to select early agronomical characteristic-associated markers (Martı´n et al. 2005) can be very useful to reduce the time and cost of the development of new genotypes (Bracci et al., 2011).

With regard the paternity analysis, SSRs are the most suitable to trace the genetic contribution of alleles from the parents to the offspring, being co-dominant and highly polymorphic markers (Mookerjee et al. 2005). The effectiveness of SSRs in the identification of paternity contribution to progeny obtained from olive breeding programs has been demonstrated by several authors (De la Rosa et al. 2004; Diaz et al. 2007). The results demonstrated that SSR analysis is a convenient technique to routinely assess the crosses made in breeding programs and to for check self-incompatibility in olive cultivars (Diaz et al. 2006). These studies have highlighted that no contamination by self-pollen was found, indicating that placing pollination bags well before anthesis is important and that emasculation to avoid selfing is unnecessary (De la Rosa et al. 2004). The analysis also revealed that the main factor affecting the success of crosses seems to be the inter-

compatibility among the parental cultivars, since this significantly influences the rate of contamination from external pollen donors. These results indicate that knowledge of cross-compatibility among cultivars is necessary to plan efficient olive breeding crosses (Diaz et al. 2007).

The possibility of associating genetic characteristics and DNA-based molecular markers is very important to select the progeny showing interesting agronomical traits and even specific organoleptic characteristics at the first stages of development which may use as a marker for future *olive oil* identification. However, this technique, called marker-assisted selection (MAS), requires some knowledge on the co-segregation of molecular markers and genetic characteristics in the progeny. (Bracci et al., 2011)

8. Conclusion

For the inefficiency of analytical parameters in showing variability among samples of the same cultivar/blend due to the environmental conditions and pressing technologies, Several DNA-based technologies and traceability analysis has been used to reveal the different origin of lots that have contributed to the olive oil blend. In this regard, DNA-based methods make an important contribution to protect high quality brand names and in turn the consumer

The knowledge of genome nucleotide sequences also could be useful to identify new sequence polymorphisms, which will be very useful in the development of many new cultivar-specific molecular markers (e.g.; Single Nucleotide Polymorphisms, SNPs) and in the implementation of more efficient protocols for tracking and protect *olive oil* origin (in POD *olive oils*).

The greatest challenges one faces while using DNA technology is the low quality and highly degraded DNA recovered from the fatty matrices and the impact of oil extraction processing on the size of the recovered DNA. DNA of low, difficult to determine content and of unknown, variable quality would potentially lead to inconsistent and consequently inconclusive results. Although, the concentration of DNA did not appear to be limiting; rather, successful PCR amplification likely depended on the ability of the DNA extraction method to free DNA from inhibitors of PCR present in the *olive oil*.

It is to be considered if the DNA is damaged, it could be not properly accessible to the DNA polymerase, which stalls at the sites of damage and the reaction may be interrupted; this being able to influence the length and significance of the synthesized amplicons. The use of proteinase K during extraction process has recommended for a better protection of DNA from degradation and increase in DNA yield, as well.

Identification of molecular markers suitable for tracing the genetic identity of olive cultivars from which oil is produced, on the other hand, has a great importance**.** For making decision, which molecular markers will be more useful in obtaining reliable results through the numerous molecular markers existing in the literature, many of them have been practically examined (including RAPDs, AFLPs, SCARs, SSRs, ISSR, SNPs, …). A combination of molecular markers (RAPD, ISSR, and SSR) to establish a relationship between small-scale-produced monovarietal and commercial *olive oil* samples for certification purposes has been proposed.

Several authors recommended sequences of DNA that show polymorphism at low hierarchical level are therefore suitable for distinguishing between individuals within the same species. They clearly pointed non-coding nuclear DNA sequences could be the best choice. Among those sequences, the microsatellites are likely the most suitable ones. However SNPs that require shorter than 100 bp DNA templates, considered to be successfully used for a wider range of olive oil identification.

In some cases of using microsatellite, the microsatellite profiles obtained with the monovarietal oil-derived DNA were generally consistent with the cultivar used, although some ambiguities were recorded likely due to contamination in monovarietal oils by other cultivars grown in the same block or contaminations occurred at the mill. Moreover, in some cases the lack of matching in leaf and oil profiles has been reported that was due to the presence of embryos in berry seeds that brought the alleles of pollinators. Other cases of mis-amplification were recorded as a missing allele, due either to the preferential amplification of one of the two alleles in oil-derived DNA templates, or to the excess of degradation of the DNA template of the miss allele, that limited the production of a sufficient number of copies of that allele to be detected. In such a case, real-time PCR assay could possibly solve this kind of problems.

To trace out the adulteration in *olive oil* using combined approach of molecular biology and bioinformatics based on unique SNPs present in conserved DNA sequence of plastid genomes of sunflower, canola and olive has been already performed. In general, plastid/chloroplasts are miniature organelles (approx. 5 X 3 μm in size) enclosed in double layer membranes. They are present in abundance (10–100 per cell, and each plastid contains about 100 copies of circular plastid genomes, average size 150 kb) and there is probability that most of the plastid organelles may be left intact due to miniature size when cold pressed to extract the oil from seeds of olive, canola and sunflower.

Moreover, plastid DNA present in extracted oil could be safe from nucleases activities due to double layer membranes and present in large number of copies in comparison to 1–2 nuclear genomic DNA which may be more prone to degradation.

In addition, a new chloroplast marker represents a valuable tool to assess the level of olive intercultivar plastome variation for use in population genetic analysis, phylogenesis, cultivar characterisation and DNA food tracking is recommended.

In summary, molecular biological techniques have become an every-day tool to solve a number of problems and questions in the section of varietal/species identification, fraud, traceability and paternity analysis.

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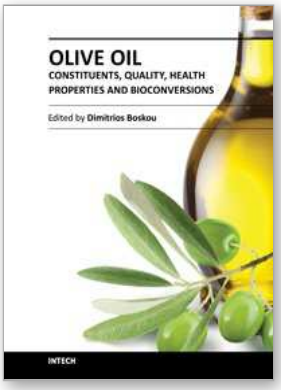

Olive Oil - Constituents, Quality, Health Properties and Bioconversions

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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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