Chapter 9

Challenges for Genetic Identification of Olive Oil

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1. Introduction

Olive oil is the oil extracted exclusively from fruit of *Olea europaea* L. only by means of mechanical methods or other physical procedures that do not cause any alteration of the glyceric structure of the oil thus preserving its characteristics and properties. The healthy properties of olive oil are well known in the Mediterranean diet, in which virgin olive oil is the main source of fat [1].

In comparison to commonly used vegetable oils, the cost of olive oil is higher. As such, olive oil comes up against adulteration with other cheaper oils as well as the use of unapproved production methods [2]. Blending premium olive oil with low quality oils (mostly pomace) or with other plant oils such as hazelnut (*Corylus avellana*), soya (*Glycine max*), almond (*Prunus dulcis*), maize (*Zea mays*), sunflower (*Helianthus annuus*) and sesame (*Sesamum indicum*) has a great negative effect on olive oil trade [3]. This defines an urgent action in confirming olive oil authenticity

In the recent years, the European Union (EU) has introduced sever regulations about virgin olive oil origin confirmation with the aim of governing its label and protecting its producers and consumers from fraudulent activities [3]. Besides, the concept of certified brands such as PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) has been recently introduced to provide more tools to protect olive oil within both EU and non EU countries. Although aforementioned tools confirms oil origin (geographical cultivation origin or place of processing), it is still to decide if olive oil PDOs and PGIs are safeguarded from fraudulent labeling [4].

The authenticity of olive oil, and particularly that of a virgin olive oil, is conventionally assessed by monitoring of several components such as sterols, phenols, fatty acids, triacylglycerols, volatile compounds and tocopherols. However, the analytical analyses have their limits and chemical composition of virgin olive oil is influenced by genetic (variety) and en-

vironmental factors (climatological and edaphologic conditions). As the composition of extra virgin olive oil is the result of a complex interaction among olive variety, environmental conditions, fruit ripening and oil extraction technology, two main approaches, botanical and geographical origin identification, are focused to trace olive oil. However, in both cases, the selection of appropriate markers is sophisticated and needs more attention [5]. This has promoted a growing interest towards the application of DNA-based markers since they are independent from environmental conditions. The evaluation of DNA nucleotide sequences can provide more precise information, which can be obtained through traditional morphological markers or chemical composition analysis. Thus, specific protocols for DNA isolation from olive oil have been developed [6-9]. However, 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 sequence of *Olea europaea* L. has deposited in NCBI (National Center for Biotechnology Information) [10].

On the other hand, the advent of molecular markers offers a powerful tool to uncover synonymy and parentages between olive cultivars, and reveals a large admixture amongst varieties of different geographic origin of olive that consequently improves PDO olive oil recognition [11]. Individual fingerprinting based on molecular markers has become a popular tool for studies of population genetics and analysis of genetic diversity in germplasm collections, including the solution of synonymy/homonymy and analysis of paternity and kinship.

In this chapter, researches for genetic identification of olive oil in introducing unequivocal identifiers for authentication and traceability of olive oil will emphasis as a crucial concept to be overcome for international olive oil trade.

2. An overview on olive oil

Olive tree (*Olea europaea* L.) represents the most important oil producing crop in the Mediterranean basin. Olive tree is a diploid species (2n = 46) that is able to survive for a long time [12-13], is outcrossing and sometimes self-incompatible which implies that seeds are produced by cross-pollination [13-15].

This species belongs to monophyletic *oleaceae* family. *Oleaceae* comprises about 600 species and 24 genera [16, 17]. Within this family, *Olea* and ten other (extant) genera constitute the subtribe *Oleinae* within the tribe *Oleeae* [16]. Thirty-three species and nine subspecies of evergreen shrubs and trees have been circumscribed in *Olea* based on morphological characters [18]. In addition, these taxa are classified in three subgenera, *Olea*, *Paniculatae* and *Tetrapilus*, the first of which has two sections (*Olea* and *Ligustroides*). Section *Olea* is formed exclusively by the olive complex (*Olea europaea*), in which six subspecies are recognized [18, 19]. This subgenus is distributed from South Africa to China, across the Saharan mountains, Macaronesia and the Mediterranean basin. The cultivated one is *Olea europaea* subsp. *europaea* var. *europaea*. that found outside of its native range as a result of human-mediated dispersal; it

has been repeatedly introduced in the New World and has become naturalized and has invaded numerous areas in Australia, New Zealand and the Pacific islands [18, 20].

Olive is the second most important oil fruit crop cultivated worldwide after oil palm. Its cultivation covers over eight million hectares of land, predominantly concentrated in the Mediterranean basin, where 70% of the olive oil produced is consumed [21]. The olive tree is a glycophytic species that shows a high tolerance to drought and salt stresses, if compared with other fruit trees that are generally salt sensitive [22]. 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 [13].

The olive oil is known for its beneficial effects on health, such as ability to reduce blood pressure and low-density lipoprotein (LDL) cholesterol, as well as for its cancer prevention, antimicrobial and antioxidant virtues [23, 24]. The international olive council Resolution no. res-3/89-iv/03 [25, 26] catagorised virgin olive oil as: (i) "Extra virgin olive oil": virgin olive oil that has a free acidity, expressed as oleic acid, of not more than 0.8 g per 100 g; (ii) "Virgin olive oil": virgin olive oil that has a free acidity, expressed as oleic acid, of not more than 2 g per 100 g; (iii) "Ordinary virgin olive oil": virgin olive oil that has a free acidity, expressed as oleic acid, of not more than 3.3 g per 100 g.

The increase in the demand for high-quality olive oils has led to the appearance in the market of olive oils, elaborated olive oil with specific characteristics. They include oils of certain regions possessing well-known characteristics, that is, olive oils with a denomination of origin, or with specific olive variety composition. Olive oils obtained from one genetic variety of olive or from several different varieties are called monovarietal or coupage, respectively. Monovarietal olive oils have certain specific characteristics related to the olive variety from which they are elaborated [5]. However, coupage olive oils are obtained from several olive varieties to achieve a special flavor or aroma [13].

3. Olive oil adulteration

Olive oil is one of the most valuable single products of the agro-food industry. It is made from diverse cultivars either mixed or single. Those ensure different tastes and typicity, and these may be also enhanced by the region of production of cultivars [8].

Recently PDO and PGI olive oils appeared that requiring precise definition of several parameters such as cultivar, geographical origin, agronomic practice, production technology, and organoleptic qualities. The quality of these monovarietal oils is associated with superior taste, consistency and colour and is directly related to the olive cultivar. Therefore, the authenticity efforts concentrated on the identification of their varietal origin as well as their adulteration with lower grade, processed olive oils [27].

That is why a well-documented traceability system has become a requirement for quality control in the olive oil chain.

DNA, being not environmentally labile, considered a great potential to be used as a means of identifying the varieties of the trees purporting to be the source of a given sample of olive oil [3].

Genetic 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 olive oil traceability is necessary for the prevention of deliberate or accidental mixing or mislabeling, which is very important in the international trade.

4. Conventional identification tools of olive oil: Chemical analysis

As the quality of an olive oil depends on the olive variety from which it is elaborated, the production of olive oils from certain varieties with appreciated quality has increased [28]. The olive variety selection is mainly based on its adaptation to different climatic conditions and soils.

Several analytical determination has improved during years, because of improving both of knowledge on olive oil composition and of analytical techniques and instrumentation. Unfortunately, adulterations, too, improved and became more sophisticated; for example, it is recognized that by careful blending high oleic oils (such as sunflower oil) the obtained product well fits the fatty acid composition of olive oil. However, they usually have a "wrong" sterol composition and those who perform frauds were able to eliminate sterols, because of this act, the absolute amount of sterols was enclosed in the standard as well as the measurement of sterol dehydration products (such as stigmastadienes).

To relate the fatty acid composition of olive oils 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) [29]. A relationship between the fatty acid composition and some specific cultivars has been observed [5].

The volatile fraction in olive oils, 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 [5].

In fact, genetic and geographic factors influence the volatile compound production of the olive fruits and affect the differentiation of olive oils according to their olive variety [30, 31]. The volatile compound contents allowed differentiation among monovarietal olive oils and even identification of the technique used for olive oil production [32].

The colour of a virgin olive oil is due to the solubilization of the lipophilic chlorophyll and carotenoid pigments present in the fruit. The green-yellowish colour is due to various pigments, that is, chlorophylls, pheophytins, and carotenoids [33]. Several researchers reported the same qualitative composition in chlorophyll and carotenoid pigments, independent of

the olive variety and the time of picking [34, 35]. 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 [36]. Lutein/β-carotene ratio has been reported as a tool to differentiate oils from a single cultivar [5].

Tocopherols and hydrocarbons are the compositional markers less studied to date to differentiate olive oils. 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 [5].

5. Need for cultivar identification, search for new approaches: Molecular markers (DNA) vs. biochemical descriptors

Olive oils labeled with their region of origin are sold at a premium price. This premium is greatest for oil from those regions associated with superior taste, consistency or colour. For cold-pressed oils (extra virgin and virgin), these properties are associated with the cultivar and the environment.

A lot of research has been carried out to assure the authenticity of olive oil through chemical analysis [37-40]. However, several difficulties have been encountered in distinguishing olives and olive-oils from different cultivars because their characteristics are strongly influenced by environmental conditions. On the other side, accurate and rapid identification of cultivars is especially important to obtain a reliable label of origin.

In such a case, some DNA-based technologies can help in revealing either the authenticity or the different origin of lots that have contributed to the olive oil. This action discourage from the adulteration with extraneous material of lower cost and value.

6. Olive oil genetic traceability

The genomic analysis of olive oil involves two main obstacles; extraction of DNA from an oily matrix and selection of appropriate molecular markers that can provide a trustable result. Overcoming these two important limits make possible the assessment of genetic traceability of olive oil.

DNA analysis offers an alternative approach, relative to other macromolecules and metabolites, due to it is less influenced by environmental and processing conditions, enables genome fingerprinting with consequent identification of variety/type composition.

Significant amounts of DNA are present in olive oil obtained by cold pressing [6]. However, the filtration process lowers DNA concentration, which tends to disappear due to nuclease degradation [7, 41]. Application of molecular markers to trace foods brought new benefit to consumers.

Several works described the application of molecular markers to genetic recognition of the cultivar composition of monovarietal olive oils. However, these works describe the application of multilocus markers, such as RAPDs or AFLPs [9, 41] or microsatellites [8, 42].

6.1. Achievements in olive oil genetic traceability between 2000 – 2006

During the years between 2000 -2006, many important events regarding genetic traceability of olive oil have been recorded. The most outstanding achievement was defining a protocol for DNA extraction from olive oil [8-9, 41]. The other success in this context refers to the deposition of several sequences of olive genome on NCBI database, including 458 nucleotides and 24 ESTs (Expressed Sequence Tags). These sequences facilitated the access to more DNA markers. For example, Cipriani *et al.,* (2002) isolated and sequenced 52 microsatellites or simple sequence repeats (SSRs) from nearly 60 positive clones obtained from two 'Frantoio' olive genomic libraries enriched in (AC/GT) and (AG/CT) repeats, respectively [43]. Furthermore, many authors have reported on SSR development in olive and several of them are currently available for DNA analysis [43-46].

These approaches offered new opportunities for researchers to perform such an analysis involving olive cultivar identification and olive oil authentication.

6.1.1. A big obstacle: DNA Extraction

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.

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 [6].

Muzzalupo *& Perri* (2002) tested some enzymatic mixtures to prevent DNA damage that occurs during crushing and malaxation [41]. They emphasized positive effect of proteinase-K treatment during the malaxation process to provide DNA amenable to random amplified polymorphic DNA (RAPD)-PCR amplification.

However, Busconi *et al.,* (2003) defined a reliable DNA extraction method via CTAB method from 50-100 mL lab-made monovarietal oil and commercial extra virgin olive oil [9]. The suggested method concerned both quantity and quality of DNA.

Breton *et al.,* (2004) have used several supports to retain DNA checking different techniques (silica extraction, hydroxyapatite, magnetic beads, and spun column) [8]. The method using magnetic beads has been introduced as the most efficient method and they claimed that the running protocol is usable in routine labs to control virgin or crude oil samples and may be used for refined oil, as well.

DNA has been extracted from cell residues recovered by oil centrifugation as reported by Pasqualone *et al.,* (2004) [42]. Doing successful DNA extraction and SSR analysis, the electrophoretic patterns showed an adequate level of amplification and were identical to those obtained from leaves and drupes of the same cultivar [42].

Pafundo *et al.,* (2005) reported optimization of AFLPs for the characterization of olive oil DNA, to obtain highly reproducible, and high quality fingerprints [47]. Her group 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 [47].

6.1.2. Molecular markers selection and PCR analysis

AFLP, RAPD, SCAR and SSR have been employed as genetic marker for various cultivar / genotype identification. Muzzalupo & Perri (2002) reached the first unambiguous and reproducible RAPD-PCR amplification of DNA recovered from virgin olive oil [41]. The presence of additional alleles in RAPD profiles deriving from monovarietal oil, missing in the leaves of original varieties were interpreted as signature of pollen DNA, but no data were provided as support of this hypothesis [41].

However, Busconi *et al.,* (2003) showed the correspondence between profiles of the DNA purified from monovarietal oil with that from the leaves of the same cultivar [9]. Although Pafundo *et al.,* (2005) found the aforementioned correspondence close to 70% [47]. Based on their findings, DNA extraction from olive oil was the limiting step for the reliability of AFLP profiles. Their results also 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.

Breton *et al.,* (2004) and Pasqualone *et al.,* (2004) used SSR to identify olive cultivars contributed in commercial olive oil samples and virgin olive oils [8, 42]. The electrophoretic patterns showed an adequate level of amplification and were identical to those obtained from leaves and drupes of the same cultivar.

6.2. Improvements in olive oil genetic traceability between 2007 – 2012

By 2007 the main obstacle in genetic traceability of olive oil, DNA extraction, has been overcome successfully; however, there are still some publications which introducing DNA extraction protocols specified for olive oil. In this period, the main activities were focused on using different molecular approaches; meanwhile the volume of sequences deposited on NCBI has being increased exponentially which enabled the researchers in profiting other molecular markers such as designing new and more effective primer pairs, on different regions over nuclear regions, such as chloroplast, mitochondrial, and plastomal sequences (NCBI database, including 1405 nucleotides, 7865 ESTs and 26 GSS (Genome Survey Sequences) hints by 18/09/2012). Also, there have been many attempts to establish a better understanding of cultivar differentiation, genetic diversity [48] and identification of new polymorphic regions.

6.2.1. A glance on DNA extraction

Until 2007 a wide range of protocols on DNA extraction from olive oil, either cold press or refined, had been introduced. Then on, the researchers have focused on the application of current molecular tools or searching for new and more appropriate ones. The general idea that has been accepted and reported by several researchers is that olive oil provides very low yields of DNA and has variable degrees of degradation which may limit the applicability of molecular markers [49, 6]. Furthermore, it has been shown that DNA is damaged by oxidation reactions, which may cause DNA lesions and base transitions with production of dangerous adducts [50, 51]. 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 [52].

6.2.2. Molecular markers selection

Many studies in recent years have employed AFLP, RAPD, ISSR, SSR, LDR/UA, qRT-PCR, SNPs, CE-SSCP, and DNA barcode as genetic tools to produce a reliable platform to identify the cultivars contributing to an olive oil. Pafundo *et al.,* (2007) suggested developing of sequence characterized amplified region (SCAR) markers [53] derived from AFLP profile of olive oil can be instrumental to simplify the determination of varietal composition of an oil sample. A procedure to visualize AFLPs of oil in agarose gel was developed to avoid the usual procedure for SCAR isolation from polyacrylamide gel electrophoresis, expensive, time-consuming, and requiring the use of radioactive isotopes [53]. Finally, Pafundo *et al.,* (2007) mentioned the high correspondence between the profiles obtained with agarose and capillary electrophoresis as an index of the reliability of the used method [53]. In addition, Montemurro *et al.,* (2008) reported a good quality of AFLP profile in oil [49]. It has been possible to improve the sensitivity of AFLP with the optimisation of DNA extraction and restriction/ligation condition.

On the other hand, the effect of olive oil storage time on the quality and quantity of DNA as an analyte for molecular traceability has been assessed via AFLP, which was used due to its multilocus nature, which allows a better discrimination of DNA composition [51]. Comparison of AFLPs was made among profiles of leaves and monovarietal olive oil stored at different times. Montemurro *et al.,* (2008) it has been detected that for some cultivars such as Taggiasca cv. although the AFLP profiles of leaf and of the oil DNA extracted after one and three weeks of storage were similar, the same profiles were quite different for DNA extracted in the one year stored oils [49]. For Carolea, some homologies were found between leaves and oil, whereas for other cultivars such as Leccino and Ogliarola Leccese these homologies were not detectable. Finally, it has been declared that nine months after production; profiles of oil DNA were highly different from the leaf profiles in all examined cultivars [49].

Another study to assess DNA stability in olive oil during storage time proposed the use of lambda DNA as a marker. In this study the progress of DNA fragmentation in olive oil has been monitored throughout a 12-month storage period. *Lambda* DNA was introduced into filtered olive oil samples in three different concentrations as a DNA marker. It has been re-

vealed that the inhibitory effect of olive DNA extracts was increased partially and gradually with the storage period of the olive oil samples used for the DNA extraction [54].

Martines-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 [55]. Among eleven RAPD primers tested only two produced reproducible bands in all olive oil samples, which demonstrate low efficiency of this tool. However, it has been shown that ISSR marker system in olive oil is more informative than RAPD. Finally, Martinez-Lopes confirmed that SSR amplification was satisfactory only when water phase DNA was used in the reaction. SSR analysis used to compare the profile of DNA isolated from monovarietal oil with that from leaves of the same cultivar [55].

SSRs have a high discrimination power and so far they are the most employed markers. Several authors [25, 42, 56-59] reported a good match between olive oil and leaf profiles but they did not report any data about repeatability of results. In addition the SSR sequence dramatically influences the efficiency of analysis, as well as the kind of oil [58]. The reproducibility of results was low confirming that the choice of SSR loci and primers is relevant for an efficient analysis. Furthermore, assigning the true size of alleles and resolution of conflicts considers as another obstacle in interpretation of SSR results.

All the authors agree that differences in size and allele drop out in oil may be due to components interfering with PCR reaction, or to the lower quantity and quality of DNA, which makes difficult the selective amplification of DNA for any allele pairs [3].

The appearance of extra alleles detected in oil addresses either the mixing with traces of other oils present on the machinery during milling process or the accidental mixing with other cultivars during harvesting, transportation and processing. In addition, DNA from the pollinators present in the genome of the seed embryo, could potentially contain alleles not present in the genome fruit pulp, invalidating the molecular traceability of olive oil [60].

Pasqualone *et al.*, (2007) demonstrated that microsatellites are useful in checking the presence of a specific cultivar in PDO oil, thus verifying the identity of the product [61]. However, they obtained only the marker profile of the main cultivar in the oil: no signal was detected for the secondary varieties [62]. Specifically, Pasqualone *et al.,* 2007 confirmed the sufficiency of a single microsatellite, GAPU103A, to distinguish Leucocarpa oil from the other samples [61]. An identification key based on the amplification profile of this microsatellite was set up to distinguish the oils from different cultivars [57]. Rotondi *et al.,* (2011) performed a comparison between genetic results, chemical and sensory properties of monovarietal olive oils and demonstrated a very good correspondence between the clustering obtained by SSR analysis and the clustering based on selected fatty acids composition [63].

Single nucleotide polymorphisms (SNPs) are molecular markers which require short DNA amplicons for genotyping [27]. In addition, they are the most abundant markers in the genome; they are stably inherited; bi-allelic in most cases and co-dominant [64]. The most significant comparative advantage of SNPs among all molecular markers is the requirement for short, even shorter than SSRs, approximately 100 nucleotides PCR templates as analytical targets. This advantage can be considered highly critical for heavily

processed food matrices such as olive oils and other plant oils due to the highly degraded nature of DNA present in these matrices. Moreover, although the identification of SNPs was considered an expensive task few years ago, the recent developments on high throughput sequencing technological platforms allows the cost- and time-efficient identification of SNPs in every plant species [64].

Consolandi *et al.,* (2008) gave precise and accurate genotype results using ligation detection reaction (LDR)/universal array (UA) on a SNP-containing DNA sequences for the genotyping of olive cultivars. In this assay, alleles are distinguished by a ligation detection reaction and, subsequently, detected by hybridization onto a universal array [6].

Bazakos *et al.,* (2012) reported a successful use of SNPs in tracing olive oil and mentioned neither paternal contribution of embryos was detected in olive oil samples nor did additional peaks in leaf samples [27].

Kumar *et al.,* (2011) proved SNPs variation in noncoding spacer region between psb-trnH and partial coding region of matK of plastid genome. This procedure enabled to discriminate the mixing of canola and sunflower oil into olive oil. This plastid based molecular DNA technology proposed to be used for rapid detection of adulteration easily up to 5% in olive oil [65]. The development of this kind of marker requires a high level of genome sequence information: it is therefore not surprising if only a few SNPs have been reported in olive, where only a small amount of sequence data was available before the year 2009.

To overcome the lack in sequence knowledge, Reale *et al.,* (2006) used both a sequence-based and an arbitrary approach to identify eight SNPs in olive [66].

As conventional PCR technique is not optimal for authentication purposes when quantification is needed, qRT-PCR has been introduced as an efficient tool allowing discarding primers with low PCR efficiency [67]. Wu *et al.,* (2008) employed a sensitive real-time PCR method using the novel fluorescence stain Evagreen (Evagreen intercalates in a sequence independent way in DNA duplexes) established for detection of olive oil, which successfully distinguished olive oil from inferior plant oils [68].

A more recent study using a CE-SSCP (Capillary Electrophoresis-Single Strand Conformation Polymorphism) method based on PCR technique was established to trace olive oil authenticity from adulteration with other vegetable oils [69]. SSCP is based on the dependence of electrophoretic mobility of a single-stranded DNA on its folded conformation, which is dependent on the nucleotide sequence. Even single base change in a sequence is likely to result in different conformations, which results in slight difference of molecular mobility [70]. The method developed was very suitable for the determination of modeled and of unknown adulterants [71].

Another novel method for identification of different species of vegetable oils based on suspension bead array has been reported by Li *et al.,* (2012) [72]. The suspension bead array as a rapid, sensitive, and high-throughput technology has a great potential to identify more species of vegetable oils with increased species of probes [72].

7. Future challenges and prospective

Future research in the concept of olive oil genetic traceability will concern the application of high-throughput platforms including functional genes, non-nuclear genes, transcriptome analysis, and developing more sophisticated SNPs detection.

Understanding the function of genes and other parts of the genome is known as functional genomics that describes the relationship between an organism's genome and its phenotype. This approach provides a more complete picture of how biological function arises from the information encoded in an organism's genome and such information will contribute in intra-species determination especially with PDO oils.

Chloroplast DNA considers as a most important non-nuclear genes and has been investigated for cultivar identification in olive oil [73]. One advantage of chloroplast DNA is the high copy number of chloroplast per cell (about 50), which is especially beneficial for refined oil sample. It is to develop suitable markers on this region and a compositional test able to identify a cultivar in a monovarietal olive oil. The designed markers will be applied in a high-throughput platform to assess and quantify the contribution of a single cultivar in commercial multivarietal oils.

Olive transcriptome will address the identification of genes differentially expressed during fruit development, with particular attention to those involved in lipid and phenolic metabolism. The provided information will discuss the case of olive oil PGI.

Improving SNPs detection using high resolution melting (HRM) RT-PCR analysis allows olive cultivar genotyping, results in an informative, easy, and low-cost method able to greatly reduce the operating time is also recommended.

Finally, Zhang *et al.,* (2012) proposed an alternative strategy would be using fast and less accurate sensor technology, such as electronic nose, as screening method and verifying suspected samples by DNA method [71].

8. Conclusions

Appropriate method, DNA-based analysis, has been developed to verify the authenticity of olive oil and detect possible adulteration to protect the consumer against any fraud practices. DNA analysis represents an attractive and alternative choice to the more classical analytical methods, because DNA, rather than the macromolecules and metabolites, is less influenced by environmental and processing conditions [74].

Although significant progress has been made in the last decade on DNA extraction from olive oil and the choice of molecular markers, still a consensus protocol, by which the mystery behind olive oil authentication reveals, has not been accepted in trade markets, yet.

At present, DNA extraction from oil is no longer problematical, and the critical point is the choice of markers. Basic criteria to evaluate the suitability of molecular markers at this pur-

pose are: i) the discrimination power; ii) the correspondence between leaf and oil-DNA profiles; iii) reproducibility and repeatability of results; iv) simplicity of analysis [3].

RAPDs and AFLPs give complex profiles that can be applicable to monovarietal oils but not to mixtures of three to four cultivars, such as those usually adopted in PDO oils [53]. Single-locus microsatellites are more effective to this aim, but they are not applicable to high-throughput screening such as microarray.

Single locus (SSRs and SNPs) are preferred to multilocus (AFLP, RAPD) markers because they are simpler to perform, more easily interpretable and can be combined in high-throughput platforms.

Since most olive cultivars are auto-incompatible (pollen could not germinate on an ovary from the same tree) the DNA extracted from oil contains alleles of the tree (fruit pulp somatic tissues) as well as alleles of the seed embryo which may contain exogenous alleles from the pollinator. Thus, Bracci *et al.,* (2011) 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 [62].

Besides, using capillary electrophoresis permits to differentiate alleles with very small differences in molecular weight and to detect a very low or partially degraded DNA, which is the case of extracted DNA from olive oil [75].

The availability of other approaches such as semi-automated SNP genotyping assay proposed to verify the origin and authenticity of monovarietal extra virgin olive oils [6].

Finally, generating an "Identity Card" which, can be used for the unequivocal identification of highly prized oil, has been considered as a potential for olive oil DNA fingerprinting [3].

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