To Grigoris and Evi my parents,
and Eftihia my sister

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CHRISTOS BAZAKOS
2007

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ABBREVIATIONS

NIF	Near Infra-Red
NMR	Nuclear Magnetic Resonance
UV	Ultra-Violet
VIS	Visible
IR	Infra-Red
IRMS	Isotope Ratio Mass Spectrometry
GC	Gas Chromatography
LC	Liquid Chromatography
TLC	Thin-Layer Chromatography
HPLC	High-Performance Liquid Chromatography
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
SCARS	Sequence Characterised Amplified Regions
AFLP	Amplified Fragment Length Polymorphism
SSR	Simple Sequence Repeats
SNP	Single Nucleotide Polymorphism
c SNP	coding SNP
r SNP	regulatory SNP
ddNTPs	dideoxynucleotide triphosphates
FRET	Fluorescence Resonance Energy Transfer
MIP	Molecular Inversion Probe

ASO	Allele-Specific Oligonucleotides
FITC	Fluorescein IsoThioCyanate
TAMRA	carboxytetramethylrhodamine
PDO	Protected Designation Origin
NAGREF	National Agricultural Research Foundation
CTAB	CetylTrimethylAmmonium Bromide-based
EtOH	Ethanol

ABSTRACT

The aim of this work is to generate a single nucleotide polymorphism database on *Olea europea* varieties, in order to detect the varietal contribution present on olive oils on a both qualitative and quantitative level.

The first objective would be to screen DNA sequences of several gene targets among many oil producing varieties for SNPs that can be used in the future for the varietal characterisation of commercial olive oils. The second objective would be to develop an SNP-based approach and test its analytical characteristics, such as reproducibility, limit of detection and limit of quantification on previously known olive oil samples. Other issues such as the influence of storage period on the quality of extracted DNA shall also be investigated.

The development of molecular markers in olive tree varieties by means of SNPs and the understanding of the mechanism under which they contribute to several phenotypic traits such as antioxidants production, resistance to biotic or abiotic stresses, oil quantity and quality shall be beneficial to all European scientists focused on that species. In particular, it shall promote olive breeding and selection schemes under a common European strategy. Therefore, National agricultural or oil institutes shall take advantage of such knowledge for the benefit of European countries. Varietal discrimination of olives through SNPs shall be useful to the European Food Agencies that shall be likely involved with aspects such as Food authenticity and traceability and especially in P.D.O. issues of olive oils.

CHAPTER 1

LITERATURE REVIEW

1. Food authenticity and fraudulence

Food authenticity is a term which simply refers to whether the food purchased by the consumer matches its description.

False description can occur in many forms such as the undeclared addition of water or the use of food additives such as colours, flavourings, and other various adulterations.

In addition, the wrong declaration of the amount of a particular ingredient found in the product and finally, the false statements about the source of ingredients (i.e., their geographic origin, plant, or animal) are some levels of adulteration that have been in existence since the beginning of trade (Kvasnicka, 2005).

1.1 The significance of authenticity

Food authenticity is a major issue for producers, processors, distributors, consumers and regulators. To begin with, the producers prefer the cultivation of superior quality products which need additional effort but the sell price is higher. Consequently, the authenticity and price of their products are the reward for their effort.

Also, for the industry the presence of non-authentic foodstuffs obviously damages profits as well as consumer confidence. Food manufacturers need to ensure that their products meet the demands of legislation in the countries where they are sold, whereas governments need to guarantee that tested and valid methods are available to meet the needs of industry and to protect the public from deception or fraudulent labelling (Kvasnicka, 2005).

Moreover, many consumers are prepared to pay a premium for products they consider to be of superior quality, whether that is due to the way of processing (as

with the case of organic food), or due to several other reasons, such as the geographical origin, which is sometimes considered as an identification criterion that is often used as a quality index (Kvasnicka, 2005). Consumer choice might also reflect lifestyle or religious concerns (e.g. vegetarianism, preference for organic products, absence of pork for Jews and Muslims), or health concerns (e.g. absence of peanuts, lactose or gluten for individuals with particular allergies) (Woolfe and Primrose, 2004). Therefore, the description and labelling of food must be honest and accurate, particularly if the food has been processed removing the ability of the consumer to distinguish one ingredient from another.

1.2 Means to detect food authentication

While adulteration practices remained quite simple, their detection by relatively straightforward analytical methods was possible. As novel and advanced technologies have become widely available, the adulteration practices have also become more complicated (Kvasnicka, 2005).

There is a need not only for new analytical methods to establish food authenticity, but also for a continuous reassessment of these techniques in order to keep pace with changing industrial practices and the increasingly devious adulteration methods of deceitful producers. In recent years, many methods, conventional or non–conventional, have been developed for detection of food adulteration.

1.2.1. Conventional methods (Non–DNA–based)

Conventional methods are not based on DNA analysis; instead this approach focuses on the analysis of many different constituents or properties of food products. Also, they separate molecules with similar chemical characteristics even from complex matrices and they are used for the determination of a series of foodstuff ingredients.

1.2.1.1 NIF (Near infra–red) technology

Near infra-red light is part of the broad spectrum of energy known as electromagnetic radiation. The infra-red region is of relatively low energy and upon interaction with molecules, causes inter-atomic vibrations. The frequency of this wave oscillation in the infra-red region is about $10^{13} – 10^{14}$ Hz, which is of the same order as the natural mechanical vibrational frequencies of many chemical groups (e.g water). As a result near infra-red measurement technology is fast, continuous, non-destructive and can be applied relatively simply in many industrial processes (Benson, 2003).

1.2.1.2. NMR spectroscopy

NMR spectroscopy is nowadays being used more and more for food analysis. Standard ^{1}H, ^{13}C and high resolution magic angle spinning (HR/MAS) NMR spectra give a wealth of chemical information on liquid foodstuffs and even semi-solid foods. In addition NMR spectra of food products can act as 'fingerprints' that can be used to compare, discriminate or classify samples (Le Gal and Colquhoun, 2003).

1.2.1.3. Spectrophotometric techniques (UV, IR)

Spectrophotometric techniques cover the ultraviolet (UV), visible (VIS) and infra-red (IR) spectra. These techniques are based upon the production or interaction of electromagnetic radiations with matter. Emission techniques measure light radiations produced when the analyte is excited by thermal, electrical, or radiant energy. Absorption techniques are based upon the decline of a beam of light radiation as a consequence of its interaction with and partial absorption by the analyte (Meurens, 2003).

NIF, NMR, IRMS and spectrophotometric techniques belong to non-destructive spectroscopic methods for discrimination between authentic and non-authentic samples (orange juice, cereal grains, vegetable oil, etc.) (Kvasnicka, 2005). They have the advantage that time consuming sample preparation steps are normally kept to a minimum (Ulberth and Buchgraber, 2000).

1.2.1.4. Chromatographic methods (GC, LC, TLC, HPLC)

Chromatographic methods have been developed for the rapid and reliable separation of molecules with extremely similar chemical characteristics even from complex matrices and they are based on the absorption and/or partition of the molecules to be separated between a mobile and a stationary phase. These procedures have found increasing acceptance and employment in food science and technology for quantitative analysis of numerous molecules such as normal constituents of foods, legal or illegal additives, pollutants, etc (Forgacs and Cserhati, 2003). They can be used either on the detection of specific adulterants or as authenticity markers (3-methylhistidine for lean meat, ratio of citric and isocitric acids in citrus juice, D-malic acid in apple concentrate, etc.) (Kvasnicka, 2005).

Chromatographic techniques most frequently used in food analysis are gas chromatography (GC) (solid stationary phase and gas mobile phase), thin-layer chromatography (TLC) (planar solid surface, liquid mobile phase moving by capillary forces or by a pump system) and high-performance liquid chromatography (HPLC) (stationary phase filled in column, liquid mobile phase moved by pump) (Forgacs and Cserhati, 2003).

1.2.1.5. Enzymatic methods

Enzymatic analysis as an analytical technique is, firstly, analysis using enzymes as reagents to measure all substances capable of being transformed by enzymes, and secondly, the determination of enzyme activities. The activity of an enzyme is measured by the rate of consumption of the reactants (substrates) or the formation of products (Henniger, 2003).

Enzymatic methods for the determination of a series of foodstuff ingredients are useful tools in routine food analysis. They allow the specific, reliable, precise and quick determination of some sugars, acids and salts, alcohols and other compounds, which occur in nature and are contained in foodstuffs (Henniger, 2003).

1.2.1.6. Enzyme immunoassays

Immunochemical methods are based on the ability of antibodies (immunoglobulins) to recognize and bind or interact with three-dimensional structures and play a major role in biochemical research. Being primarily a part of the immune system in most classes of vertebrates (Stanworth and Turner, 1979), immunoglobulins have been utilized as the key substances in any immunoassay for more than 40 years now (Martlbauer, 2003).

One representative example of the enzyme immunoassay method is ELISA. The purpose of ELISA is to determine if a particular protein is present in a sample and if so, how much. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polysterene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjunction. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

Besides biomedical research and clinical chemistry, enzyme immunoassays have been used in a broad range of applications in food analysis. One major use of enzyme immunoassays is the detection of proteins occurring in food that allow either the identification of the animal species or give an indication on adulteration of processed food.

1.2.2. Non–conventional methods (DNA–based)

Non–conventional methods are based on DNA analysis and a variety of methods exist whereby the residual DNA content of food material can be used to

obviously identify the nature of the product. At the same time non–conventional methods overcome many problems of conventional methods such as denaturation of proteins because of the heating and processing of foodstuff (Lockley and Badsley, 2000).

1.2.2.1. Species-specific genes

This method is based on genes specific for each species. There are some genes that can be detected only in one species using DNA hybridization. Probes comprising labelled total genomic DNA from a given species would hybridize to DNA from the same species with little cross–reactivity. Species specific binding of the probes to the targets was believed to result from the hybridization of complementary repetitive sequences (Chikuni *et. al*, 1990).

Furthermore, species-specific PCR-primers are based on species-specific genes which mean that these primers offer opportunities to study the population and evolutionary biology of various plant species (Taberlet et. al., 1991).

1.2.2.2. Polymorphisms

Polymorphisms are genetic variations in a population's DNA. Genetic variation refers to the variation in a population or species, and includes the nuclear, mitochondrial, ribosomal genomes as well as the genomes of other organelles. New genetic variation is caused by genetic mutation, which may take the form of recombination, migration and/or alterations in the karyotype (the number, shape, size and internal arrangement of the chromosomes).

Molecular markers give information about differences in the sequence of DNA, with a compromise between precision and convenience. Some markers could also be sensitive to environmental interferences which can mask genetic diversity. The advent of PCR amplification and its application to molecular marker detection, first initiated around 1989 (Weber and May, 1989), have brought about several advantages such as wider accessibility for specific genomic regions, the requirement

for lower amounts of DNA and a greater facility in operations (Marmiroli *et. al*, 2003).

RAPD, *SCAR*, *AFLP*, *SSR* and *SNPs* are the main molecular markers that are used on food authenticity.

Random Amplified Polymorphic DNA (*RAPD*) is a dominant PCR-based molecular marker that was developed in the early of 1990s (Williams *et al.*, 1990). This molecular marker has in common the use of one, usually, or two primers of random sequence to amplify multibanded fingerprints from a complex genome (Marmiroli *et. al*, 2003).

A) This genetic marker has been used in many studies because it is fast with low cost and only a small amount of DNA is needed. On the other hand it has low reproducibility and it is not useful in different germplasm.

One example of the application of the RAPD molecular marker was the identification of marine mammals in processed sea food products (Martinez and Danielsdottir, 2000).

B) Sequence Characterised Amplified Regions (SCARS) were first described in 1993 (Paran and Michelmore, 1993), shortly after the invention of RAPD markers. A SCAR is identified by two specific primers that amplify a well-defined genetic locus, derived by sequencing a single RAPD amplicon. When the target RAPD amplicon has been purified, they are sequenced and the sequence information is used to design long primers, consisting of 24-25 nucleotides. The polymorphism between SCAR bands is determined by variation in length of the sequence between the two primers, or by lack of annealing. SCARs are usually a dominant marker but in some cases therefore SCARs can be co-dominant.

C) Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995) are based on the detection by PCR amplification of restriction fragments generated from a genome. The amplification of the restriction fragments is carried out by ligating specific adapters to the fragments' ends and utilizing primers complementary to the adapters. The origin of polymorphism in AFLP is the same as in RFLP. Both of them are based on changes in the restriction site sequence or deletions/insertions between

two adjacent restriction sites. AFLPs are also dominant markers and they are more laborious to produce.

D) Simple Sequence Repeats (SSR) are also called microsatellites, and consist of 1-10 bp repeated in tandem, with a variable number of repetitions (Tautz, 1989). They are very common in eukaryotic genomes and they are highly polymorphic due to variation in the number of repeats. Microsatellites can be detected by amplification from specific primers annealing to the unique flanking sequences and this can make them amenable to be transferred to related species (Marmiroli *et. al*, 2003).

E) Single Nucleotide Polymorphism (SNP) are single base changes in the DNA sequences, the most abundant type of mutation. The informational content is not very high, but it can be increased by the occurrence of several adjacent SNPs in one sequence. They can be identified by sequencing, and then a procedure for identifying them with PCR is designed. The search for SNP can be more effectively carried out by hybridization on chips in macro- or microarrays, using oligonucleotide probes (Lemieux *et al.*, 1998).

i) SNP classification

Several types of SNPs are distinguished, according to their assignment to the structural element of genomic DNA or their functional effect.

SNPs are classified:

a) depending on the SNP position in a gene, into exon, intron and promoter ones;
b) into regulatory SNPs and designated rSNPs, which are oligonucleotide substitutions involving regulatory regions that control gene expression;
c) depending on the presence of data on the functional effect of the single-nucleotide substitutions, into anonymous SNPs (functional effect is unknown), candidate SNPs (presumably having a functional effect), and protein SNPs (single-nucleotide substitutions, resulting in a change in the protein function or expression) (Khlestkina and Salina, 2006).

ii) Main advantages and limitations of SNPs

In contrast to multiallelic markers, analysis of biallelic SNP markers can be practically fully automated. Several thousands of SNPs can be analyzed simultaneously by the application of DNA microarrays. Thus, using modern

technologies, the effectiveness of SNP analysis can be many times higher than that of other methods of DNA analysis. Moreover, none of the other types of DNA polymorphism has such diverse and numerous methods of analysis as SNP. Because of this, SNP markers can be used in projects with different financial levels.

Single-nucleotide polymorphism is the most common type of DNA polymorphism. For instance, in the wheat genome, the SNP density ranges from 1 per 370 bp (Procunier *et al.* 2003) to 1 per 540 bp (Somers *et al.*, 2003). High occurrence of SNP loci in the genome opens a possibility to develop, on their basis, molecular genetic maps of very high density, required for isolating and studying genes for resistance to various diseases as well as other agronomically valuable plant genes. In addition, owing to the advantages of SNP markers, they can be widely used for certification of cultivars and lines.

High SNP frequency also involves, coding and regulatory, regions of genomic DNA (cSNP and rSNP). This feature opens a perspective for effective development of diagnostic SNP markers.

They are without their limitations, however, and might provide marginal additional, or even less, utility in some applications.

iii) Comparisons of some methods of SNP analysis

A. Enzyme-Coupled SNP typing

Most current SNP typing methods utilize enzyme reactions as a key transformation in a typing scheme because enzyme-catalyzed reactions show high fidelity in recognition of a single base difference between wild-type and mutant DNAs. Figure 1.1 shows a typical example of SNPs. The wild-type has an A–T base pair at the polymorphic site, whereas the mutant DNA has a G–C base pair (Nakatani, 2004).

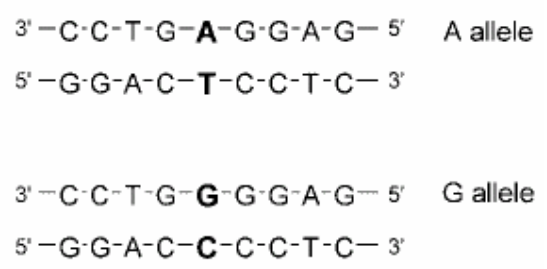


Figure 1.1 Single-nucleotide polymorphisms. Wild-type DNA (upper) has an A–T base pair, whereas mutant DNA has a G–C base pair at the polymorphic site.

A. 1. Single-nucleotide primer extension

A general scheme for SNP typing with a single nucleotide primer extension is shown in Figure 1.2 (Syvanen, 1999).

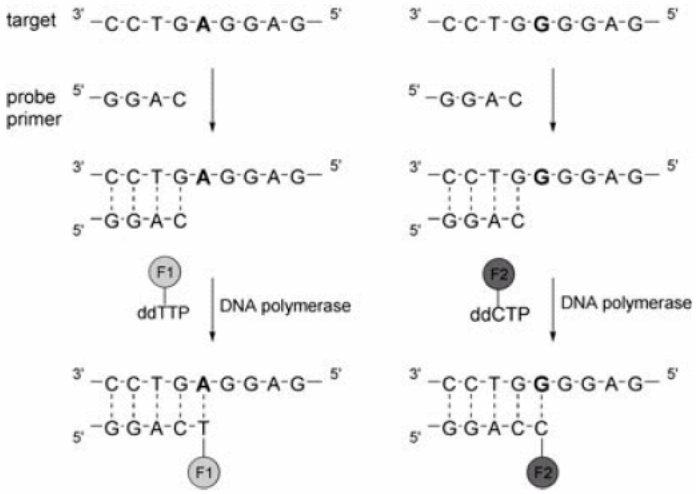


Figure 1.2. Single-nucleotide primer extension of wild-type and mutant DNAs with labeled ddNTPs.

The probe primer that is common for both the wild and the mutant targets hybridizes on the 3' side to a polymorphic site. For the fluorescence detection method, dideoxynucleotide triphosphates (ddNTPs) that are to be incorporated into a primer are labelled with fluorescent dyes that have different emission maxima among each other. In the primer extension, ddTTP is incorporated into the 3' end of the primer hybridized to the wild-type, whereas ddCTP will be incorporated into the primer hybridized to the mutant. Observation of fluorescence specific to ddTTP or ddCTP in the extended primer defines the base at the polymorphic site (Nakatani, 2004).

A. 2. The Invader® assay

One method that effectively uses the enzyme reactions in SNP typing is the Invader assay (Figure 1.3) (Kwiatkowski *et al.*, 1999).

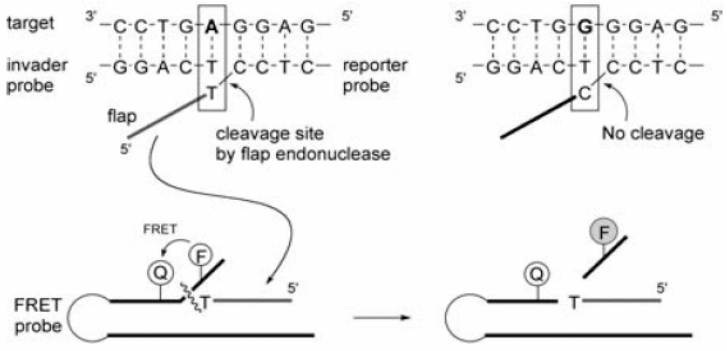


Figure 1.3. Schematic representation of the Invader® assay.

The assay utilizes two oligonucleotides, called "reporter" and "invader" probes, and an enzyme called flap endonuclease. The reporter probe consists of an allele-specific sequence and an additional sequence called a flap at the 5' side of the probe. The reporter probe hybridizes to the target on the 5' side to the SNP site at the allele-specific sequence. The flap sequence is not complementary to the target sequence at all. The invader probe is also allele-specific and hybridizes to the 3' side to the SNP site. The nucleotide at the end of the invader probe is located just opposite the nucleotide to be identified in the target. When the invader and the reporter probes, which are both specific to the wildtype, hybridize to the wild-type DNA, two thymine bases are located opposite the adenine at the SNP site. In contrast, a cytosine base in the reporter probe and a thymine base in the wild-type specific invader probe are located opposite a guanine at the SNP site in the mutant DNA. The flap endonuclease recognizes the former DNA structure and cleaves the reporter probe at the base opposite the SNP site. In Invader assays, the cleaved flap sequence is designed to be another invader probe in a signal-producing system. The flap sequence in the second reporter probe (FRET probe) is labeled with a fluorescent dye. The fluorescence of the dye is quenched by fluorescence resonance energy transfer (FRET) to the quencher attached to the stem part in the reporter probe. Cleavage of the flap in the FRET probe by the flap endonuclease results in the disruption of FRET, and thus an increase in

the fluorescence. As a hybridized complex produced from the FRET probe specific to the wild type is not recognized by the enzyme, the intensities of the fluorescence signals do not increase. Cleavage of the flap is a catalytic process, and the cleaved probe can be displaced by other reporter probes. In principle, it is conceivable that Invader assays, only if there is enough DNA yield, could be carried out without PCR amplification of the target prior to the assay (Nakatani, 2004).

A. 3. The TaqMan® assay

Another important method of enzyme-coupled SNP typing is the TaqMan® assay (Figure 1.4). (Livak *et al.,* 1995)

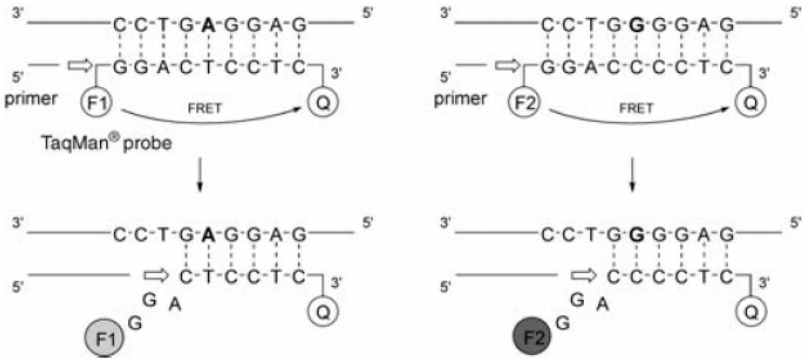


Figure 1.4. The TaqMan® assay

This assay is an allele-specific hybridization method (Saiki *et al.*, 1989) coupled with PCR amplification. In addition to a primer set for the PCR reaction, this assay uses TaqMan probes that are allele-specific and labeled with fluorescent dyes and quenchers. The TaqMan probe hybridizes to the sequence containing the polymorphic site in the target. In the hybrid, the fluorescence of the dye is quenched by FRET. A primer hybridizes to the 3' side to the polymorphic site and is elongated by the Taq polymerase. During the polymerase reactions, the TaqMan probe is replaced by an elongating primer and digested by the exonuclease activity of the polymerase; this results in the termination of FRET by the separation of the fluorescent dye from its quencher. The TaqMan probe specific to the wild-type DNA does not hybridize to the mutant DNA, especially in the presence of the competitive

mutant specific probe, and, therefore, will not be digested by the enzyme in the absence of the consensus target DNA (Nakatani, 2004).

A. 4. Ligase-assisted assay

Ligase-assisted assay is one of the important methods for enzyme-coupled SNP typing (Tobe *et al.*, 1996, Baron *et al.*, 1996). A unique typing method that involves circular DNA formation by a combination of gap-fill polymerization and a subsequent ligation has been developed (Hardenbol, 2003). The assay uses molecular inversion probes (MIPs; Figure 1.5).

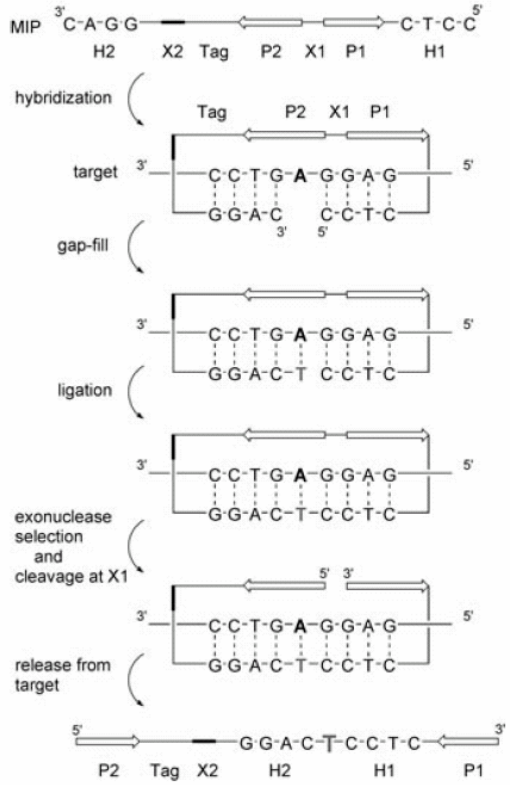


Figure 1.5. The molecular inversion probe assay.

The MIP consists of two regions hybridized to the target sequence (H1 and H2), two primer sites (P1 and P2), and a Tag sequence hybridized to the arrayed sequence in the detection step. The sites and X1 and X2 are incorporated to cleave the circularized probe. X1 in Figure 1.5 denotes a uracil, which is removed by uracil DNA glycosylase. Upon hybridization of MIP to the target, a gap appears between H1and H2 and located opposite the polymorphic site. The gap is filled by polymerization with each one of four

dNTPs followed by ligation to produce a circular probe. The gap is not filled with uncomplimentary dNTPs to the nucleotide base at the polymorphic site. The remaining probes in a linear form are digested by exonuclease treatments. Having completed the selection, the circularized probe is cleaved at the X1 site (uracil) by uracil DNA glycosylase; this makes the probe inverted to the linear form, with two primer sites being located at each termini. The inverted probe is amplified by PCR and hybridized to the arrayed sequence at the Tag sequence. Hybridization is detected by use of a fluorescence-labelled oligonucleotide complementary to one in the primer sequence. This assay needs only one probe for typing two alleles, thus making multiplex genotyping extremely simple (Nakatani, 2004).

B. Non-enzymatic SNP typing methods

The major advantage of enzyme-coupled SNP typing is the high fidelity of the base sequence recognition. This implies that non-enzymatic SNP typing methods should be comparable in their ability to recognize base sequences or should use other principles for the efficient discrimination of the mutant DNA from the wild-type. Allele-specific hybridization (Saiki *et al.*, 1989) is a standard method employed in the non-enzymatic discrimination of the mutant from the wild-type. This method utilizes allele-specific oligonucleotides (ASO) that hybridize to the target sequences containing the polymorphic site. Hybridization of ASO that is specific to the wild-type target produces a fully matched duplex, whereas a duplex containing a single mismatched site would be produced upon hybridization with mutant DNA (Nakatani, 2004).

One critical issue in the ASO hybridization method is the difficulty in choosing the appropriate conditions for hybridization that discriminate a fully matched duplex from a single mismatched duplex (Nakatani, 2004).

Another important issue regarding non-enzymatic SNP typing is cost. Most typing methods utilize fluorescence detection, which requires fluorescent labelling of either the target DNA or hybridization probes. Fluorescent labelling of target DNA can be easily carried out during PCR amplification with fluorescently labelled dNTPs or primers. The latter could be synthesized by standard oligo DNA synthesis with fluorescently labelled

phosphoramidites. However, fluorescently labelled dNTPs and phosphoramidites are expensive, and potentially increase the total costs of SNP typing (Nakatani, 2004).

The last issue in non-enzymatic SNP typing is the necessity for PCR amplification prior to the assay.

B. 1. Strand-exchange methods

In practice, it is difficult to define the hybridization conditions for SNP typing by ASO hybridization (Howell *et al.*, 1999) especially for large-scale and simultaneous assay formats. A unique SNP-typing method employing a kinetic rather than a thermodynamic differentiation of a fully matched duplex from a single mismatched duplex has been reported (Figure 1.6) (Kim *et al.*, 2003).

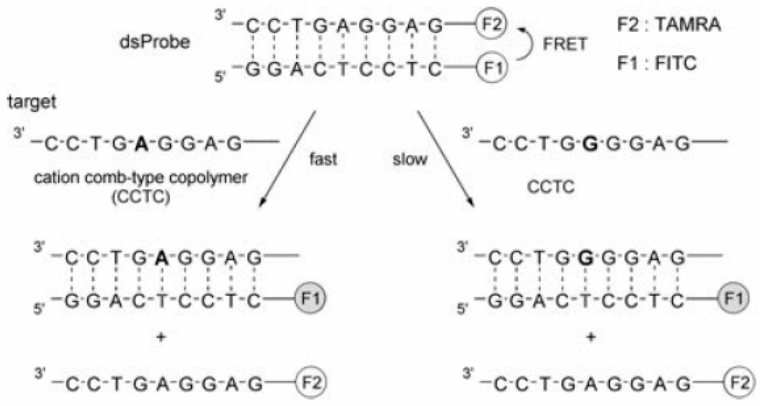


Figure 1.6 Strand-exchange methods with unlabeled targets.

The method is based on the discovery that a cation comb-type copolymer (CCTC) accelerates the strand exchange of a fully matched duplex with a matched complementary strand but not with a single mismatched strand. The method utilized a double-stranded probe, in which one strand was labeled with FITC (fluorescein isothiocyanate) while the other complementary strand was labelled with carboxytetramethylrhodamine (TAMRA). In the double-stranded probe, the fluorescence of FITC was suppressed by FRET to TAMRA. The double-stranded probe was incubated with the target sample that can hybridize to the FITC-labelled strand by displacing the TAMRA-labelled strand. CCTC accelerates the strand-exchange reaction when the target has the sequence that

is fully complementary to the FITC-labelled strand. Strand exchange resulted in an increase in the fluorescence intensity of FITC by termination of FRET. The time-course measurements of increasing intensity determine the allele type (Nakatani, 2004).

B. 2. Chemically-modified fluorescence-probe methods

B. 2. 1. Based on FRET chemistry

Oligomers that have a fluorescence probe and intercalating quencher in close vicinity on the same strand have been developed for discriminating a perfectly matched duplex from a singly mismatched duplex (Figure 7) (Yamane, 2000).

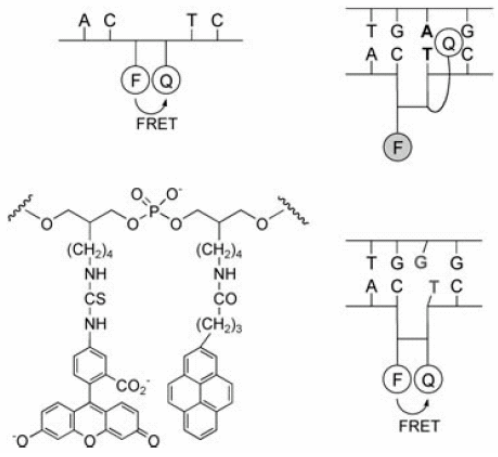


Figure 1.7 FRET detection by modified ASO.

In a single-stranded state, the fluorescence of fluorescein was quenched by a quencher pyrene. When hybridized to the perfectly matched sequence, pyrene intercalated into the duplex; this resulted in the emission of fluorescence from fluorescein due to the disruption of FRET. In the case of hybridization with a mismatched sequence, pyrene intercalation is inhibited by the mismatched base pair. As a consequence, the fluorescence remains quenched. These probes have been termed MagiProbeB. The performance for match–mismatch discrimination is modulated by the ability of the intercalative binding of the quencher (Nakatani, 2004).

B. 2. 2. Modified nucleotide-based method

Fluorescent nucleotide-base analogues have been investigated as probes reporting dynamics of DNA (Rist *et al.*, 2002). Because the fluorescence is sensitive to the microenvironment surrounding the fluorescence chromophore, these nucleotide base analogues have been utilized to report the hybridization (Nakatani, 2004).

Table 1.1. Comparison of molecular markers

Marker	PCR	Abundance	Reproducibility	Automation	Cost
RFLP	No	↓ –	↑	↓	↑
RAPD	Yes	– ↑	↓	=	↓
SCAR	Yes	↑	↑	=	=
AFLP	Yes	↑	↑	– ↑	=
SSR	Yes	↑	↑	– ↑	↓
ISSR	Yes	↑	↑	– ↑	↓
SNP	Yes	↑ ↑ ↑	↑	↑	↓

(═: Moderate) [Henry *et al*, 2005; The Handbook of Plant Genome Mapping]

1.3. The case of olive oil

Olive (*Olea europaea* L.) is a typical crop species of the Mediterranean countries. It is an outcrossing diploid species (2n = 46) that belongs to the family of Oleaceae and shows differences in self-incompatibility depending on the cultivar considered (Cuevas *et al.*, 2001; Wu *et al.*, 2002; Diaz *et al.*, 2006b). Olive tree cultivation originated from the Eastern countries of the Mediterranean Basin, most likely from Syria in the prehistoric age (Zohary and Spiegel-Roy, 1975). The cultivation extended from East to West following both arches of the Basin (Hatzopoulos *et al.*, 2002). Though olive is cultivated mainly in Mediterranean countries, in the last few years growth of olive production has expanded throughout the world to countries such as Australia, Canada, China, Peru, Chile, Argentina, and the United States (Reale *et al.*, 2006).

1.3.1. The significance of olive oil

Olive oil is the principle source of fat in the traditional Mediterranean diet and the beneficial effects of its constituents in human health have been extensively reported (Wahrburg *et al.*, 2002). It is characterized by a high content of mono-unsaturated fatty acids. The main component of olive oil is oleic acid which ranges from 55 to 83% of total fatty acids. The fatty acid composition of olive oil is influenced by different factors, such as the variety of the olive tree, agricultural and climate factors (Bockisch, 1993). Moreover olive oil contains some substances such as tocopherols and phenolic compounds that are reported to exert beneficial effects on health (Visioli and Galli, 1995; 1998).

During the last ten years, the contribution from areas surrounding the Mediterranean Basin to the average production and exportation of olive oil worldwide account for 99% and 98%, respectively (International Olive Oil Council). These statistics, along with reported evidence of many beneficial effects on human health make olive oil one of the most important food commodities globally.

1.3.2. Adulteration of olive oil

Olive cultivation and harvest difficulties, in combination with its high nutritional value, make it vulnerable to fraud. The main types of adulteration of olive oil are:

1) Economic adulteration, which mostly refers to the mixing of extra virgin grade with either lower grades and/or other vegetable oils. For the detection of such fraud a lot of conventional analytical methods (paragraph 1.2.1) have been applied, mostly in combination with multivariate analysis methods (Aparicio, 1997).
2) Minimally processed, which refers to non-refined or cold pressed olive oils. Virgin olive oil is obtained from the olive using only those mechanical means that do not alter the oil in any way (Bianchi G., 2002). The oil has not undergone any treatment other than washing, decanting, centrifuging and filtering. The definitions 'extra virgin' and 'virgin' are based on a European marketing standard that includes fatty acid content and organoleptic characteristics (Woolfe and Primrose, 2004).
3) Characterization and denomination of geographical region. The olive oil can also have a designation of origin when it meets the specific characteristics associated with a region. Olive oils labeled with their region of origin sell at a premium price. This premium is greatest for oil from those regions associated with superior taste, consistency or colour. In reality, only the cultivar is of relevance because only certain cultivars are grown in each locality (Woolfe and Primrose, 2004). There are certain olive cultivars like the Greek cultivar "Koroneiki" or the Italian "Frantoio", used mainly for oil production, since the oil quantity and quality is superior when compared to others (Hatzopoulos *et al.*, 2002). This means that apart from the detection of adulterants, there is also a need for identification of the variety of the olives where the olive was extracted from. This is because in Europe several traditional products are protected by two regulations; the EEC 510/2006, which is based on designations of origin and geographical indication (Off. J. Eur. Communities, 2006) and the EEC 2082/92, which describes specific product types (Off. J. Eur. Communities, 1992). Both regulations introduced the Protected

Designation of Origin (PDO) of traditional products, one of which is olive oil. In addition to that, a more recent European Regulation (2815/98) came into force stating that the origin of an olive oil sample is not to be determined by the region where the olive trees are grown, but by the place where the olives are milled instead (Off. J. Eur. Communities, 1998). That makes the PDO certification even more difficult and olive trade very profitable (Busconi *et al*. 2003).

1.3.3. Means of detection of fraud on olive oil

Olive oil authenticity has become a focal point attracting the attention of producers, consumers and policy makers. Although in most cases adulteration of olive oil does not pose a threat to public health (with a few prominent exceptions, e.g. the Spanish Toxic Oil Syndrome (World Health Organization Monograph, 1992) or contamination with weed seeds (Singhal *et al.*, 1997), fundamental rights of consumers (right or correct information and buying "value-for-money") are violated by fraudulent malpractice. Fraud is only economically viable if a cheaper commodity can be made to resemble a genuine, more expensive product (Ulberth and Buchgraber, 2000).

Many different chemical and biochemical techniques have been developed for determining the authenticity of olive oil and in recent years methods based on DNA analysis have become more important. This is because some techniques, such as immunoassays, work well with raw foods but lose their discrimination efficiency when applied to highly processed foods. Also many techniques do not easily distinguish between closely related materials at the chemical level. For example, olive and hazelnut oils are similar chemically so the usual analytical methods cannot be applied to detect the adulteration of olive oils with hazelnut oil (Woolfe and Primrose, 2004). Neither conventional chemical methods, nor the analysis of biomorphological traits are always able to detect country or region of origin of olive oil due to environmental effects on the chemical composition and phenotype (Alessandri et al., 1997; Barone et al., 1994). On the other hand DNA analysis has discriminating power because ultimately the definition of a variety or species is dependent on the sequence

of the DNA in its genome. As a result genetic identity seems to be the only possibility for identifying the cultivar and the products deriving from it (Busconi *et al.*, 2003). The introduction of the polymerase chain reaction (PCR; Mullis and Faloona, 1987) has accelerated the development of new reliable and informative DNA fingerprinting procedures.

Even though the number of laboratories working with olive is restricted mainly to the Mediterranean countries, molecular markers have been established in less than a decade to set the basis in order to distinguish, characterize or identify cultivars, to estimate germplasm variability and to trace olive origin. Since molecular markers are not environmentally affected they are superior to biochemical markers and able to contribute to the resolution of problems such as mis-identification in germplasm collections, nursery certification, varietal protection, and/or guaranteed labeling for certification of geographical origin. Moreover, molecular markers could be infinite (Hatzopoulos *et al.*, 2002).

In less than a decade a large number of molecular markers and techniques have been used in olive. Five major molecular marker techniques were applied on cultivar identification:

A) Random Amplified Polymorphic DNA (RAPD)

The pioneer work on distinction of olive cultivars using molecular markers was published in 1995 (Fabbri *et al.*, 1995). Seventeen oil and table olive cultivars originating from throughout the Mediterranean area were screened using RAPD markers. The results indicated a high degree of polymorphism in olive germplasm. Using the same methodology, there are many works on distinction of olive cultivars (Cresti *et al.*, 1997; Vergari *et al.,* 1996; Wiesman *et al.*, 1998; Mekuria *et al.*, 1999; Gemas *et al.*, 2000; Claros *et al.*, 2000; Besnard *et al.*, 2001; Hess *et al.*, 2000; Vargas and Kadereit., 2001; Sanz-Cortez *et al.*, 2001; Besnard and Berville, 2000; Belaj *et al.*, 2001). This accumulated data on olive RAPD analysis from different cultivars or genotypes gives information for genetic relations, olive origin and dispersal, and could provide the initial basis for cultivar distinction and germplasm evaluation of agronomic interest (Hatzopoulos *et al.*, 2002).

B) Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995)

AFLP allows for the simultaneous screening of a large number of loci, without any need for preliminary sequence knowledge. For these advantages and for their high reliability, AFLPs have been widely used for genotyping in a large number of crops, including olive (Angiolillo *et al.*, 1999, Sanz-Cortez *et al.*, 2003). Busconi *et al.* previously reported an AFLP fingerprinting of olive oil partially superimposable and agreeable with the cultivar from which the oil was made. To improve the traceability of olive oil, in terms of costs and efficiency, the conversion of fragments of AFLP patterns to a simple, codominant PCR-based marker could be advisable, in order to obtain a sequence characterized amplified region (SCAR), such as those derived from RAPD markers developed from olive (Busconi *et al.*, 2003, Hernandez *et al.*, 2001).

C) Simple Sequence Repeats (SSRs)

Because of their small size, they can be detected and correctly amplified, even in case of degraded DNA such as the DNA extracted from olive oil (Pasqualone *et al.*, 2000; 2001; 2004). Furthermore, the use of microsatellites in characterizing olive cultivars from various regions have been described by many scientists (Sefc, 2000; Carriero *et al*., 2002; Belaj *et al.*, 2004; Diaz *et al.*, 2006a; Hess *et al.*, 2000; Vargas and Kadereit, 2001).

D) Single nucleotide Polymorphisms (SNPs)

So far only AFLP (Pafundo *et al.*, 2005) and microsatellites (Pasqualone *et al*., 2004) have been used for oil samples. A very promising molecular marker that could significantly contribute to olive oil characterisation is the single nucleotide polymorphism (SNP). The frequency and nature of SNP in plants is beginning to receive considerable attention. A number of reports in *Arabidopsis thaliana* (L.) and maize (*Zea mays* ssp. *mays* L.) have provided estimates of sequence diversity in these species. In soybean (*Glycine max* L. Merr.), which is an autogamous species, the analysis of DNA variation has been mainly confined to single genes or DNA fragments with the goal of defining gene structure, function, or evolutionary relationships (Zhu *et al.*, 2002).

In olive, SNPs were used for the first time to classify 51 olive cultivars, with satisfactory results (Diaz Bermudez, 2005). Recently, olive cultivar identification was further enhanced by the SNP discovery among 65 olive varieties. The markers (SCAR, SNP) developed and used in this study could differentiate 72% of the olive samples tested. When duplications (cultivars with the same names) are excluded, 77% of the cultivars could be differentiated (Reale *et al.*, 2006). This means that more gene targets and a larger number of SNPs are essential for varietal identification.

1.4. Aim of this project

The aim of this work is to generate a single nucleotide polymorphism database on Greek *Olea europea* varieties, in order to detect the varietal contribution present on olive oils at both a qualitative and quantitative level.

The first objective is to study the effect of olive oil storage on the stability of residual DNA and the formation of PCR inhibitors. The second objective is to screen DNA sequences of several gene targets, not only among many oil producing varieties but also fruit producing varieties, for SNPs that can be used in the future for the varietal characterisation of commercial olive oils. The third objective is to develop an SNP-based approach. Other issues such as the discrimination of olive oil with other edible oils shall also be investigated.

The development of molecular markers in olive tree varieties by means of SNPs and the understanding of the mechanism under which they contribute to several phenotypic traits such as antioxidant production, resistance to biotic or abiotic stresses, oil quantity and quality shall be beneficial to all European scientists focused on that species. In particular, it shall promote olive breeding and selection schemes under a common European strategy. Therefore, national agricultural or oil institutes shall take advantage of such knowledge for the benefit of European countries. Varietal discrimination of olives through SNPs shall be useful to the European Food Agencies that are likely to be involved with aspects such as food authenticity and traceability, especially in P.D.O. issues of olive oil.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

The olive tree leaf material used in this study derived from a collection maintained at the Institute of Subtropical Plants and Olive, National Agricultural Research Foundation (NAGREF), Chania, Greece, Kostelenos olive nurseries, Poros Trizinias, Greece and the National Agricultural Research Foundation (NAGREF), Chalkidiki, Greece. The olive oils (Adramytini, Valanolia, Kalamon, Koroneiki, Megaritiki, Throumbolia) were provided by NAGREF. The olive fruits used to produce olive oil were collected from trees located in: 1) Agia Paraskevi, Lesvos, Greece, (Adramytini, Valanolia) 2) Agios Mamas, Chalkidiki, Greece, (Lianolia Kerkyras) 3) Poros, Trizinia, Greece (Gaidourelia) 4) Sparti, Peloponnisos, Greece (Kothreiki) 5) Gerakini, Chalkidiki, Greece (Table 2.1). The vegetable oils (soya oil – Kore A.E, and sesame oil – Afoi Haitoglou ABEE) were bought from Chalkiadakis Super market, almond oil – "Emile Noel, Pont-Saint-Esprit, France" was bought from Gaia. Avocado oil, sesame oil, almond oil and walnut oil were bought from Dianthos.

Table 2.1 The providers of the plant material. (L) leaves; (O) Oil; (F) Fruit.

	OLIVE VARIETY	PROVIDER							
		NAGREF CHANIA		NAGREF CHALKIDIKIS		KOSTELENOS		LESVOS	FARMERS
		L	O	L	F	L	F	L	F
1	ADRAMYTINI	×	×	×		×		×	
2	AGOUROMANAKO	×				×			
3	AMYGDALOLIA			×		×			
4	ASPROLIA LEFKADOS					×			
5	VALANOLIA		×	×		×		×	
6	VASILAKADA			×		×			
7	GAIDOURELIA	×				×	×		
8	GALATISTAS					×			
9	DAFNELIA					×			
10	THIAKI					×			
11	THROUMBOLIA	×	×	×		×			
12	KALAMON	×	×	×		×			
13	KALOKAIRIDA					×			
14	KAROLIA					×			
15	KARYDOLIA					×			
16	KARYDOLIA CHALKIDIKIS	×		×		×			
17	DOPIA SPETSON					×			
18	KLONARES KOROPIOU					×			
19	KOTHREIKI			×		×			×
20	KOLYMPADA					×			

Table 2.1 (continued)

	OLIVE VARIETY	PROVIDER							
		NAGREF CHANIA		NAGREF CHALKIDIKIS		KOSTELENOS		LESVOS	FARMERS
		L	O	L	F	L	F	L	F
21	KOLIREIKI					×			
22	AMFISSIS - KONSERVOLIA	×		×		×			
23	KORONEIKI	×	×	×		×			
24	KOUTSOURELIA	×				×			
27	MARONEIAS					×			
28	MASTOEIDES	×	×			×			
29	MATOLIA					×			
30	MAVROLIA MESSINIAS					×			
31	MEGARITIKI			×		×			
32	ZAKINTHOU					×			
33	PIKROLIA					×			
34	RACHATI					×			
35	SMERTOLIA					×			
36	STROGGYLOLIA					×			
37	TRAGOLIA					×			
38	TSOUNATI			×					
39	PATRINI			×					
40	CHONDROLIA CHALKIDIKIS			×					×
41	NISIOTIKI					×			

2.2 Production of olive oil

Olive drupes (1Kg) were collected by hand at an appropriate ripening stage, washed with fresh tap water to remove any dust and foreign material and further used for olive oil extraction in a laboratory micro-scale. The seeds were manually removed from the flesh. Olive flesh was smashed with mortar and pestle for 20 minutes until a homogenous pulp, slightly covered with an oily layer, was formed. The paste was transferred in a plastic tray and malaxed with the pestle for 20 minutes. The paste was centrifuged in falcon tubes (1/3 filled) for 30 minutes at 2800g. The oil was collected in clean falcon tubes covered right around with aluminium foil to prevent photo-oxidation. The acidity of produced olive oil was determined by measuring the total fatty acids according to the official method, thus evidencing their "extra virgin" grade.

The olive oils from NAGREF were obtained by crushing drupes from single known cultivars by means of a laboratory-scale olive mill available at the Institute of Subtropical Plants and Olive.

2.3 DNA extraction

2.3.1 DNA extraction from olive, sunflower, hazelnut, walnut, corn, soya, almond, avocado, sesame and cotton leaves

Genomic DNA was extracted from young leaves by using a standard cetyltrimethylammonium bromide-based protocol. Approximately 100mg of plant material was used for each DNA extraction. The leaves had previously been ground with liquid nitrogen in a pre-cooled mortar and pestle, transferred to 2ml Eppendorf tubes and stored at -80°C. 500µl of CTAB buffer (see Appendix) was prepared, just before use, for each sample and was mixed with the tissue. The mixture was incubated at 65^{o}C for 30 minutes in a water bath. During incubation, the tubes were vortexed briefly every 10 minutes after which they were immediately transferred on ice for 5 minutes. 500µl of cold chloroform was added into the lysate of each sample and they were shaken for 10 minutes. They were centrifuged at 16100g for 5 minutes.The top layer was transferred into another 1.5ml Eppendorf tube. 300µl of cold isopropanol was added and the samples were put on ice for 10 minutes. Then the samples were

centrifuged at 16100g for 10 minutes. The supernatant was discarded and 300µl of ethanol containing ammonium acetate (see Appendix) was added. The mixture was centrifuged at 16100g for 10 minutes. The supernatant was discarded and finally the air-dried pellet was dissolved in 100µl sterile distilled deionized water. They were kept at 4^{o}C overnight. On the second day, 2µl of RNase was added to each sample and they were incubated at 50^{o}C for 30 minutes. During incubation, they were vortexed briefly every 10 minutes. Then, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) was added into each DNA sample. They were mixed for 1 minute until they turned milky. Then, they were centrifuged at 16100g for 3 minutes and the supernatants were transferred into new 1.5 ml Eppendorf tubes. An equal volume of chloroform was added to each DNA sample and they were mixed until they turned milky. They were centrifuged at 16100g for 3 minutes and the supernatants were transferred into new 1,5 ml Eppendorf tubes. Then, 2.5 volumes of absolute ethanol and 0,1 volume of Na-acetate 3M pH 5,2 was added on each sample. They were mixed and incubated at -20 ^{o}C for 1 hour. Then, they were centrifuged at 16100g for 15 minutes and the supernatant was discarded. 1 volume of 75% ethanol was added to each sample and they were centrifuged at 16100g for 5 minutes. The supernatant was discarded and finally the air-dried pellet was dissolved in 50 µl sterile distilled deionized water.

In addition, genomic DNA was extracted from young leaves by using the DNeasy® Plant Mini Kit, Qiagen, Cat. No 69104. According to the protocol of the above kit, 400 µl of Buffer AP1 and 4 µl of RNaseA stock solution (100 mg/ml) were added to a maximum of 100mg of ground tissue (wet weight) and were vortexed vigorously. The mixture was incubated for 10 minutes at 65^{o}C. It was mixed 2-3 times during incubation by inverting the tube. Then, 130µl of Buffer AP2 was added to the lysate, after which it was mixed and incubated for 5 minutes on ice. The lysate was centrifuged for 5 minutes at 20000g. Then, the lysate was applied to the QIAshredder® Mini Spin Column placed in a 2ml collection tube and it was centrifuged for 2 minutes at 20000g. The flow-through fraction was transferred to a new Eppendorf tube without disturbing the cell-debris pellet. 1,5 volumes of Buffer AP3/E was added to the cleaned lysate and it was mixed by pipetting. 650µl of the the mixture was applied to the DNeasy® Mini Spin Column sitting in a 2ml collection

tube. It was centrifuged for 1 minute at 6000g and the flow-through was discarded. The DNeasy® Mini Spin Column was placed in a new 2ml collection tube, 500μl Buffer AW was added and it was centrifuged for 1 minute at 6000g. The flow-through was discarded and the collection tube was reused. 500μl Buffer AW was added to the DNeasy® Mini Spin Column and it was centrifuged for 2 minutes at 20000g. The DNeasy® Mini Spin Column was transferred to a 1.5 ml Eppendorf tube and 100μl of Buffer AE was pipetted directly onto the DNeasy membrane. It was incubated for 5 minutes at room temperature (15-25 ^{o}C) and then it was centrifuged twice for 1 minute at 6000g.

2.3.2 DNA extraction from olive oil

Residual DNA from olive oil samples was isolated by using different kits (Table 2.2).

Table 2.2 Extraction kits for olive oils

KIT	OLIVE OIL VARIETY
QIAamp®	1 , 5 , 7 , 11 , 12 , 19, 23 , 26 , 28 , 40
PROMEGA	7 , 40
GENESCAN	26 , 40

One of the kits that was used for DNA extraction from vegetable oils was the Wizard Magnetic DNA Purification System for Food by PROMEGA, as modified by Breton *et al* (2004). A total volume of 40ml vegetable oil was fractioned in four aliquots of 10ml each. 2ml of Lysis Buffer A was added. The tubes were vigorously shaken for 1 minute. 1ml of Lysis Buffer B was then added. The tubes were again vigorously shaken for 1 minute. 25 μl of RNAse A was added. The tubes were once again vigorously shaken for 1 minute and they stayed at room temperature for 10 minutes. A 3ml sample of Precipitation Buffer G was added, vigorously shaken. Then, they were spun at 4000g for 20 minutes. The oil (upper phase) was removed with a 10ml pipette and discarded. Only 5cm of oil remained on top of the interface. The lysate was pooled from all four tubes in a clean 50ml tube. 150μl of Wizard Magnetic DPSF was added and vortexed briefly. 0.9 volumes of isopropanol was added and mixed in well. The solution was slowly stirred at room temperature for 1 hour. The beads were maintained in the tube with a magnet, whereas the solution was discarded.

The beads were rinsed 3 times with 1-2 ml of 70% ethanol and subsequently dried at 37^{o}C for 15 minutes. Beads were then suspended in 200 ml of water and were placed in 65^{o}C for 5 minutes. This released DNA, and the beads were eliminated from the DNA solution with the magnet as previously described.

Another kit that was used for DNA extraction from vegetable oils was the DNA Extractor Fat by GENESCAN. 2g of oil was weighed in a suitable tube. 10ml of hexane and 1ml of Lysis buffer for lecithin was added and mixed vigorously (approximately 30 seconds until the samples were dissolved). They were centrifuged for 15-20 minutes at 4000g until the phases were separated. The inter-phase and the aqueous phase were transferred into a sterile 2ml reaction vessel. 1 volume of chloroform was added and mixed well by vortexing. Then, they were centrifuged for 5 minutes at maximum speed for the separation of organic and aqueous phases. The aqueous upper phase was transferred into a new sterile 1.5 ml reaction vessel. 2ml of glycogen solution and 0.8 volumes of isopropanol were added. Then, they were incubated for 30 minutes at room temperature. The extracted DNA was precipitated by centrifugation for 10 minutes at maximum speed. The supernatant was carefully discarded. 500 µl of 75% EtOH was added to the pellet and mixed by vortexing. The extracted DNA was precipitated by centrifugation for 5 minutes at maximum speed. The supernatant was carefully discarded. The DNA pellet was dissolved in 25µl 0,2x TE buffer. Then, the samples were applied to the DNA extractor cleaning columns. Firstly, the resin in the column was resuspended by vortexing. The cap was loosened by an one-fourth turn and the bottom closure was snapped off. The column was placed in a 1.5ml micro-centrifuge tube for support. The column was pre-spun at 735g for 2 minutes. The flow-through liquid was removed and it was short-spun again for 30 seconds. The column was placed in a new 1.5ml tube. The cap was removed and discarded. The samples were slowly applied to the top center of the resin. The column was spun at 735g for 2 minutes. The purified sample was collected in the bottom of the support tube.

The last kit that was used for DNA extraction from vegetable oils was the Stool kit by Qiagen. 50ml of vegetable oil was centrifuged at maximum speed for 10 minutes using a 2ml microtube. The ASL buffer was warmed in a 70^{o}C waterbath and 2ml was added to the 2ml microtube. It was vortexed well and it was put for 2

minutes in a 70°C waterbath. 1.6ml of solution was pipetted into a 2ml Eppendorf tube. It was incubated for 5 minutes in a 70°C waterbath and vortexed. Then, it was centrifuged at maximum speed for 2 minutes. 1.2ml of the supernatant was pipetted into a new 2ml Eppendorf tube and 1 tablet of InhibitEX was added to each tube. It was vortexed until the tablet was completely suspended and incubated 1 minute at room temperature. It was centrifuged at maximum speed for 6 minutes. All the supernatant was pipetted into a 1.5ml Eppendorf tube and centrifuged at maximum speed for 6 minutes. 15µl of ProteinaseK was pipetted in a new 1.5ml Eppendorf tube. 200µl supernatant and 200µl buffer AL were added and vortexed. It was incubated in a 70°C waterbath for 10 minutes and spun briefly. 200µl EtOH 100% was added to the lysate and vortexed. All the solution was applied to the QiAamp spin column. It was centrifuged at maximum speed for 2 minutes. The column was placed in a new collection tube and the filtrate was discarded. 500µl of buffer AW1 was added and centrifuged at maximum speed for 2 minutes. 500µl more of Buffer AW1 was added and centrifuged at maximum speed for 6 minutes. The column was transferred to a 1.5ml Eppendorf tube and 200µl of Buffer AE was added directly to the column membrane. It was incubated for 5 minutes at room temperature and centrifuged for 2 minutes to elute DNA.

2.4 Effect of olive oil storage on its DNA: λDNA

In order to check the effect of storage on olive oil quality, different quantities (0.1µg, 1µg, 2µg, 5µg, and 10µg) of λDNA (New England, Biolabs, N.3011S, LOT: 145) were added to filtered olive oil, and then stored under a controlled environment similar to that of a supermarket.

DNA extraction was carried out using QIAamp kit, protocol No. 2, after 2 days, 10 days, 20 days, 40 days, 3 months, 6 months, 9 months, and 1 year.

2.5 Spectrophotometric quantification

Spectrophotometric estimation of DNA was not efficient since the olive oil DNA was very degraded and could not be detected or quantified by spectrophotometry.

The PCR product was quatified by the ImageJ program which is a public domain image processing and analysis program developed in Java.

2.6 Primer design

To check the DNA fragmentation effect, 3 pairs of primers were designed: PL1-PL2, PL1-PL3, PL2-PL4. To monitor the PCR inhibitory effect, 1 primer pair was used: Spo7aF-Spo7aR. To check the presence of olive oil DNA, 3 different chloroplastic pairs of primers were used: Plant(A1)-Plant(A2), OliveCPF-OliveCPR, rbcLF-rbcLR.

In the case of finding target-genes for sequencing 3 different pairs of primers were designed: Cyclo2F-Cyclo2R and Cyclo3F-Cyclo3R from Cycloartenol synthase; and Lupeol2F-Lupeol2R from Lupeol synthase. In addition, 2 different pairs of internal primers, Cyc2aF-Cyc2aR and Lup2F-Lup2R, were designed to give shorter fragments than cyclo2F-cyclo2R and lupeol2F-lupeol2R respectively. These primers were used for PCR amplification of the DNA of olive oils.

In the case of vegetable oils, chloroplastic primers Plant(A1) – Plant(A2) (Taberlet *et al.*, 1991) were designed to monitor the difference between the species according to the amplicons' fragment length.

PCR primers used throughout this study were designed with PrimerExpress® 2.0 software (Applied Biosystems). After the designing procedure, a BLASTn (Altschul *et al.*, 1990) search was carried out to ensure the uniqueness of the complementary DNA region against newly designed primers. SNaPshot™ primers were designed to give an annealing temperature of approximatelly 50°C and were blasted against the amplicon target to ensure that they do not anneal onto any extra sites.

The sequence of all the primers used in this study, as well as their annealing temperature and the size of the band amplified, is presented in Table 2.3.

Table 2.3 All the primers that used in this study

NAME OF PRIMER	PRIMER SEQUENCE (5' TO 3')	ANNEALING TEMPERATURE	TARGET	SIZE OF AMPLIFIED FRAGMENT (bp)
PL-1	GACGCACTGAATACGCTGAA	56	Bacteriophage λ genome	107
PL-2	TCGCATTCTTCGGTTGTTT	56		
PL-1	GACGCACTGAATACGCTGAA	59	Bacteriophage λ genome	415
PL-3	CTCGCATATCAGGAAGCACA	59		
PL-2	TCGCATTCTTCGGTTGTTT	56	Bacteriophage λ genome	691
PL-4	AGAGGCAGCAAAATCATCAGA	56		
Spo7a-F	TTGCTGAGGGAATTCGGAAA	55	Nuclear sporulation protein	401
Spo7a-R	GTAGCCCGCCTGCGAGGC	55		
Plant(A1)	CGAAATCGGTAGACGCTACG	60	Intraspacer chloroplast region trnL-trnF	480-700
Plant(A2)	GGGGATAGAGGGACTTGAAC	60		
OliveCPF	GGCTTTTAAGTGCGGCTAGA	55	Chloroplast region	104
OliveCPR	TTCCCTTTCAGGATCAGTCG	55		
rbcLF	AATCTTCTACTGGTACATGGA	50	Chloroplast region	172
rbcLR	GTAAACATGTTAGTAACAG	50		
Lupeol2-F	CTAACTCGATGGCCGTTTTCTAA	60.4	Lupeol synthase	501
Lupeol2-R	GCAACTCAAATGAATGAATCATGAT	60.4		
Lup2-F	GCAACTCAAATGAATGAATC	50		
Lup2-R	AACTCATGTTTTGTAGGTG	50		
Cyclo2-F	ATTCTTTTGGCTACTTGGACATCTTT	60.4	Cycloartenol synthase	901
Cyclo2-R	AACCCTCAGCTGTGCAATCTG	60.4		
Cyc2a-F	ATTCTTTTGGCTACTTGGACATCT	50		257
Cyc2a-R	GGAGATTGTAGCAATGTTGATATG	50		
Cyclo3-F	ATTTCAGATTGCACAGCTGAGG	60.4		901
Cyclo3-R	ATCCTGAGGAAATCATCTCCATTT	60.4		

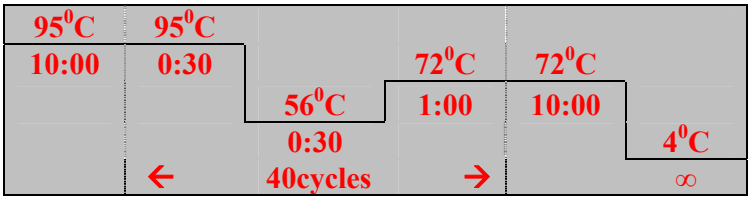
2.7 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was used to amplify λDNA, olive oil DNA, vegetables oil DNA and finally DNA from leaves. The denaturation temperature depended on the polymerase enzymes that were used. For Taq Polymerase the denaturation temperature was 94^0C for 4 minutes, but for AmpliTaq Gold Polymerase the denaturation temperature was 95^0C for 10 minutes. The annealing temperatures depended on the primer and finally the extension period depended on the fragment length. For fragment lengths over 650bp, the extension time was 90 seconds but for fragment lengths under 650bp the extension time was 60 seconds.

2.7.1 PCR on λDNA

The conditions for denaturation, annealing and polymerization of the PCR reactions are described in Table 2.4.

Table 2.4 PCR reaction program on λDNA



The PCR reactions were set at a 10μl total volume, as described in Table 2.5

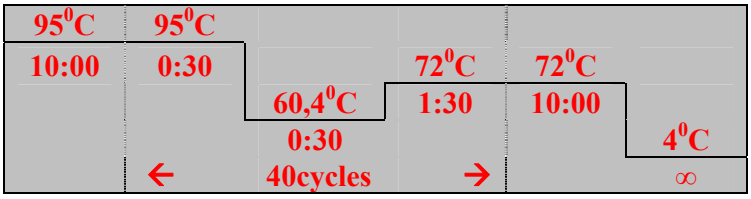
Table 2.5 PCR reaction on λDNA

Compounds	Volume
AmpliTaq Gold Buffer	1 μl
$MgCl_2$	1 μl
dNTPs	0,2 μl
Primer Forward	0,3 μl
Primer Reverse	0,3 μl
AmpliTaq Gold Polymerase	0,1 μl
DNA	1 μl
H_2O	6,1 μl
Total Volume	10 μl

2.7.2 PCR on olive leaf DNA

The conditions for denaturation, annealing and polymerization of the PCR reactions are described in Table 2.6.

Table 2.6 PCR reaction program on leaf DNA



The PCR reactions were set at a 100µl total volume, as described in Table 2.7

Table 2.7 PCR reaction on leaf DNA

Compounds	Volume
AmpliTaq Gold Buffer	10 µl
$MgCl_2$	10 µl
dNTPs	2 µl
Primer Forward	3 µl
Primer Reverse	3 µl
AmpliTaq Gold Polymerase	1 µl
DNA	4 µl
H_2O	67 µl
Total Volume	100 µl

2.7.2.1 Agarose gel electrophoresis

The DNA fragments were mixed with 6x loading buffer (see Annex) and separated by a horizontal gel electrophoresis apparatus (Horizon® 58, Life Technologies™, GIBCO BRL) through 2% agarose gel in 1xTAE (see Appendix), with a final concentration of ethidium bromide of 10mg/ml (see Appendix), at a constant voltage pf 100V.

2.7.2.2 Imaging technique

The ethidium-stained DNA bands were visualised using an UV transilluminator (TFX-20M, Life Technologies™, GIBCO BRL) and then photographed using a Kodak Camera (Digital Science, Electrophoresis Documentation and Analysis, System 120).

2.7.2.3 Purification of PCR product

Purification was conducted with the StrataPrep® DNA Gel Extraction Kit, STRATAGENE (Catalog #400766). 300µl of DNA Extraction Buffer for each 100µl of gel volume was added to a 1.5ml microcentrifuge tube. The mixture was heated at 50^{o}C for at least 10 minutes. It was being mixed occasionally. When the gel had dissolved, the mixture was transferred to a microspin cap that was seated in a 2ml receptable tube. The cap of the 2ml receptable tube was snapped onto the top of the microspin cup. The tube was centrifuged at maximum speed for 30 seconds. The liquid was discarded but the microspin cap was retained and it was replaced in the 2ml receptable tube. 750µl of 1X wash buffer was added to the microspin cap. The cap of the receptable tube was snapped onto the top of the microspin cap and it was centrifuged at maximum speed for 30 seconds. The wash buffer was discarded and the microspin cap was retained and placed back in the 2ml receptable tube. It was centrifuged at maximum speed for 30 seconds. The microspin cap was transferred to a fresh 1.5ml microcentrifuge tube. 30µl of elution buffer was added directly onto the top of the fiber matrix at the bottom of the microspin cap. The tube was incubated at room temperature for 5 minutes. It was centrifuged at maximum speed for 1 minute. The microspin cap was discarded and the DNA was kept in -20 ^{o}C.

2.7.2.4 Sequencing PCR

Two PCR reactions were set up for each sample. A single forward and reverse PCR reaction was performed to generate single-stranded DNA. The samples were sequenced using a BigDye Version 3 dideoxyterminator sequencing kit (PE Biosystems).

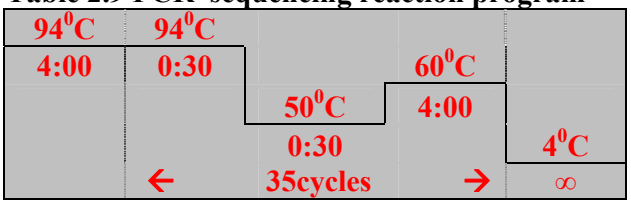
The PCR reactions were set at a 15µl total volume, as described in Table 2.8.

Table 2.8 PCR sequencing reaction

Components	Forward	Reverse
DNA	8.8µl	8.8µl
Primer forward [1pM]	3.2µl	----
Primer reverse [1pM]	----	3.2µl
BigDye	3µl	3µl
Total	15µl	15µl

The conditions for denaturation, annealing and polymerization of the PCR reactions are described in Table 2.9

Table 2.9 PCR sequencing reaction program



2.7.2.5 Purification of PCR product

The sequencing PCR product was purified to get rid of the unused components of the PCR reaction. 15µl of total volume PCR reaction (without mineral oil) was transferred into a 1.5ml Eppendorf tube containing 85µl of 75% isopropanol. The samples were shortly vortexed and left for precipitation at room temperature for 30 minutes. The solution was centrifuged for 20 minutes at 4°C with 14.000rpm. The supernatant was discarded carefully. The pellet was rinsed by 250µl of 75% isopropanol and dried at 90°C for 1 minute.

Just before loading the samples, the DNA pellet was resuspended in 12µl of TSR (Template Suppressing Reagent, PE Biosystems) by vortexing and short spinning twice. The DNA was denatured by heating at 95°C for 5 minutes.

2.7.2.6 Sequencing

The samples were loaded for sequencing in a PERKIN ELMER, ABI Prism ™ 310 Genetic Analyzer (PE Biosystems). Sequencing traces obtained were aligned against each other by using three methods: visual inspection, BioEdit software (Hall, 1999) and SeqDoc software (Crowe, 2005). Some of the SNPs were further confirmed through single base extension analysis by using the SNaPshot™ kit, according to the manufacturers' instructions.

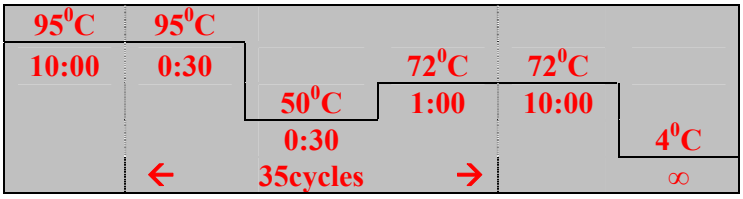
2.7.3 Single-base extension (SNaPshot™) analysis

PCR amplification of target DNA was carried out as described above. When olive oil DNA was used, it was previously diluted down 20 times with molecular grade water. Five microliters of the PCR product was then mixed with 2 µL ExoSAP-IT® (USB Corporation) and incubated at 37°C for 45 min and then at 80°C for 15 min. For the primer extension reaction, a mixture containing 5µL of SNaPshot Reaction Mix, 3µL of previously cleaned up PCR product, 1µL of 2pM SNP primer and 1µL water was incubated for 25 cycles at 95°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec and then incubated at 4°C for further treatment. The whole primer extension product (10µL) was mixed with 1µL shrimp alkaline phosphatase (USB Corporation) and incubated at 37°C for 60 min and then at 75°C for 15 min. The mixture was then stored at -20°C until further use. After this post-extension step, 0.5µL of the sample was mixed with 0.5µL of GeneScan™ -120 LIZ™ size standard and 9µL of Hi-Di™ formamide and then incubated at 95°C for 1 min and put on ice prior to being loaded into the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

2.7.4 PCR on olive and other vegetable oils

The conditions for denaturation, annealing and polymerization of the PCR reactions are described in Table 2.10.

Table 2.10 PCR reaction program on olive and other vegetable oils



The PCR reactions were set at a 50µl total volume, as described in Table 2.11

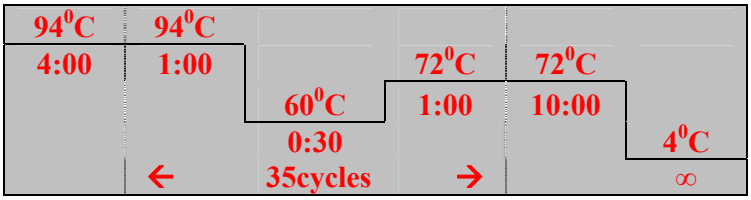
Table 2.11 PCR reaction on olive and other vegetable oils

Compounds	Volume
AmpliTaq Gold Buffer	5 µl
$MgCl_2$	5 µl
dNTPs	1 µl
Primer Forward	1.5 µl
Primer Reverse	1.5 µl
AmpliTaq Gold Polymerase	0.25 µl
DNA	5 µl
H_2O	30.75 µl
Total Volume	50 µl

2.7.5 PCR on vegetables leaves

The conditions for denaturation, annealing and polymerization of the PCR reactions are described in Table 2.12.

Table 2.12 PCR reaction program on vegetable leaves



The PCR reactions were set at a 50µl total volume, as described in Table 2.13.

Table 2.13 PCR reaction program on vegetable leaves

Compounds	Volume
Taq Buffer	**5 µl**
$MgCl_2$	**2.5 µl**
dNTPs	**1 µl**
Primer Forward	**1.5 µl**
Primer Reverse	**1.5 µl**
Taq Polymerase	**0.2 µl**
DNA	**0.5 µl**
H_2O	**37.8 µl**
Total Volume	**50 µl**

2.8 Capillary electrophoresis – Lab-on-a-chip technology

The PCR products of vegetable oils and vegetable leaves were further analysed on a capillary elecrophoresis system with a lab-on-a-chip technology. The capillary elecrophoresis system was Experion™ DNA 12K Analysis Kit by BIO-RAD.

2.8.1 Priming the chip

The Experion DNA 12K was removed from its packaging and placed on the chip platform. 9µl of filtered gel-stain solution was pipetted into the highlighted well-labeled GS. The priming station was carefully closed and the pressure was set to C while the time was set at 30 seconds. At the end of the priming, the chip was inspected for bubbles or evidence of incomplete priming.

2.8.2 Loading the samples and DNA 12K ladder into the chip

The chip was placed on a clean surface and 9µl of the gel-stain solution was pipetted into the other 3 wells labeled GS. 5µl of the loading buffer was pipetted in each sample well (1-11) and the ladder well, L. The wells were inspected for excessive bubble formation by pipetting. 1µl of DNA 12K ladder was pipetted into the well-labelled L. Additionally, 1µl of each sample was pipetted into each of the 11

sample wells. The chip was placed in the Experion vortex station for 60 seconds. The run was started immediately. When the chip run was completed, the electrodes were cleaned with 800 µl DNase-free water filled in the cleaning chip

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Effect of olive oil storage on the stability of residual DNA and the formation of PCR inhibitors

Extractability of high quality DNA from olive oils of several quality grade still remains an important issue that has to be studied in depth in order to establish reliable PCR-based methods. In this study, a first attempt was made to monitor the effect of storage on the integrity of the DNA found in olive oil, by using spiked λDNA as a model molecule.

3.1.1 PCR inhibitors on olive oil

The amount of PCR inhibitors being present in the oil samples during the storage period was monitored by examining the effect of adding DNA extracts from olive oil to a standard PCR, targeted at a yeast amplicon. To elucidate that question by using the olive DNA template instead, as extracted from oil samples, would be very difficult since the concentration of intact target molecule would probably differ among oil samples. As a result, any differences in the amplification signals would be due to factors whose quantitative contribution could not be defined. Therefore, the suggested approach was carried out by setting up a range of PCR reactions containing yeast DNA, yeast-specific primers and 30% (v/v) of olive oil DNA extract. A similar approach has previously been used elsewhere for coffee extracts [P-19]. The number of PCR cycles used was such that the end point of the reaction was in the exponential phase of the PCR, thus the amplicon could be quantified. The specificity of yeast primers employed in this study was initially tested against λ phage and olive leaf DNA as controls. The results illustrated in Figure 3.1 exhibit an amplification signal only in the case where yeast DNA was applied, thus suggesting the specificity of such primers.

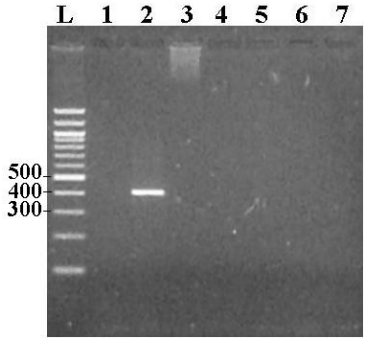


Figure 3.1 Specificity of yeast PCR primers as revealed from an agarose gel electrophoresis. Lane L) 100 bp DNA ladder; Lane 1) Non template control; Lane 2) yeast template; Lane 3) bacteriophage template; Lane 4) olive leaf template. Extracted DNA from spiked oil samples, originating from phage and olive was applied in the remaining lanes in several volumes. Lane 5) undiluted; Lane 6) diluted with water 1:10; Lane 7) diluted with water 1:50.

The decrease in the quantity of yeast amplicon as visualised with gel electrophoresis is illustrated in Figure 3.2.

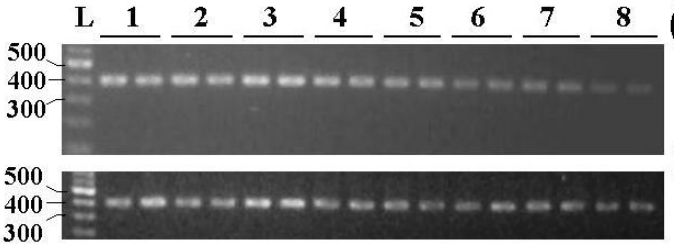


Figure 3.2. Agarose gel electrophoresis illustrating the inhibitory effect of DNA extracts from oils, stored during one year, on yeast PCR. Two replicate sets of oils were used and 2 PCRs per oil sample were applied. Lane L) 100 bp DNA ladder; Lanes 1-8) correspond to oil sampling after 2, 10, 20, 40, 90, 180, 270, 360 days of storage, respectively.

The amplification signal was then semi-quantified through densitometry and its respective evolution is shown in Figure 3.3.

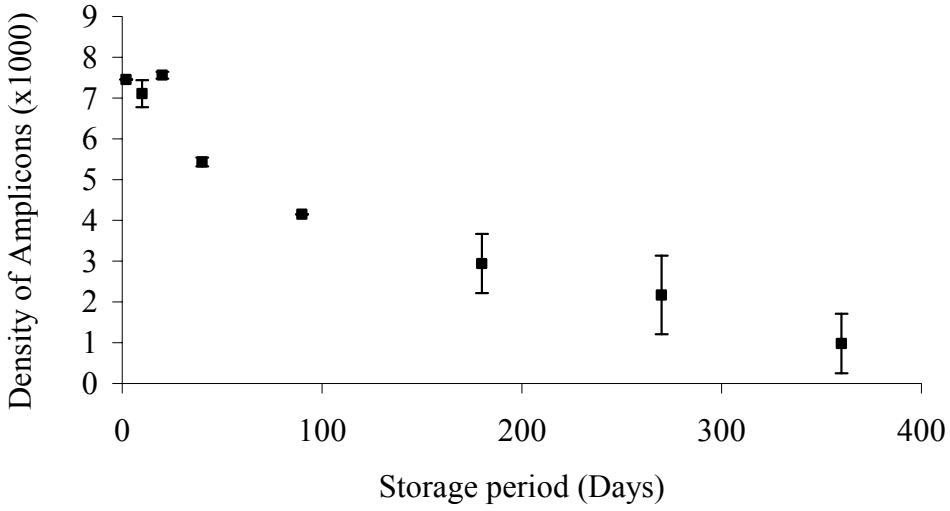


Figure 3.3 Relative quantification of PCR amplicons of Figure 3.2, as given from calculations on densitometry with ImageJ software.

From these results it seems that the inhibition of yeast PCR starts taking place after 20 days of storage period and gradually increases thereafter.

3.1.2 Effect of olive oil storage on residual DNA stability

To monitor any potential DNA fragmentation effect throughout the storage of olive oil at "supermarket" conditions, filtered oils were spiked with λDNA in 3 different concentrations. The reason for spiking olive samples with DNA was the extremely low quantity of the endogenous DNA molecules found in olive oil, and also the possible inconsistency of the successful amplification signals. However, the PCR ability of olive DNA template from the same filtered olive samples was possible (data not shown), the success of which is in agreement with other study (Pasqualone *et al.*, 2005). However, such a low DNA quantity would potentially lead to inconsistent and consequently inconclusive results. Therefore, the decision of employing an exogenous DNA such as that from λ phage was based on the fact that it is a linear molecule and probably it does not exhibit the degree of folding as an eukaryotic DNA does when it interacts with nucleoproteins (Freitfelder, 1987). In addition to that, extracted DNA

from plant tissues also carries compounds such as polyphenols and polysaccharides, which are normally not found in commercial λDNA extract. Therefore, the degree of folding, along with the presence of such impurities may play a possible protective role on the DNA. In addition to that, quality and quantity of such compounds may also depend on the DNA extraction protocol to be followed and/or they may be tissue-dependent as well. Moreover, it is not known if they are part of the fraction which normally exists in filtered oils. This is the reason why olive leaf DNA was not employed instead. The integrity of λDNA was tested through PCR amplification on DNA extracts from stored olive oil, with λ phage-specific primer pairs specially designed for targets of 107, 415 and 691 bp length, respectively. Figure 3.4 illustrates the success of the amplification of the 107 bp fragment originating from λ phage which was successful during the whole storage period in all three sets of samples spiked with different λDNA concentrations.

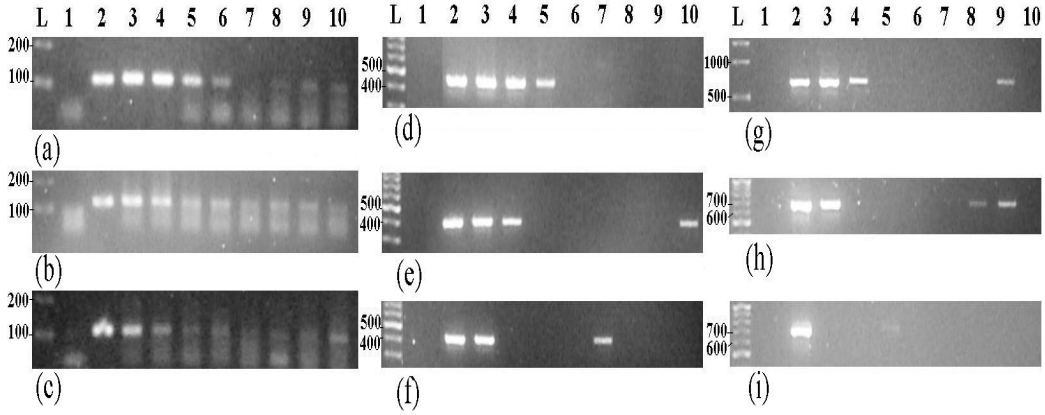


Figure 3.4. Agarose gel electrophoretic results of PCR amplicons generated with λDNA-specific primers and DNA template extracted from the spiked olive samples, as these were stored for one year. PL-1 and PL-2 PCR primers: a) 200 ng/mL λDNA, 107 bp amplicon; b) 40 ng/mL λDNA, 107 bp amplicon; c) 2 ng/mL λDNA, 107 bp amplicon. PL-1 and PL-3 PCR primers d) 200 ng/mL λDNA, 415 bp amplicon; e) 40 ng/mL λDNA, 415 bp amplicon; f) 2 ng/mL λDNA, 415 bp amplicon. PL-2 and PL-4 PCr primers g) 200 ng/mL λDNA, 691 bp amplicon; h) 40 ng/mL λDNA, 691 bp amplicon; i) 2 ng/mL λDNA, 691 bp amplicon.

With respect to the other two fragments, amplification seemed to fail after a storage period of 20 and 10 days for the targets of the 415 and 691 bp lengths, respectively. This amplification threshold is likely to be reduced gradually to one sampling day less, along with the decrease in spiking *λ*DNA concentration. In any case, amplification signal is gradually reduced throughout the whole storage period. These results indicate a likelihood of DNA degradation, along with the production of PCR inhibitors. However, the former seems to happen at such a rate which is probably not enough to degrade DNA molecules of approximately 100 bp length, whereas the latter was further confirmed through the PCR experiment on yeast DNA.

It is interesting to note that the production of PCR inhibitors along with the DNA fragmentation effect during storage seem to be in positive correlation with results stated elsewhere. Some of these are the increase in primary (storage in dark) and secondary (storage in light) oxidation products, triglyceride oligopolymers and polar compounds (Caponio *et al.*, 2005), including oleic acid percentage (Morello *et al.*, 2004), as these were affected by storage conditions. Conversely, they are likely to be in negative correlation with the decrease of compounds exhibiting an antioxidant and vitamin activity, such as *α*-tocopherol, *β*-carotene (Caponio *et al.*, 2005), phenols and chlorophylls (Vacca *et al.*, 2006), again due to storage. For an in-depth understanding of the mechanism of a presumed DNA fragmentation at a molecular level, one would potentially have to reveal first how and in which state DNA is found in the olive oil matrix. It has been stated elsewhere that olive oil can be considered as a fine emulsion of a small quantity of water in a large quantity of oil phase (Bianco *et al.*, 1998). There is strong evidence suggesting that the stability of that emulsion is partially due to endogenous amphiphilic components along with polypeptide molecules (Sotiroudis *et al.*, 2005). Moreover, endogenous emulsifiers in virgin olive oil consist of glycerides, polyphenols and phospholipids (Boskou, 1996). Considering that DNA is water soluble, its likelihood partition in the water phase, especially during malaxation, could possibly explain the fact that DNA is still present in extra virgin olive oil, even after any filtration step. However, the precise mechanism of DNA route from fruit-to-oil remains to be elucidated and further experiments on DNA integrity have to be carried out.

3.2 Construction of a Greek Single Nucleotide Polymorphism (SNP) database on olive varieties

Due to relevant European regulations on traditional products with Protected Designation of Origin (P.D.O.), there is a need for identification of the variety of the olives where the olive was extracted from. Towards that direction, several research groups have started developing molecular biology techniques for the analysis of olive oil samples.

The aim of this work was the development of a Greek database of single nucleotide polymorphism (SNP) markers for olive tree cultivars. Greek varieties were chosen because Greece is one of the main olive oil producing countries (International Olive Oil Council). Moreover, there has never existed any other study with so many Greek varieties until now. The construction of this Greek database will probably improve the authentication and discrimination of Greek olive oils. In addition, the samples were collected not only from one region, but from 3 different cultivating regions. This will probably support not only the discrimination of olive varieties but it may also determine the region where the trees were grown.

3.2.1 DNA extraction from leaves

The plant material used throughout this study was young healthy leaves from the most important Greek olive varieties. The leaves were young and the DNA was extracted immediately after the collection of the leaves by using a standard cetyltrimethylammonium bromide-based protocol. The DNA from leaves that were provided by NAGREF Chania was extracted in a previous study (data not shown). The results of DNA extraction from leaves that were provided by the Kostelenos nurseries is shown in Figure 3.5. In Figure 3.6 the DNA extraction from leaves that were provided by NAGREF of Agios Mamas are shown. The quantification of DNA extraction was made by different concentrations of λDNA that were run on agarose gel at the same time with the DNA extraction of the samples

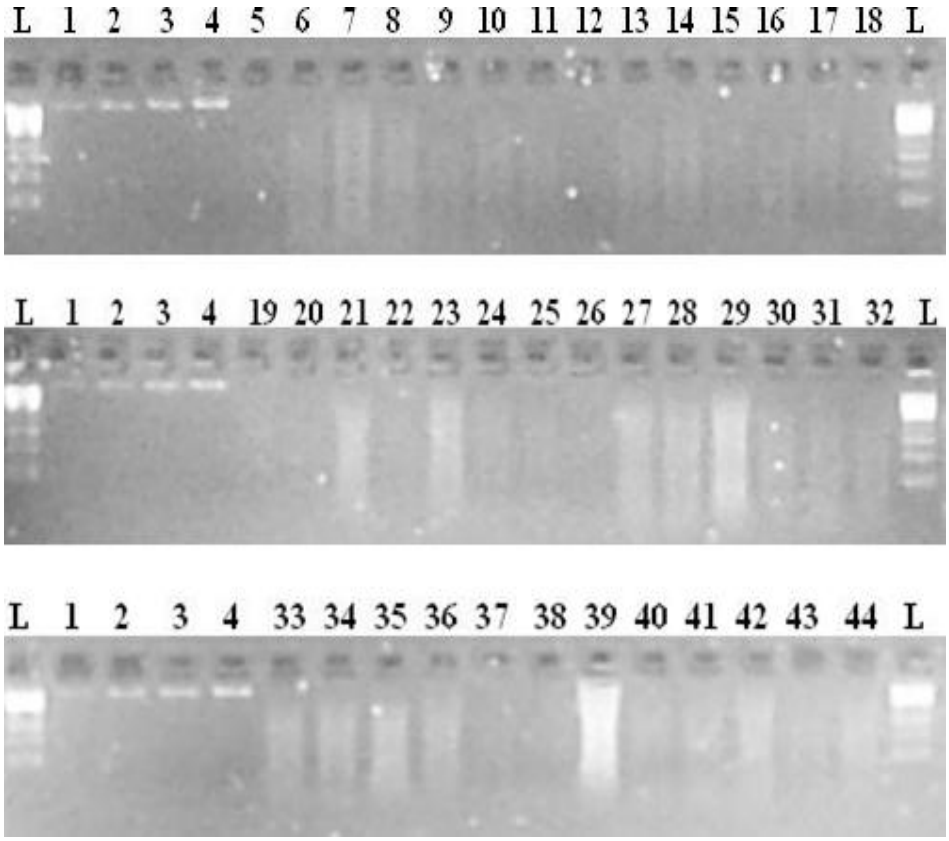


Figure 3.5 Agarose gel electrophoretic results of DNA extraction from leaves that were provided by the Kostelenos nurseries: L) DNA Ladder 1kb; 1) 5ng/μl λDNA; 2) 10ng/μl λDNA; 3) 15ng/μl λDNA; 4) 25ng/μl λDNA; 5) Agouromanako; 6) Adramytini; 7) Amygdalolia; 8) Asprolia Lefkados; 9) Valanolia; 10) Vasilakada; 11) Gaidourelia; 12) Galatistas; 13) Dafnelia; 14) Thiaki; 15) Throumbolia; 16) Kalamon; 17) Kalokairida; 18) Karolia; 19) Karydolia; 20) Dopia Spetson 21) Karydolia Chalkidikis; 22) Klonares Koropiou; 23) Kothreiki; 24) Kolympada; 25) Kolireiki; 26) Amfissis; 27) Koroneiki; 28) Koutsourelia; 29) Lianolia Kerkyras; 30) Maroneias; 31) Mastoeides Koini; 32) Mastoeides Megali; 33) Matolia; 34) Mavrolia Messinias; 35) Megaritiki; 36) Zakinthou; 37) Pikrolia; 38) Rachati; 39) Smertolia; 40) Stroggylolia; 41) Tragolia; 42) Nisiotiki; 43) Agouromanako GPL; 44) Chondrolia Chalkidikis.

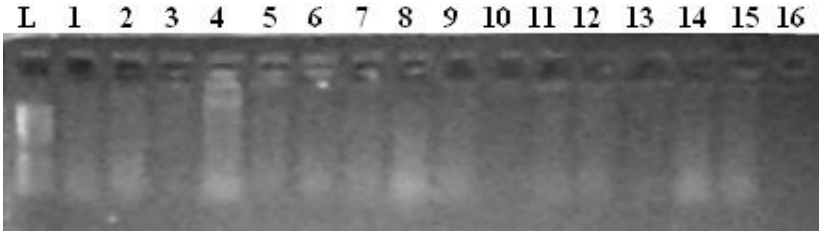


Figure 3.6 Agarose gel electrophoretic results of DNA extraction from leaves that were provided by NAGREF of Agios Mamas : L) 1 Kbp DNA ladder; 1) Lianolia Kerkyras; 2) Karydolia Chalkidikis; 3) Koroneiki; 4) Vasilikada; 5) Patrini; 6) Chondrolia Chalkidikis; 7) Amfissis; 8) Megaritiki; 9) Adramytini; 10) Tsounati; 11) Kalamon; 12) Amygdalolia; 13) Kothreiki; 14) Valanolia; 15) Manaki; 16) Throumbolia.

The quality of the DNA extraction, as shown in Figures 3.5 and 3.6, wasn't good enough, probably because of the high numbers of polysaccharides, polyphenols, carbohydrates and other inhibitors. This result was expected, because most of the pellets were jelly before the hydration, which probably proves the presence of polysaccharides. The quality of DNA extraction was checked by PCR amplification. Most of the PCRs worked except for samples 10, 11, 12, 15, 18, 20, 24, 25, 26, 27, 33, 36, 38, 40 from Figure 3.5 and samples 3, 7, 9, 10, 11, 12, 13, 16 from Figure 3.6. The DNA extraction was repeated for these varieties by using the DNeasy® Plant Mini Kit, Qiagen, Cat. No 69104. The extracted DNA was of high quality (data not shown) and the PCR worked very well.

3.2.2 PCR Amplification

The extracted DNA from all the collected olive varieties was used for PCR amplification of Cycloartenol synthase (cyclo2F-cyclo2R and cyclo3F-cyclo3R) and Lupeol synthase (lupeol2F-lupeol2R) (Spaniolas, 2007). The genes *cycloartenol synthase* and *lupeol synthase*, respectively, were suggested by Reale *et al*. (2006). Lupeol synthase is a multifunctional enzyme that forms other triterpene alcohols, including beta-amyrin, as minor products. Cycloartenol synthase is involved in the biosynthesis of brassinosteroids (Herrera et. al. Phytochemistry 1998).

PCR products were loaded on an agarose gel (Figure 3.7) and they purified by the StrataPrep® DNA Gel Extraction Kit, STRATAGENE (Catalog #400766).

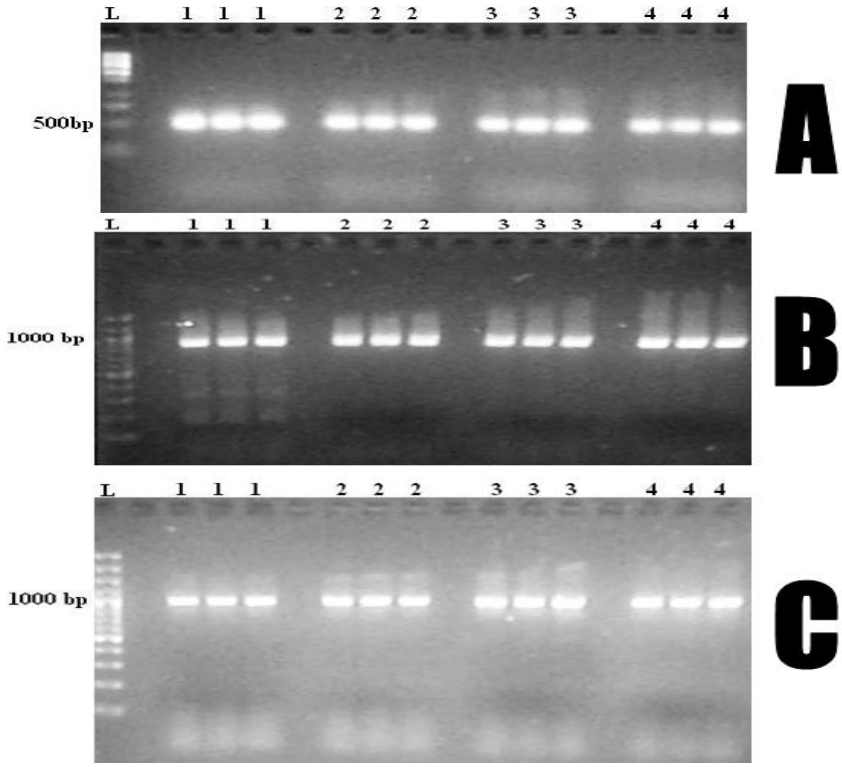


Figure 3.7 PCR products: A) Lupeol synthase (Lupeol2F- Lupeol2R); B) Cycloartenol synthase (Cyclo2F- Cyclo2R); C) Cycloartenol synthase (Cyclo3F- Cyclo3R).

Sometimes the signal was not shown, so the PCR was repeated because a good signal for amplified DNA is very important for the sequencing results. In most cases the signal was more likely better when the initial volume of 100µl of the reaction was divided into two reactions of 50µl. However, when the signal still remained low, the extracted DNA was diluted 10 to 50 times until a good signal was achieved. The dilution factors probably reduced the inhibitors of the reaction. The use of standard cetyltrimethylammonium bromide-based (CTAB) protocol appeared to be the biggest problem in PCR amplification because probably many of the inhibitors hadn't been removed during phenol-chloroform purification. The use of the DNeasy® Plant Mini Kit results in lower yield DNA but the quality was much better than the CTAB method. Most of the PCR worked without dilutions of the DNA and the signal was good enough.

3.2.3 Sequencing

Sequencing was carried out directly on PCR amplicons rather than on cloned fragments. This choice was based on the fact that through direct sequencing we should be able to monitor heterozygous SNPs along with homozygous ones, whereas through cloning we would need a lot of sequenced colonies from each variety in order to uncover heterozygocity. However, cloning is likely more appropriate for haplotype mining purposes.

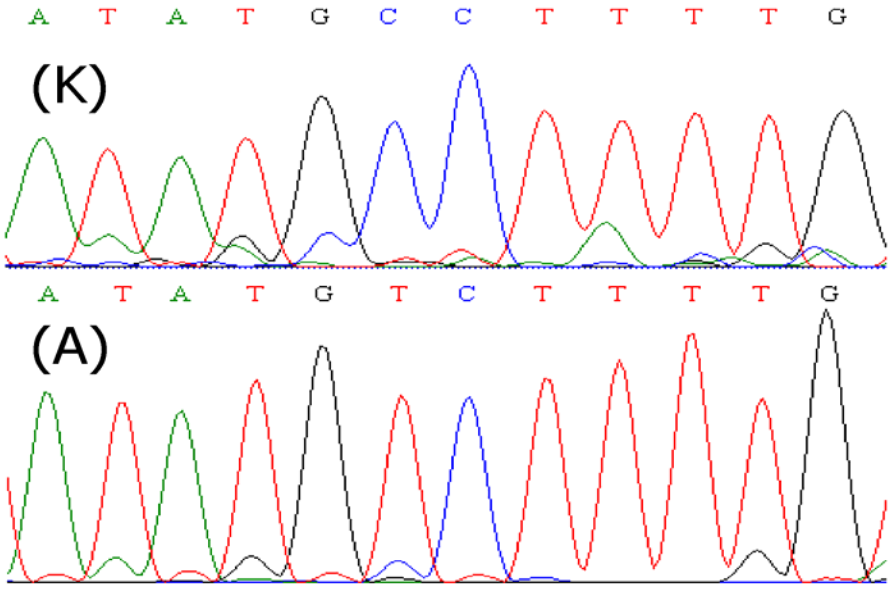


Figure 3.8 Alignment of Koroneiki (K) and Adramytini (A) sequencing traces

3.2.3.1 The Greek SNP database

The SNP discovery quest was based on DNA fragments of approximately 500 and 1800 bp that correspond to the genes *lupeol synthase* (Table 3.1) and *cycloartenol synthase* (Table 3.2 and 3.3) , respectively.

This number of SNPs is in agreement with the findings by Reale *et al.* (2006), although 2 out of 3 proposed genes were analysed, and employed different PCR primers that led to longer fragments than those amplified by the above group.

Table 3.1 Greek SNP Database of the Lupeol synthase (Lupeol2F-Lupeol2R) fragment

	1		2				3		4	5		6			7	8		9	10	11	12	13		14	
A/A	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
SNP No.	**Aggouromanako (NAGREF Chania)**	**Aggouromanako (Kostelenos)**	**Adramytini (Lesvos)**	**Adramytini (Kostelenos)**	**Adramytini (NAGREF Chania)**	**Adramytini (NAGREF Agios Mamas)**	**Amygdalolia (Kostellenos)**	**Amygdalolia (NAGREF Agios Mamas)**	**Amfissis (NAGREF Agios Mamas)**	**Amfissis (Kostelenos)**	**Asprolia Leukados (Kostellenos)**	**Chondrolia (Polygyros)**	**Chondrolia (NAGREF Chania)**	**Chondrolia Chalkidikis (NAGREF Agios Mamas)**	**Dafnelia (Kostelenos)**	**Gaidourelia (NAGREF Chania)**	**Gaidourelia (Kostelenos)**	**Galatistas (Kostelenos)**	**GPL**	**Koutsourelia (NAGREF Chania)**	**Konservolia (NAGREF Chania)**	**Kerkyras (NAGREF Chania)**	**Koroneiki (NAGREF Chania)**	**Koroneiki (Kostelenos)**	**Koroneiki (NAGREF Agios Mamas)**
1	CC		CC	CC	CC	CC	CC	CC	CG?	CG	CG	CC	CC	CC	CG	CC	CC		CC	CC	GC	CC	CC	CC	
2	CC		CC	CC	CC	CC	CC	CC	CT	CC	CC	CT	TC	CT	CC	TC	TC	TC	CC	CC	CC	CC	TC	TC	CT
2+				CC	CC	CC		CC	CC	CC	CC	CA		CA	CC		CA	CA?	CC		CC	CC		CC	AC?
3-		AA		AA	AA	AA		AA	AA	AA	AA	AA		AA	AA		AA	GA?	AA		AA			AG	AG
3	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AT	AA	AT	AA	AA?	AT	AT?	AA	AA	AA		AA	AT	AT
4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA		AG	AA	AA
5	AG ?	GG	AG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	AG ?	GG	GG	GG	AG ?	GG		AG	GG	GG
6	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AC	AC	AC	AA	AC	AC	AC	AA	AA	AA		CC	AC	AC?
7	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TC	TC	CT	TT	TC	TC	TC	TT	TC	TT		CC	CC	CC
8	GA	GG	GA	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GA	GG	GG	GG	GA	GG		GA	GG	GG
9				AA	AA	AA	AA	AA	AA	AA	AA	AA		AA	AA			AG	AA		AA			AG	AG

Table 3.1 (continued)

	15			16		17	18		19		20	21	22	23			24			25	26		27
A/A	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*	*43*	*44*	*45*	*46*	*47*	*48*
SNP No.	**Kalamon (Kostelenos)**	**Kalamon (NAGREF Agios Mamas)**	**Manaki (Kostelenos)**	**Kothreiki (NAGREF Agios Mamas)**	**Manaki (NAGREF Agios Mamas)**	**Kolymbada (Kostelenos)**	**Kalokairida (Kostelenos)**	**Karydolia (Kostelenos)**	**Karydolia Chalkidikis (NAGREF Agios Mamas)**	**Karydolia Chalkidikis (Kostelenos)**	**Klonares (Kostelenos)**	**Karolia (Kostelenos)**	**Kolireiki (Kostelenos)**	**Lianolia Kerkyras (Kostelenos)**	**Lianolia Kerkyras (NAGREF Agios Mamas)**	**Mastoides (NAGREF Chania)**	**Mastoides A (Kostelenos)**	**Mastoides B (Kostelenos)**	**Mastoides (NAGREF Agios Mamas)**	**Matolia (Kostelenos)**	**Megaritiki (Kostelenos)**	**Megaritiki (NAGREF Agios Mamas)**	**Dopia Spetson (Kostelenos)**
1	CC	CC	CC	CC	CC		CC	CC	CC	CC	CG	GC	CG		CC	CG		GC	CG	GC	CC	CC?	
2	CC	CC	CC	CT	CC	CC	CC	CC	CT	CT	CC	CC	CC		CC	TT	CC	CC	CC	CC	CT	CT	
2+	CC	CC	CC	CA	CC				AC	CA		CC	CC		CC		CC	CC	CC	CC	CC	CA	
3-	AA	AA	AA	AG	AA				AA	AA		AA	AA	AA	AA			AA	AA	AA	AG	AG?	
3	AA	AA	AA	AT	AA	AA	AA	AA	AT	AT	AA	AA	AA	AA	AA	AA		AA	AA	AA	AT	AT	
4	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA		AA	AA	AA	AA	AA	
5	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	AG ?		GG	GG	GG	GG	GG	GG
6	AA	AA	AA	AC	AA	AA	AA	AA	AC	AC	AA	AA	AA	AA	AA	AA		AA	AA	AA	AC	AC	AC
7	TT	TT	TT	CT	TT	TT	TT	TT	CT	CT	TT	TT	TT	TC	CT	TT		TT	TT	TT	TC	CT	TC
8	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GA		GG	GG	GG	GG	GG	GG
9	AA	AA	AA	AG	AA		AA	AA	AA	AA	AA	AA	AA	AA	AA			AA	AA	AA	AG	AG	AG

Table 3.1 (continued)

A/A	28	29	30	31	32	33		34		35	36		37			38		39
	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64		65
SNP No.	**Nissiotiki A (Kostelenos)**	**Patrini (NAGREF Agios Mamas)**	**Pikrolia (Kostelenos)**	**Rachati (Kostelenos)**	**Smertolia A (Kostelenos)**	**Stroggylolia (Kostelenos)**	**Throumbolia (NAGREF Chania)**	**Throumbolia (Kostelenos)**	**Throumbolia (Agios Mamas)**	**Tragolia (Kostelenos)**	**Thiaki (Kostelenos)**	**Valanolia (Lesvos)**	**Valanolia (Kostelenos)**	**Valanolia (NAGREF Agios Mamas)**	**Vassilikada (Kostelenos)**	**Vassilikada (NAGREF Agios Mamas)**		**Zakynthou (Kostelenos)**
1			CC	CG?	CC	CC?		CG	CG	CC	CC	CG	CG	CG	CC	CC		CC
2	CT		CC	CC	CC	CC?	CC	CC	CC	CC	CC	TC	TC	CT	CC	CC		CC
2+	CA?	CC	CC	CC	CC	CA?		CC	CC	CC			CA	CA	CC	CC		CC
3-	AG	AA	AA	AA	AA	AA		AA	AA	AA			AA	AA	AA	AA		AA
3	AT	AA	AA	AA	AA	AA?	AA	AA	AA	AA	AA	AT	AT	AT	AA	AA		AA
4	AA	AA	AG?	AA?	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA		AA
5	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	AG	GG	GG	GG	GG		GG
6	AC	AA	AA	AC?	AA	AC?	AA	AA	AA	AA	AA	AC	AC	AC	AA	AA		AA
7	CC	CC	CC		TT	TC	TT	TT	TT	TC	TT	TC	TC	CT	TC	CT		TC
8	GG	GG	GG		GG	GG	GG	GG	GG	GG	GG	GA	GG	GG	GG	GG		GG
9	AG		AA		AA	AA	AA	AA	AA	AA			AA	AA	AA	AA		AA

Table 3.2 Greek SNP Database of the Cycloartenol synthase (Cyclo2F-Cyclo2R) fragment

	1				2		3		4		5	6			7	8	9		10		11		12
A/A	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*
SNP No.	**Adramytini (Lesvos)**	**Adramytini (NAGREF Chania)**	**Adramytini (Kostelenos)**	**Adramytini (NAGREF Agios Mamas)**	**Aggouromanako (NAGREF Chania)**	**Aggouromanako (Kostelenos)**	**Amygdalolia (Kostelenos)**	**Amygdalolia (NAGREF Agios Mamas)**	**Amfissis (Kostelenos)**	**Amfissis (NAGREF Agios Mamas)**	**Asprolia Leukados (Kostelenos)**	**Chondrolia (NAGREF Chania)**	**Chondrolia (Polygyros)**	**Chondrolia (NAGREF Agios Mamas)**	**Dafnelia (Kostelenos)**	**Galatistas (Kostelenos)**	**Gaidourelia (NAGREF Chania)**	**Gaidourelia (Kostelenos)**	**GLP (Kostelenos)**	**Koroneiki (NAGREF Chania)**	**Koroneiki (Kostelenos)**	**Koroneiki (NAGREF Agios Mamas)**	**Karolia (Kostelenos)**
1	AT		TA	AT	AT	TA	AA	AA	AT	AT	AA	AT	TA	AT	TA	AA	AA	AA	AA	AT	AT	AT	AT
2	AT	AT	TA	AT	AT	TA	AA	AA	AT	AT	AA	AT	TA	AT	TA	AA	AA	AA	AA	AT	AT	AT	AT
3	AG	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
4	AC	AC	AC	AC	AC	AC	AA	AA	AC	AC	AA	AC	AC	AC	AC	AA	AA	AA	AA	AC	AC	AC	AC
5	AC	AC	AC	AC	AC	AC	AA	AA	AC	AC?	AA	AC	AC	AC		AA	AA	AA	AA	AA	AA	AA	AC
6	GG	GG	GG	GG	GG	GG	GG	GA?	GG	GG	GG?	GG	GG	GG	GG	GA	GG		AG	GG	GG	GG	AG?
7	TC	TC	CT	CT	TC		TT	TT	TC	CT	TT?	TC	TC	TC	TC		TT		TT	TC	TC	CT	
7a	GG	GG			GG		GG		GG			GG	GG		GG		GG		GG	GG	GG		GG
8	TC	TC	CT	CT	CT		CC	CC	TC	CT		TC?	CC?		TC	CC	CC		CC	TC	TC	CT	TC
9	AG	AG	AG	GA	GA		GG	GG	AG	GA	GG	AG	AG		AG	GG	GG	GG	GG	AG	AG	AG	AG
10	TG	TG	TG	GT	TG		GG	GG	TG	TG	GG	TG?	TG		TG	GG	GG	GG	GG	TG	TG	TG	TG
11	TT	TT	TT	TT	TT		TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TC	TC	CT	TT

Table 3.2 (continued)

	13		14	15		16	17		18	19	20	21		22		23	24			25			26	27
A/A	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*	*43*	*44*	*45*	*46*	*47*
SNP No.	**Karydolia (Kostelenos)**	**Karydolia Chalkidikis (NAGREF Agios Mamas)**	**Kerkyras (NAGREF Chania)**	**Koutsourelia (NAGREF Chania)**	**Koutsourelia (Kostelenos)**	**Konservolia (NAGREF Chania)**	**Kalamon (Kostelenos)**	**Kalamon (NAGREF Agios Mamas)**	**Kalokerida (Kostelenos)**	**Kolireiki (Kostelenos)**	**Karolia Chalkidikis (Kostelenos)**	**Kolymbada (Kostelenos)**	**Manaki (Kostelenos)**	**Kothreiki (NAGREF Agios Mamas)**	**Manaki (NAGREF Agios Mamas)**	**Klonares Koropiou (Kostelenos)**	**Lianolia Kerkyras (Kostelenos)**	**Lianolia Kerkyras (NAGREF Agios Mamas)**	**Mastoides (NAGREF Chania)**	**Mastoides A (Kostelenos)**	**Mastoides B (Kostelenos)**	**Mastoidis (NAGREF Agios Mamas)**	**Mavrelia Messinias (Kostelenos)**	**Maronias (Kostelenos)**
1	AA	AT	AT	AT	TA	AT		AA	TA	AT	AT	AA	TA	AA	AT		AA	AA	AT	AT	AT	AT	AT	AA
2	AA	AT	AT	AT	TA	AT		AA	TA	AT	AT	AA	TA	AA	AT		AA	AA	AT	AT	AT	AT	AT	AA
3	AA	AA	AA	AA	AA	AA		AA	AA	AA	AA	AA	AA	AA	AA		AA	AA	AA	AA	AA	AA	AA	AA
4	AA	AC	AC	AC	AC	AC		AA	AC	AC	AC	AA	AC	AA	AC		AA	AA	AC	AC	AC	AC	AC	AA
5	AA	AC		AA	AA	AC		AA	AC	AC	AC	AA	AC	AA	AC		AA	AA	AA	AA	AA	AA	AC	AA
6	AG	GG	GG	GG	GG	GG		GG	AG	GG	GG	AG	GG	GG	GG		GG	GG	GG	GG	GG?	GG	GG	GG
7		CT		TC	CC	TC		TT	TC		TC	TT	TC	TT	CT		TT	TT	TC			CT		TT
7a			GG	GG	GG	GG			GG	GG	GG	GG	GG				GG		GG		GG?			GG
8		CT	CC	TC	TC	TC		CC	TC	TC	TC?	CC	TC	CC	CT		CC	CC	TC			CT		CC
9		GA	GG	AG	AG	AG	GG	GG	AG	AG	AG	GG	AG	GG	GA	GG	GG	GG	AG	GA?	AG	GA		GG
10		GT	GG	TG	TG	TG	GG	GG	TG	TG	TG	GG	TG	GG	GT		GG	GG	TG	TT?	TG	GT		GG
11		TT	TT	TC	TC	TT	TT	TT	TT	TT	TC	TT	TT	TT	TT		TT	TT	TC	CT?	TC	CT		TT

Table 3.2 (continued)

	28	29		30	31	32	33	34	35	36	37		38		39	40		41		42		43
A/A	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
SNP No.	Matolia (Kostelenos)	Megaritiki (Kostelenos)	Megaritiki (NAGREF Agios Mamas)	Mavrolia (Kostelenos)	Nistiotiki (Kostelenos)	Dopia Spetson (Kostelenos)	Patrini (NAGREF Agios Mamas)	Pikrolia (Kostelenos)	Rahati (Kostelenos)	Smertolia (Kostelenos)	Stroggylolia (Kostelenos)	Throumbolia (NAGREF Chania)	Throumbolia (Kostelenos)	Throumbolia (NAGREF Agios Mamas)	Thiaki (Kostelenos)	Tragolia (Kostelenos)	Valanolia (Lesvos)	Valanolia (Kostelenos)	Valanolia (NAGREF Agios Mamas)	Vassilikada (Kostelenos)	Vassilikada (NAGREF Agios Mamas)	ZakInthou (Kostelenos)
1	TA	AA	AA	TA	AA	AA	AT	AT	AT	TA	AT	AT	AT	AT	TT	AT	AA	AA	AA	AT	AT	AA
2	TA	AA	AA	TA	AA	AA	AT	AT	AT	TA	AT	AT	AT	AT	TT	AT	AA	AA	AA	AT	AT	AA
3	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AG	AA	AA	AA	AA	AA
4	AC	AA	AA		AA	AA	AC	AC	AC	AC	AC	AC	AC	AC	CC	AC	AA	AA	AA	AC	AC	AA
5	AC	AA	AA		AA	AA	AA	AC	AA	AC	AC	AC		AC	CC	AA	AA	AA	AA	AC	AC	AA
6	GG	GA	GA?		GG	AG	GG	GG	GG	GG	AG	GG		GG	GG	GG	AG		GA	GG	GG	GG
7			TT		TT	TT		CT?		TC	CC?	TC		CT	CC		TT	TT	TT	CT	CT	TT
7a	GG	GG			GG	GG			GG	GG	GG	GG			GC	GG	GG					GG
8	TC	CC	CC		CC	CC		CT	TC	TC	TC	TC		CT	TT	TC	CC	CC	CC	CT	CT	CC
9	AG	GG	GG		GG	GG		GA	AG	AG	AG	AG	AG	GA	AA	AG	GG	GG	GG	GA	AG	GG
10	TG	GG	GG		GG	GG		TG	TG	TG	TG	TG	TG	GT	TT	TG	GG	GG	GG	GT	TG	GG
11	TT	TT	TT		TT	TT		TT	TT	TT	TT	TT	TT	TT	TT	TC	TT	TT	TT	TT	TT	TT

Table 3.3 Greek SNP Database of the Cycloartenol synthase (Cyclo3F-Cyclo3R) fragment

	1				2			3			4		5		6		7	8	9		10	11		12
A/A	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*
SNP No.	**Adramytini (Stelios)**	**Adramytini (NAGREF Chania)**	**Adramytini (Kostelenos)**	**Adramytini (NAGREF Agios Mamas)**	**Aggouromanako (NAGREF Chania)**	**Aggouromanako (Kostelenos)**	**Amygdalolia (Kostelenos)**	**Amygdalolia (NAGREF Agios Mamas)**	**Amfissis (Kostelenos)**	**Amfissis (NAGREF Agios Mamas)**	**Asprolia Leukados (Kostelenos)**	**Chondrolia (NAGREF Chania)**	**Chondrolia (Polygyros)**	**Chondrolia (NAGREF Agios Mamas)**	**Dafnelia (Kostelenos)**	**Galatistas (Kostelenos)**	**Gaidourelia (NAGREF Chania)**	**Gaidourelia (Kostelenos)**	**GLP (Kostelenos)**	**Koroneiki (NAGREF Chania)**	**Koroneiki (Kostelenos)**	**Koroneiki (NAGREF Agios Mamas)**	**Karolia (Kostelenos)**
12	TC				TC				CT				CT?				CC			TC	CT		
13	TC	TC	TC		TC				CT			TC	CT?		CT		TT	TT		TC	CT		CT
14	CC	CC	CC		CC				CC			CC	CC		CC		CC	CC		TC	CT		CC
15		AC	AC						AA				CA										AA

Table 3.3 (continued)

A/A	13		14	15		16	17		18	19	20	21		22		23	24			25			26	27
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
SNP No.	Karydolia (Kostelenos)	Karydolia Chalkidikis (NAGREF Agios Mamas)	Kerkyras (NAGREF Chania)	Koutsourelia (NAGREF Chania)	Koutsourelia (Kostelenos)	Konservolia (NAGREF Chania)	Kalamon (Kostelenos)	Kalamon (NAGREF Agios Mamas)	Kalokerida (Kostelenos)	Kolireiki (Kostelenos)	Karolia Chalkidikis (Kostelenos)	Kolymbada (Kostelenos)	Manaki (Kostelenos)	Kothreiki (NAGREF Agios Mamas)	Manaki (NAGREF Agios Mamas)	Klonares Koropiou (Kostelenos)	Lianolia Kerkyras (Kostelenos)	Lianolia Kerkyras (NAGREF Agios Mamas)	Mastoides (NAGREF Chania)	Mastoides A (Kostelenos)	Mastoides B (Kostelenos)	Mastoidis (NAGREF Agios Mamas)	Mavrelia Messinias (Kostelenos)	Maronias (Kostelenos)
12				TC	CT	TC				CT		CC					CC		TC					
13	TT		TT	TC	CT	TC	TT		CT	CT	CT	TT	CT			TT	TT		TC					
14	CC		CC	TC?	CT	CC	CC		CC	CC	CC	CC	CC			CC	CC		TC					
15	CA		AC				CA?		??	AA	AA	AC	CA			CA	AC							

Table 3.3 (continued)

	28	29		30	31	32	33	34	35	36	37		38		39	40		41			42		43
A/A	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
SNP No.	**Matolia (Kostelenos)**	**Megaritiki (Kostelenos)**	**Megaritiki (NAGREF Agios Mamas)**	**Mavrolia (Kostelenos)**	**Nistiotiki (Kostelenos)**	**Ntopia Spetson (Kostelenos)**	**Patrini (NAGREF Agios Mamas)**	**Pikrolia (Kostelenos)**	**Rahati (Kostelenos)**	**Smertolia (Kostelenos)**	**Stroggylolia (Kostelenos)**	**Throumbolia (NAGREF Chania)**	**Throumbolia (Kostelenos)**	**Throumbolia (NAGREF Agios Mamas)**	**Thiaki (Kostelenos)**	**Tragolia (Kostelenos)**	**Valanolia (Lesvos)**	**Valanolia (Kostelenos)**	**Valanolia (NAGREF Agios Mamas)**	**Vassilikada (Kostelenos)**	**Vassilikada (NAGREF Agios Mamas)**	**Zakinthou (Kostelenos)**
12		CC			CC					CT?	CT?	TC				CT	TT					CC
13		TT			TT	TT		CT		CT?	CT?	TC	CT		CC	CT	CC			CT		TT
14		CC			CC	CC		CC		CC	CC	CC	CC		CC	CT	CC			CC		CC
15		CA			CA	CA		AA		CA	AA		CA		AA							CA

There are 60 Greek varieties (Therios, 2005). Of these varieties, 43 were sequenced and the majority of them worked well. The sequencing of nuclear DNA without cloning met many difficulties because it was not easy, high-quality amplified DNA to be achieved. Sequencing regions with relatively higher signal-to-noise ratio were excluded from the actual analysis and 27 candidate SNP positions were found.

According to this SNP database of Greek olive varieties an initial discrimination was carried out. Based on the SNPs found so far, one can choose an appropriate combination either for genotyping among the varieties used or for a "yes or no"-type of request. For the former issue (an example is given in Appendix C), the number of SNPs as resulting from *lupeol synthase* and *cycloartenol synthase* targets, seems to be inefficient to discriminate among all the varieties. More genes are required to be sequenced and analyzed. As far as the latter question is concerned, 19 of 43 varieties could be efficiently tested for fraud. These varieties are: Amfissis, Asprolia Lefkados, Adramytini (Lesvos), Agouromanako (NAGREF Chania), Gaidourelia, Kalokairida, Koutsourelia, Koroneiki, Kothreiki, Klonares Koropiou, Kerkyras, Mastoeides, Rachati, Thiaki, Tragolia, Throumbolia, Stroggylolia, Valanolia and Vasilikada. Some of the main oil-producing Greek varieties are 9 out of the above 19 varieties (Koroneiki, Kothreiki, Agouromanako, Koutsourelia, Asprolia Lefkados, Adramytini, Valanolia, Throumbolia) (Therios, 2005). The combinations of SNP positions could discriminate these varieties from all the other 43. (Table 3.4)

Table 3.4 Discrimination of olive varieties according to the SNP positions

OLIVE VARIETY	SNPs POSITIONS
Amfissis	1,2,3 (lupeol2) and 15 (cyclo3)
Asprolia Lefkados	1 (lupeol2) and 1 (cyclo2)
Adramytini (Lesvos)	1,5,8 (lupeol2) and 3 (cyclo2)
Agouromanakolia (NAGREF Chania)	2,7,8 (lupeol2)
Gaidourelia (NAGREF Chania)	1,2,7,8 (lupeol2)
Gaidourelia (Kostelenos)	2,2+,3-,7(lupeol2) and 6,7(cyclo2)

Table 3.4 continued

OLIVE VARIETY	SNPs POSITIONS
Kalokairida	1,2,2+,7,8 (lupeol2) and 5,6,7(cyclo2)
Koutsourelia (NAGREF Chania)	6,7,8 (lupeol2)
Koutsourelia (Kostelenos)	6,7 (cyclo2)
Koroneiki (NAGREF Chania)	6 (lupeol2)
Koroneiki (Kostelenos and NAGREF Ag.Mamas)	6,3 (lupeol2) and 4,9 (cyclo2)
Kothreiki (NAGREF Ag. Mamas)	6 (cyclo2)
Klonares Koropiou	1,2,2+ (lupeol2) and 9 (cyclo2)
Kerkyras	1,2,2+ (lupeol2) and 8,9,10 (cyclo2)
Mastoeides (Metzidakis)	2 (lupeol2)
Mastoeides (Kostelenos and NAGREF Ag. Mamas)	1,2,2+ (lupeol2) and 5 (cyclo2)
Rachati	1,6 (lupeol2) and 3 (cyclo2)
Thiaki	1 (cyclo2)
Tragolia	1,2,7,8,9 (lupeol2) and 4,5,6 (cyclo2)
Throumbolia	1,3- (lupeol2)
Strogylolia	1,2,7,8,9 (lupeol2) and 4,5,6 (cyclo2)
Valanolia (Lesvos)	5,6 (lupeol2)
Valanolia (Kostelenos and NAGREF Ag. Mamas)	1,6 (lupeol2) and 3 (cyclo2)
Vasilikada	1,2,7,8,9 (lupeol2) and 4,5,6 (cyclo2)

For the discrimination of Thiaki, Mastoeides (NAGREF Chania), Kothreiki (NAGREF Ag. Mamas) and Koroneiki (NAGREF Chania), only one SNP is enough, since their zygosity state seems to be unique for those cultivars.

One more important result is the discrimination of some varieties in relation to the region of origin. Valanolia and Adramytini samples from Lesvos, which is the origin of these varieties, can be discriminated from Valanolia and Adramytini samples from both NAGREF Ag. Mamas and Chania, due to the SNP(5) and SNP(3) of *lupeol synthase*, respectively. Moreover, Koroneiki and Mastoeides samples from Chania can be discriminated from Koroneiki and Mastoeides samples from NAGREF Ag. Mamas and Kostelenos nurseries due to the SNP(6) and SNP(2) of *lupeol synthase*, respectively. This also occurs between Gaidourelia samples of NAGREF Chania and Kostelenos (SNP(8) of *lupeol synthase*), Koutsourelia samples of NAGREF Chania and Kostelenos (SNP(7) of *cycloartenol synthase*), Agouromanakolia samples of NAGREF Chania and Kostelenos (SNP(8) of *lupeol synthase*), Amfissis samples of NAGREF Ag. Mamas and Kostelenos (SNP(2) of *lupeol synthase*), Chondrolia samples of NAGREF Chania and NAGREF Ag. Mamas (SNP(3) of *lupeol synthase*) and Megaritiki samples of Kostelenos nurseries and NAGREF Ag. Mamas (SNP(3) of *lupeol synthase*). The above SNP positions probably offer discrimination based on the region of origin.

Furthermore, groups can be formed by the varieties that cannot be discriminated (Table 3.5). Chondrolia Chalkidikis and Karydolia Chalkidikis have the same SNPs but they can be discriminated from all the other varieties by SNP(2), SNP(3) of *lupeol synthase* and SNP(4) of *cycloartenol synthase*. The same occurs with Konservolia and Dafnelia with SNP(1), SNP(2), SNP(2+) of *lupeol synthase* and SNP(5) of *cycloartenol synthase*. Also, Zakynthou and Lianolia Kerkyras can be discriminated from the other varieties by SNP(6), SNP(7) and SNP(8) of *lupeol synthase*. One more pair is Amygdalolia and Kalamon with no difference in SNP positions between them, but they can be discriminated by SNP(1), SNP(2), SNP(2+) of *lupeol synthase* and SNP(6), SNP(8), SNP(9), SNP(10) of *cycloartenol synthase.* The Galatistas and Dopia Spetson pair can be discriminated by SNP(7), SNP(8), SNP(9) of *lupeol synthase* and SNP(1), SNP(2) of *cycloartenol synthase.* The last 3 groups which contain more than 2 varieties are: Throumbolia (NAGREF Chania), Karolia, Kolyreiki and Matolia, with no difference in SNP positions between them but they can be discriminated from the other varieties by SNP(1), SNP(2), SNP(3-) and SNP(6) of *lupeol synthase,* SNP(6), SNP(9) and SNP(15) of *cycloartenol synthase*.

The next group includes GPL, Kolympada and Karydolia also with no difference in SNP positions between them but they can be discriminated from the other varieties by SNP(1), SNP(2) and SNP(2+) of *lupeol synthase* and SNP(6), SNP(8), SNP(9), SNP(10) of *cycloartenol synthase.* The last group is the largest including 5 varieties: Agouromanakolia (Kostelenos), Adramytini (Kostelenos, NAGREF Chania, NAGREF Ag. Mamas), Manaki (Kostelenos, NAGREF Ag. Mamas), Smertolia and Throumbolia (NAGREF Chania). For the discrimination of these varieties, all the 27 SNP positions are necessary to be used.

The SNP marker developed and used in this study could differentiate 62% of the olive samples tested. The percentage of the differentiation is smaller than Reale *et al.* 2006 but in their study, varieties from different countries were analysed. In this study all the varieties are Greek, which means less polymorphism between them. Another important result is that probably 80% of the samples, whose origin is from different regions, can be discriminated. This result assists not only the varietal discrimination but also the discrimination of origin.

Table 3.5 Discrimination of olive varieties according to the SNP positions

OLIVE VARIETY	SNPs POSITIONS
Chondrolia Chalkidikis - Karydolia Chalkidikis	2,3 (lupeol2) and 4 (cyclo2)
Konservolia - Dafnelia	1,2,2+ (lupeol2) and 5 (cyclo2)
Zakinthou - Lianolia Kerkyras	6,7,8 (lupeol2)
Amygdalolia - Kalamon	1,2,2+ (lupeol2) and 6,8,9,10 (cyclo2)
Galatistas - Dopia Spetson	7,8,9 (lupeol2) and 1,2 (cyclo2)
Throumbolia(NAGREF Chania) - Kolireiki - Karolia - Matolia	1,2,3-,6 (lupeol2), 6,9 (cyclo2) and 15 (cyclo3)
GPL, Kolympada, Karydolia	1,2,2+ (lupeol2) and 6,8,9,10 (cyclo2)
Agouromanakolia(Kostelenos) - Adramytini(Kostelenos, NAGREF Chania, NAGREF Ag. Mamas) - Manaki(Kostelenos, NAGREF Ag. Mamas) - Smertolia – Throumbolia (NAGREF Chania)	All SNP positions

3.3 Exploitation of SNP database on varietal olive oil authenticity

The SNP database was used for the authenticity of 10 different varieties of olive oil. The varieties of these olive oils were Chondrolia Chalkidikis, Valanolia, Adramytini, Koroneiki, Kalamon, Throumbolia, Mastoeides, Lianolia Kerkyras, Gaidourelia and Manaki.

3.3.1 DNA extraction from olive oils

As mentioned above, DNA extraction from extra virgin olive oils is not an easy process, because of the low DNA quantity in olive oil and the presence of many inhibitors. In order to choose the method that combines good quality and quantity of extracted DNA, a preliminary comparison study was carried out between three DNA extraction kits. These kits were the DNA*Extractor* Fat (GENESCAN), Wizard Magnetic DNA Purification System for Food (PROMEGA) and QIAamp Stool kit (QIAGEN). Chondrolia Chalkidikis extra virgin olive oil and sunflower oil were used for this study. The olive oil was not filtered in contrast with sunflower oil that was filtered.

The quality and the quantity of the extracted DNA from olive oil and sunflower oil was very degraded and could not be detected or quantified by spectrophotometry. It is likely that the degradation of the extracted DNA does not avoid the PCR amplification but the only limitation is probably the fragmentation of the oils' DNA. For this reason, primers cyc2aF-cyc2aR and rbcLF-rbcLR that amplify short fragments were chosen for olive oil and sunflower oil, respectively. Moreover, the chloroplastic target of rbcL-F/R primers is more easily detectable than genomic targets, due to its high copy number per cell.

In order to reduce the inhibitors of the extracted DNA from olive oils, many dilution factors (undiluted, 1:5, 1:7, 1:10, 1:15, 1:20, 1:50) were chosen. Also, two replicates per extraction kit were carried out (Figures 3.9 and 3.10).

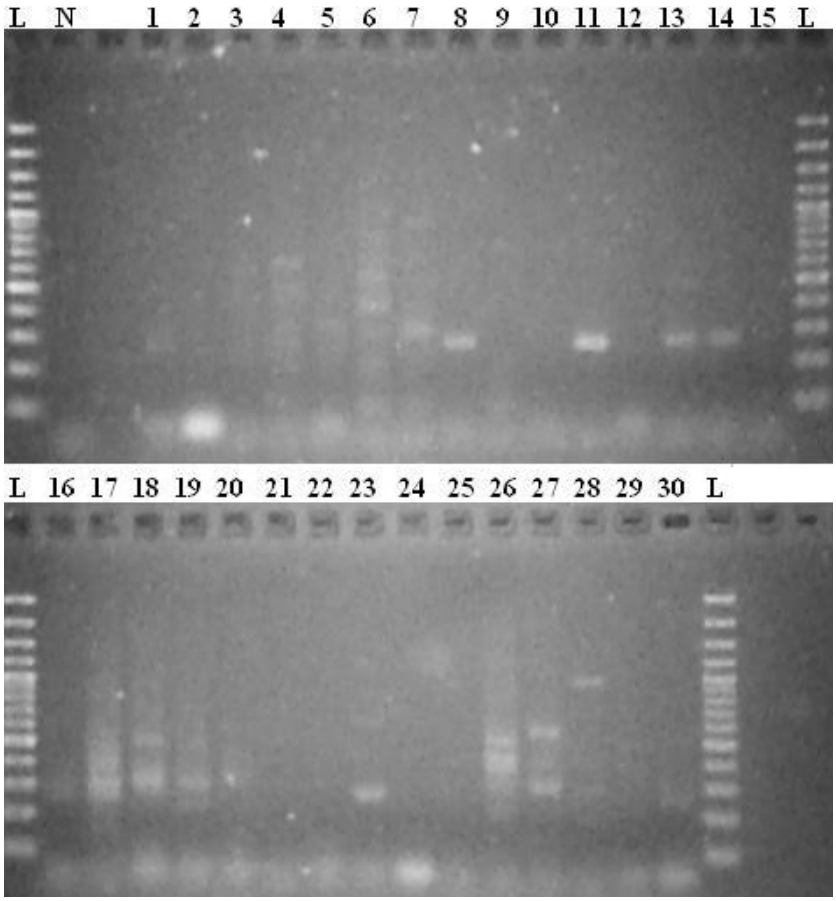


Figure 3.9 Comparison of 3 DNA extraction kits on olive oil using cyc2aF-cyc2aR primers. A) DNA Extractor Fat by GENESCAN (G); B) Wizard Magnetic DNA Purification System for Food by PROMEGA (P); C) Stool kit by QIAGEN (Q) : L) 100 bp DNA ladder; N) Non-template control; 1) G1 (undiluted); 2) G1 (1:20 dilution); 3) G1 (1:10 dilution); 4) G1 (1:5 dilution); 5) G1 (1:50 dilution); 6) P1 (undiluted); 7) P1 (1:5 dilution); 8) P1 (1:10 dilution); 9) P1 (1:20 dilution); 10) P1 (1:50 dilution); 11) Q1 (undiluted); 12) Q1 (1:5 dilution); 13) Q1 (1:10 dilution); 14) Q1 (1:20 dilution); 15) Q1 (1:50 dilution); 16) G2 (undiluted); 17) G2 (1:5 dilution); 18) G2 (1:10 dilution); 19) G2 (1:20 dilution); 20) G2 (1:50 dilution); 21) Q2 (undiluted); 22) Q2 (1:5 dilution); 23) Q2 (1:10 dilution); 24) Q2 (1:20 dilution); 25) Q2 (1:50 dilution); 26) P2 (undiluted); 27) P2 (1:5 dilution); 28) P2 (1:10 dilution); 29) P2 (1:20 dilution); 30) P2 (1:50 dilution)

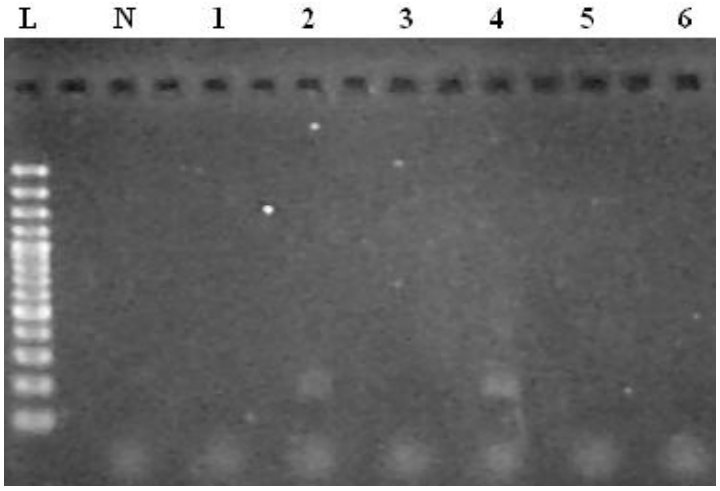


Figure 3.10 Comparison of 3 DNA extraction kits on sunflower oil using rbcLF-rbcLR primers. A) DNA Extractor Fat by GENESCAN (G); B) Wizard Magnetic DNA Purification System for Food by PROMEGA (P); C) Stool kit by QIAGEN (Q) : L) 100 bp DNA ladder; N) Non-template control; 1) G1; 2) G2; 3) Q1; 4) Q2; 5) P1; 6) P2

Comparing the results of Figure 3.9, the best amplified fragment was given by the first undiluted replicate (Q1) from the Qiagen kit. In contrast, the second undiluted replicate from the Qiagen kit (Q2) didn't give any amplified fragment. Furthermore, the 1:10 and 1:20 dilutions from Q1 and the 1:10 dilution from Q2 were worked. The results show that the PCR is inconsistent but Qiagen kit is the only one that didn't give any artifacts during PCR amplification. Moreover, the stability of Qiagen kit was not only proven in sunflower oil (Figure 3.10) but also in other laboratory work (data not shown). One more reason that Qiagen kit was the choice method is the lower cost in comparison with the Promega and Genescan kits.

3.3.2 PCR on olive oils

According to the SNP database, SNPs 1-9 from the Lupeol synthase target seem to be efficient enough to discriminate all the olive oil varieties (Valanolia, Adramytini, Koroneiki, Kalamon, Throumbolia, Mastoeides, Lianolia Kerkyras and Manaki), with the only exception of Chondrolia Chalkidikis against Gaidourelia.

That, however, could be easily resolved by the contribution of SNPs 1-3 from the Cycloartenol synthase target.

For the PCR amplification of olive oil DNA, new primers were designed for the specific fragment that contains the above SNP positions. These primers are Cyc2a-F/R and Lup2-F/R for Cycloartenol synthase and Lupeol synthase, respectively. The DNA was extracted from olive oils by the Qiagen kit, except Gaidourelia, which was extracted by the Promega kit. Many dilution factors were used but mainly the undiluted ones worked (Figures 3.11 and 3.12). The amplification of some samples was repeated many times because of the PCR inconsistency.

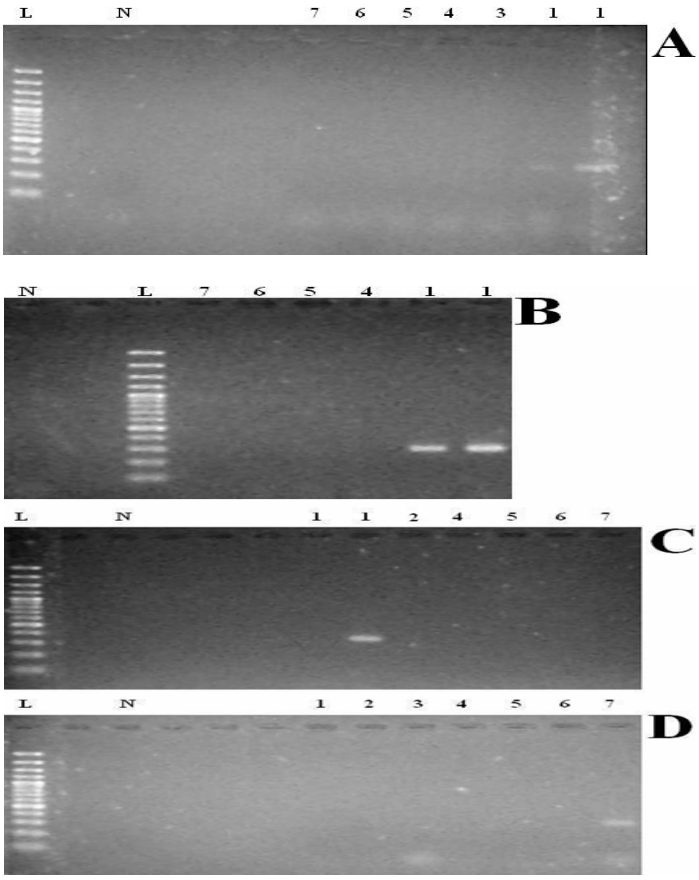


Figure 3.11 Amplicons of olive oil DNA using Lup2F-Lup2R primers. (A) Chondrolia Chalkidikis oil; (B) Kalamon oil; (C) Koroneiki oil; (D) Mastoeides oil; (E) Lianolia Kerkyras oil; (F) Gaidourelia oil; (G) Adramytini oil; (H) Valanolia oil; (I) Throumbolia oil; (J) Manaki oil; : L) 100 bp DNA ladder; N) Non-template control; 1) undiluted; 2) 1:5 dilution; 3) 1:7 dilution; 4) 1:10 dilution; 5) 1:15 dilution; 6) 1:20 dilution; 7) 1:50 dilution.

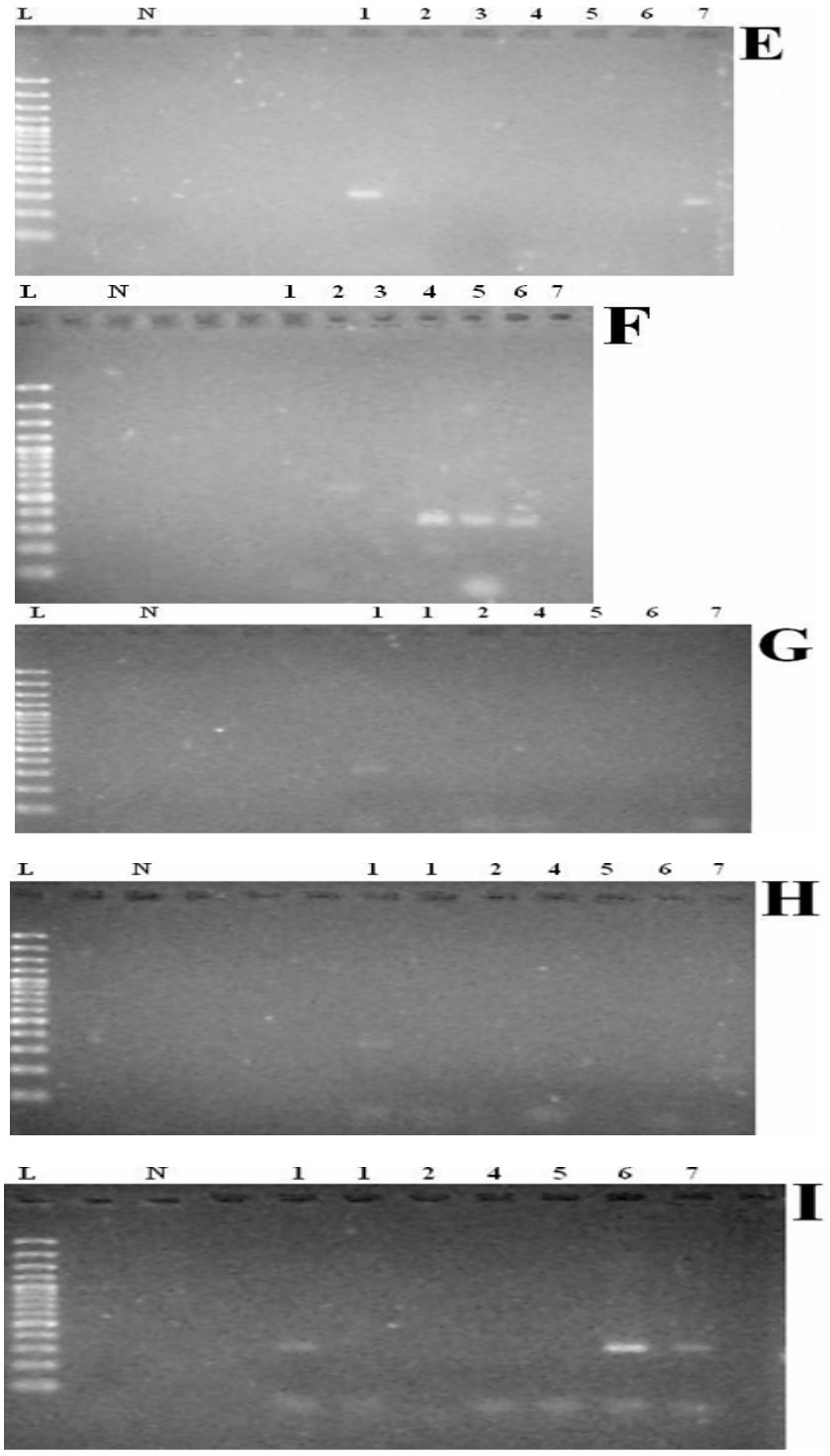


Figure 3.11 (continued)

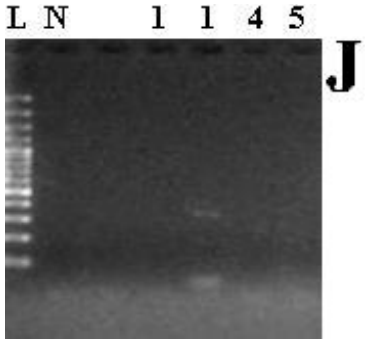


Figure 3.11 (continued)

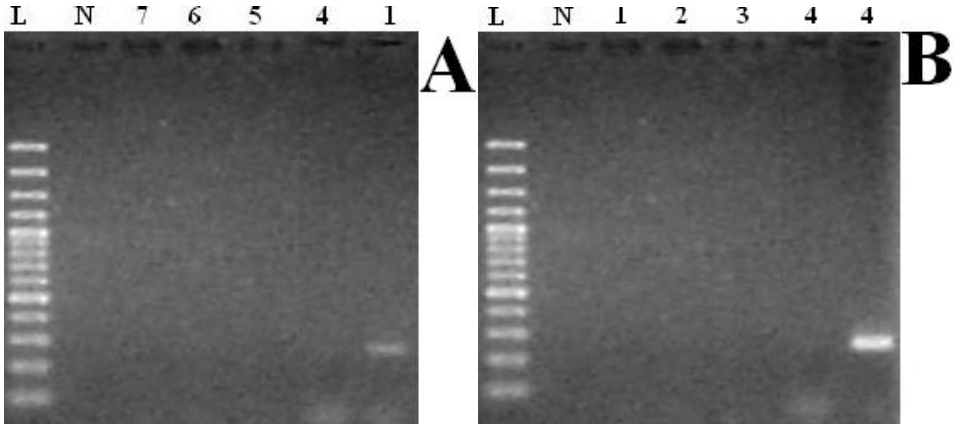


Figure 3.12 Amplicons of olive oils DNA using Cyc2aF-Cyc2aR primers. (A) Chondrolia Chalkidikis oil; (B) Gaidourelia oil; : L) 100 bp DNA ladder; N) Non-template control; 1) undiluted; 2) 1:5 dilution; 3) 1:7 dilution; 4) 1:10 dilution; 5) 1:15 dilution; 6) 1:20 dilution; 7) 1:50 dilution.

Moreover, PCR amplification of olive leaf DNA from the same varieties was carried out (Figure 3.13) with the purpose of verifying the results of SNaPshot (see paragraph 3.3.3).

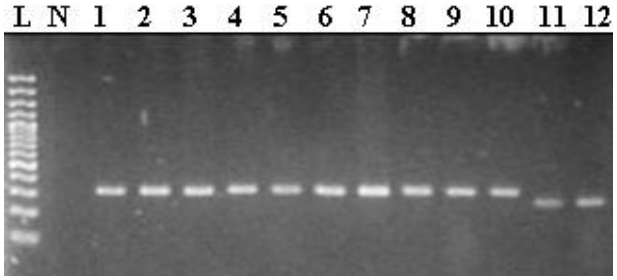


Figure 3.13 Amplicons of olive leaf DNA using Lup2F-Lup2R primers: L) 100 bp DNA ladder; N) Non-template control; 1) Chondrolia Chalkidikis (Farmer of Chalkidiki); 2) Valanolia (Lesvos); 3) Adramytini (Nagref Chania); 4) Koroneiki (Nagref Chania); 5) Kalamon (Kostelenos); 6) Throumbolia (Nagref Chania) ; 7) Mastoeides (Nagref Chania); 8) Lianolia Kerkyras (Nagref Agios Mamas); 9) Gaidourelia (Kostelenos); 10) Manaki (Kostelenos). Amplicons of olive leaf DNA using Cyc2aF-Cyc2aR primers: 11) Chondrolia Chalkidikis (Farmer of Chalkidiki); 12) Gaidourelia (Kostelenos).

3.3.3 Single-nucleotide Base Extension (SNaPshot^{TM})

The principles of the SBE assay rely on the amplification of target DNA that includes the SNP under detection and the annealing of a primer adjacent to the 3' – end of the SNP. A mixture of dideoxy-nucleotides (each labeled with different fluorescent dye) along with the SNP primer and target amplicon are subjected to several amplification cycles. During this process, once the complementary dideoxy-nucleotide is incorporated on the 3' – end of the SNP primer, the amplification terminates because the [OH^-] group responsible for the formation of phosphorus bridges no longer exists. Consequently, the type of fluorophore detected on the capillary electrophoresis pinpoints which nucleotide was initially incorporated and therefore identifies the SNP due to complementarity (Syvanen, 1999). In reality, the molecule that fluoresces after the incorporation of dideoxy-nucleotides is the SNP primer itself, extended to one extra base, complementary to the SNP locus. This is usually 15 - 35 nucleotides in total length. That virtually means that the length of the PCR target can be just as long as is required for a successful annealing between amplicon and SNP primer. In practice, that could be smaller than 100 bp. That feature could prove very useful for the analysis of heavily processed foods, where the DNA residual is fragmented.

Other adulterated food models have shown that the SNaPshot™ assay exhibits a very satisfactory limit of detection, while it needs just a small quantity of amplicon in order to give a quantifiable signal (data not shown). This is very important for olive oil that gives a very small amount of DNA and subsequently a small concentration of PCR product.

3.3.3.1 Analysis of SNaPshot ™ results

The samples have been prepared for SNaPshot analysis but they will be analyzed in a future work.

3.4 Discrimination of olive oil with other edible plant oils

One of the main adulterations of olive oil is the mixing of extra virgin grade with either lower grades and/or other vegetables oils. A first attempt was made for the discrimination of olive oil with other edible plant oils using universal chloroplastic primers. The amplified fragments were further analysed on a capillary electrophoresis system (Experion™ DNA 12K Analysis Kit by BIO RAD) using lab-on-a-chip technology which provides higher specificity than agarose gel.

3.4.1 Analytical target

As mentioned in 3.3.1, chloroplastic targets are preferred for edible plant oils that are heavily processed. For the discrimination of olive oil with other edible plant oils, the intraspacer chloroplast region (trnL – trnF) was chosen (Taberlet *et al*., 1991) Plant-A1/A2 primers give different size fragments in this region for most species (Figure 3.14 and 3.15).

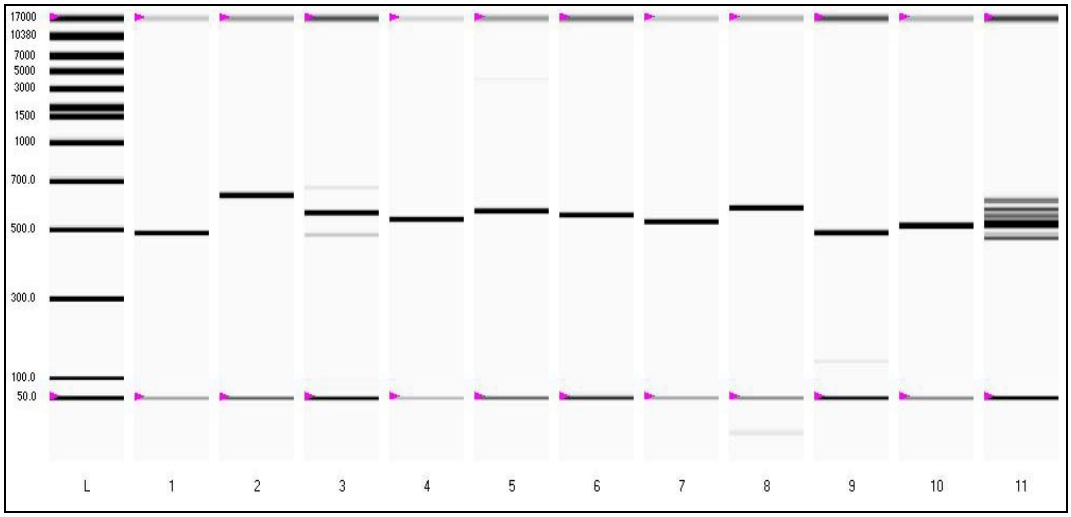


Figure 3.14 Capillary electrophoresis gel. PCR amplicons of leaf DNA using PlantA1/A2 primers: L) Ladder; 1) sunflower; 2) cotton; 3) soya; 4) sesame; 5) walnut; 6) almond; 7) avocado; 8) hazelnut; 9) corn; 10) olive; 11) pooled amplicons (sunflower, cotton, soya, sesame, walnut, almond, avocado, hazelnut, corn).

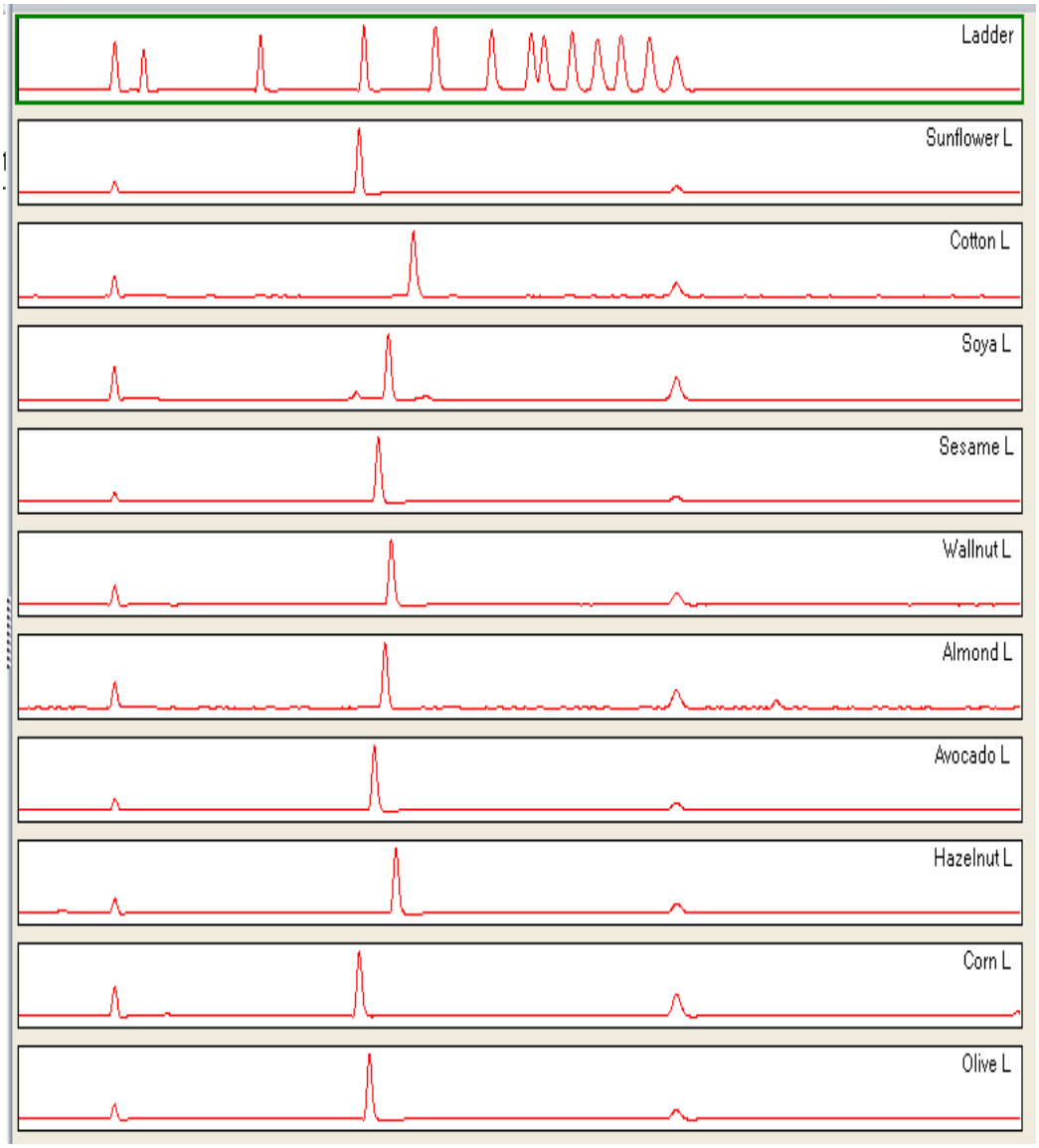


Figure 3.15 Capillary electrophoresis electropherogram of Figure 3.13

The aim of this capillary electrophoresis gel was to find the size of the amplified fragment for each species. Knowledge of the size can discriminate among species when an adulteration has occurred.

The results show that only six of the ten species can be discriminated when they are mixed (Figure 3.16).

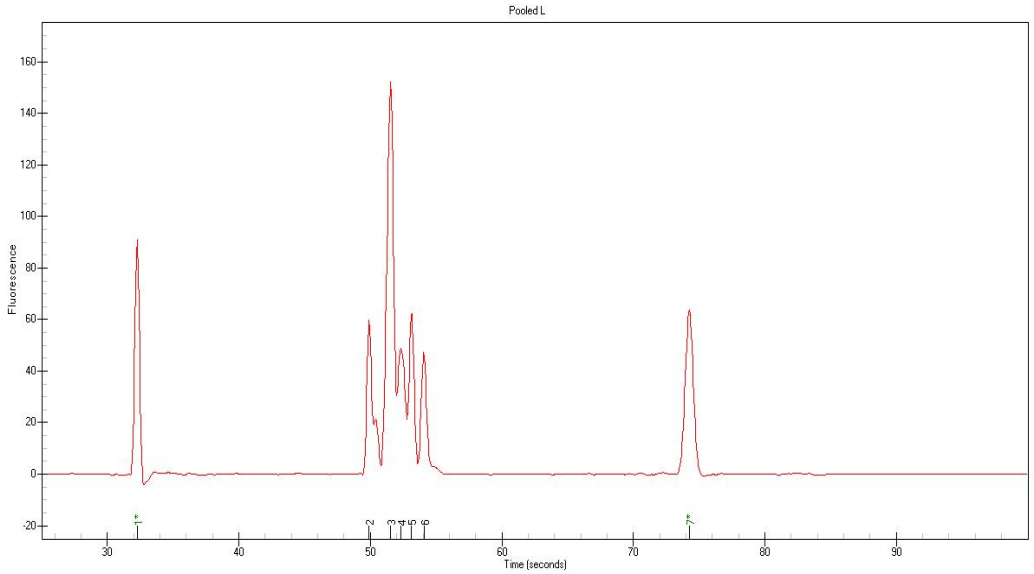


Figure 3.16 Capillary electrophoresis electropherogram with pooled amplicons of sunflower; cotton; soya; sesame; walnut; almond; avocado; hazelnut; corn; olive;

The six peaks that are visible on electropherogram of Figure 3.16 prove the existence of only six species because the remaining species have probably the same or are of the similar size to some of them. The above work was repeated four times and the average with the standard deviation was calculated to prove which species have approximately the same size (Figure 3.17).

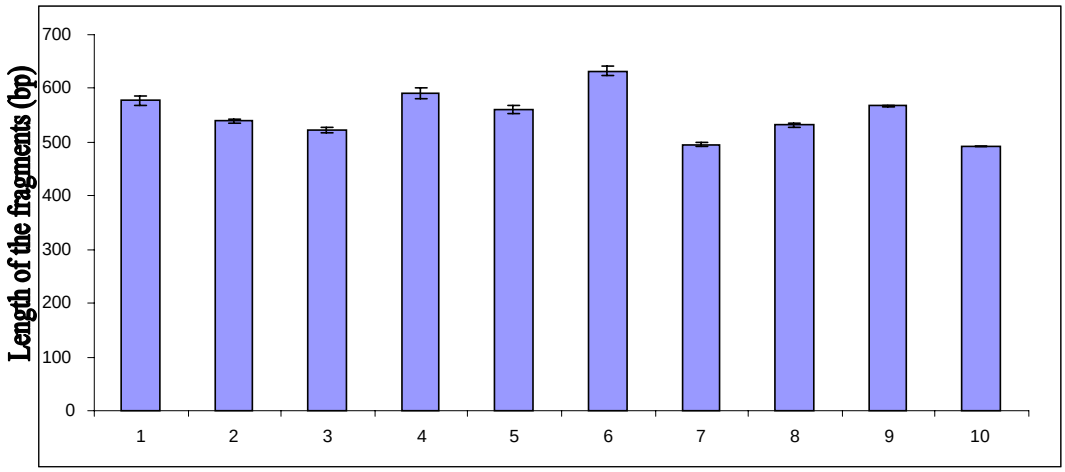


Figure 3.17 Averages and standard deviation of four different capillary electrophoresis runs: L) Ladder; 1) walnut; 2) sesame; 3) olive; 4) hazelnut; 5) almond; 6) cotton; 7) corn; 8) avocado; 9) soya; 10) sunflower.

Analyzing Figure 3.17, it is easily noticed that corn and sunflower more or less have the same fragment size. There is also no difference in size between avocado and sesame. Moreover, the standard deviation of walnut overlaps that of hazelnut which results in unreliable size difference. Finally, fragment size of olive is the same as for avocado. The difference between olive fragment size and the other species is shown in Figure 3.18.

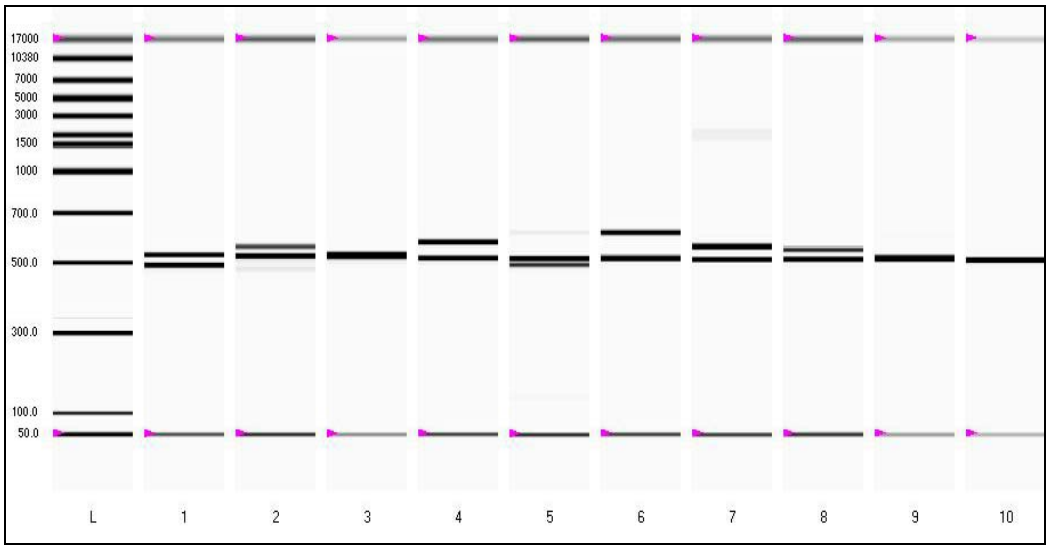


Figure 3.18 Capillary electrophoresis gel. PCR amplicons of leaf DNA using PlantA1/A2 primers: L) Ladder; 1) sunflower and olive; 2) soya and olive; 3) sesame and olive; 4) hazelnut and olive; 5) corn and olive; 6) cotton and olive; 7) walnut and olive; 8) almond and olive; 9) avocado and olive; 10) olive.

Figure 3.17 is verified by Figure 3.18. Olive can probably be discriminated among the species, except for avocado and sesame. The similarity between olive and sesame was not really expected because, according to Figure 3.17, the difference between olive and sesame is approximately 30bp. A careful study of the electropherogram (Figure 3.19) shows that there are two peaks but the sesame signal is much stronger than that of olive. The low signal for olive's peak is likely due to a pipetting error during the mixing, or it could be that the quantity of the sesame amplicons is higher than that of olive.

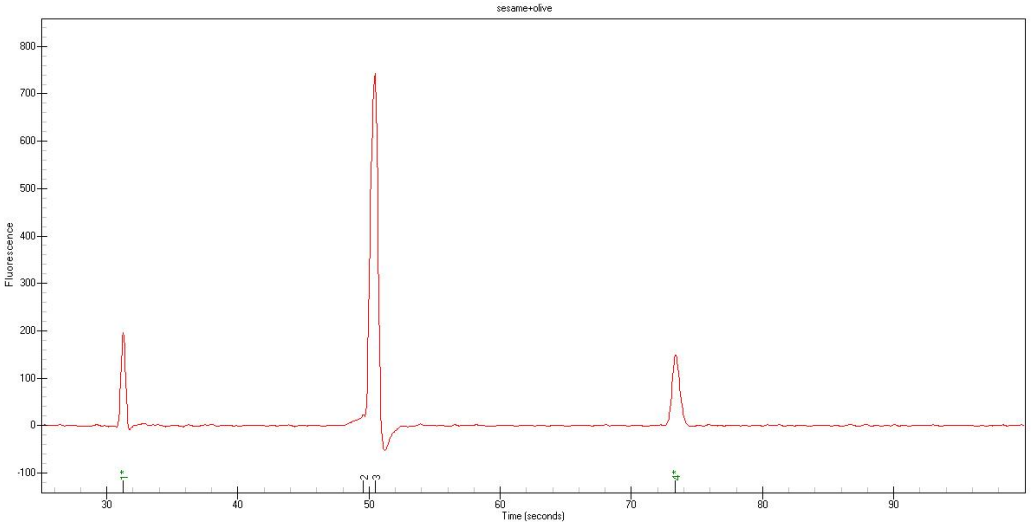


Figure 3.19 Capillary electrophoresis electropherogram of sesame and olive amplicons mixture

One more object for study was the limits of adulteration that can be detected. For this purpose, mixtures of olive and cotton amplicons in different concentrations were made (Figure 3.20). Cotton amplicons were chosen because they have a bigger size difference than that of olive.

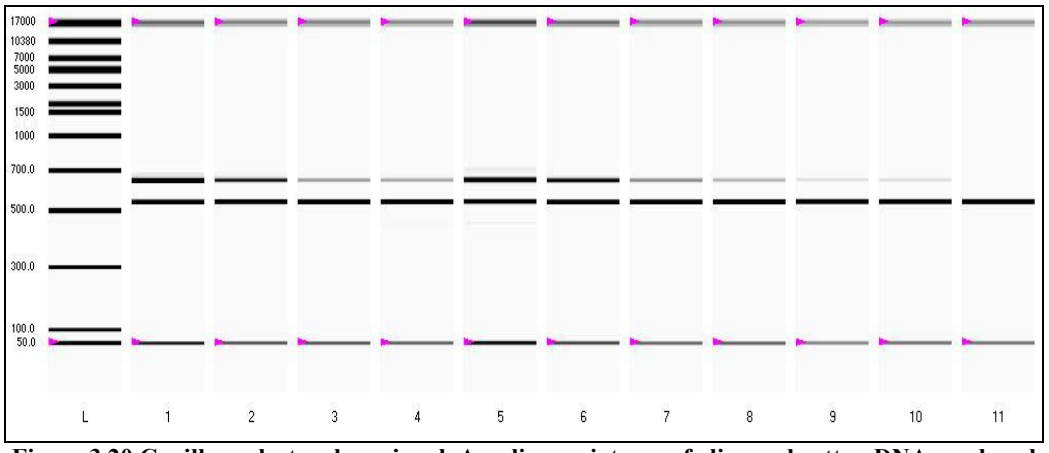


Figure 3.20 Capillary electrophoresis gel. Amplicon mixtures of olive and cotton DNA produced with Plant-A1/A2 primers. L) Ladder; 1) 50% olive - 50% cotton; 2) 70% olive – 30% cotton; 3) 90% olive – 10% cotton; 4) 95% olive – 5% cotton; 5) 50% olive - 50% cotton; 6) 70% olive – 30% cotton; 7) 90% olive – 10% cotton; 8) 95% olive – 5% cotton; 9) 99% olive – 1% cotton; 10) 99% olive – 1% cotton; 11) 100% olive

The results of Figure 3.20 show that even 1% of cotton amplicons are detectable. Furthermore, a standard deviation curve was designed in order to show the linearity of the results. The relative coefficient is equal to 99.3% (Figure 3.21).

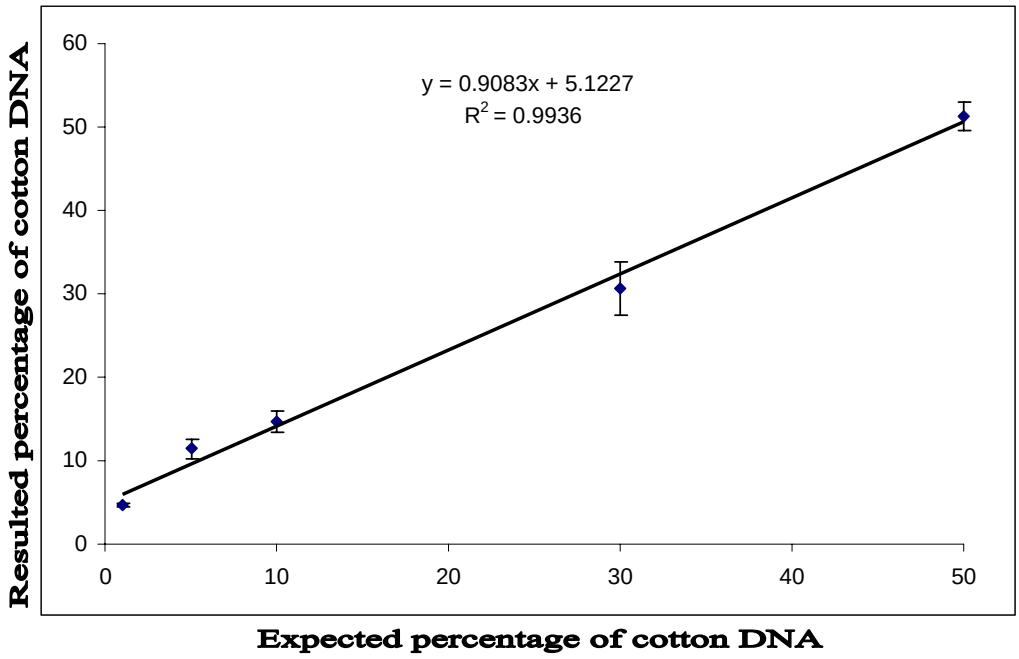


Figure 3.21 Standard deviation curve and relative coefficient of the limit of detected adulteration

3.4.2 Application on edible oils

Based on the results of DNA from leaves, the study was continued on edible plant oils from walnut, soya, sesame, almond, avocado and olive. The plant oils used for this study were not certified. The amplicons of these oils were analysed on capillary electrophoresis (Figure 3.22). Some plant oils were provided from more than one market, but they were not certified for their authenticity.

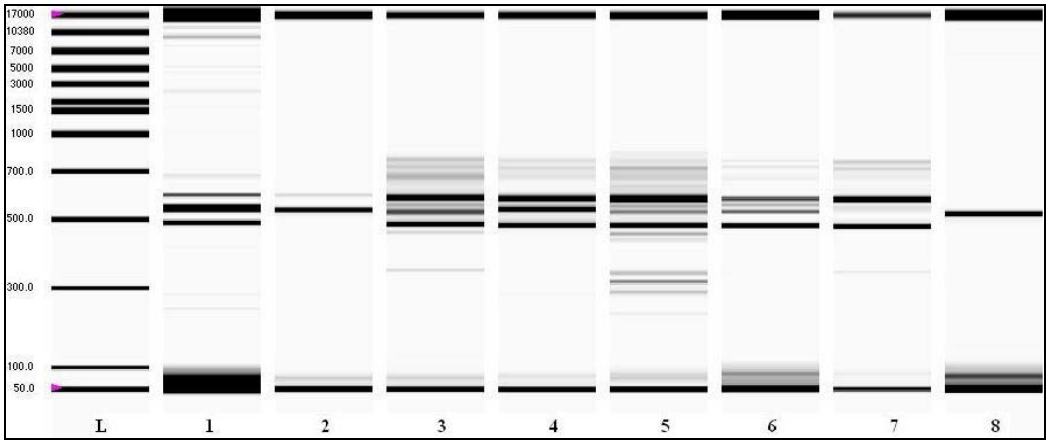


Figure 3.22 Capillary electrophoresis gel. PCR amplicons of oils' DNA produced with Plant-A1/A2 primers: L) Ladder; 1) Soya oil (Kore A.E); 2) Sesame oil (Haitoglou); 3) Sesame oil (Dianthos); 4) Avocado oil (Dianthos); 5) Almond oil (Dianthos); 6) Almond oil (Emile Noel); 7) Walnut oil (Dianthos); 8) Lianolia Kerkyras olive oil.

The gel illustrated in Figure 3.22 shows more than one band, which was not expected, for most of the oils. This probably means that some oils are adulterated with other oils. Adulteration is mainly detected on plant oils from Dianthos and almond oil from Emile Noel.

Firstly a comparison was made between Lianolia Kerkyras olive oil and leaves to test the reliability of the capillary electrophoresis (Figure 3.23). Lianolia Kerkyras olive oil was extracted in our laboratory and it is 100% pure. According to Figure 3.23 the size of olive oil is the same as that for olive leaves, which means that this method is likely to be reliable.

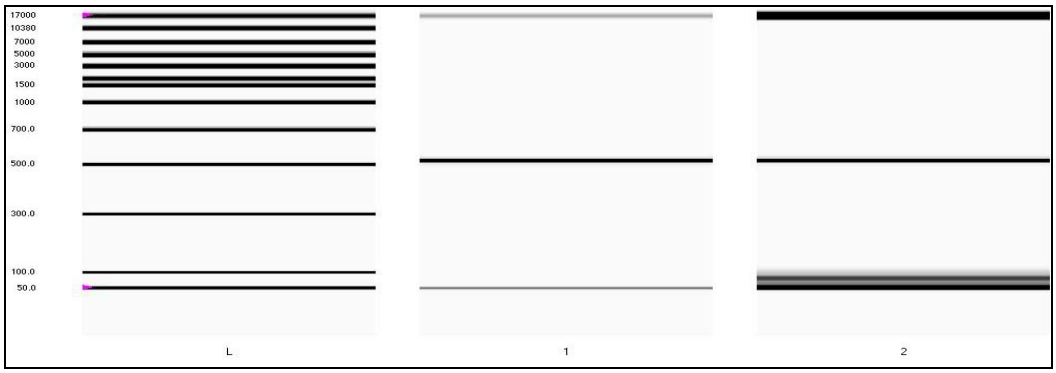


Figure 3.23 Capillary electrophoresis gel. Comparison between PCR products, produced with Plant-A1/A2 primers: 1) Lianolia Kerlyras leaves and 2) Lianolia Kerkyras oi

A comparison was then made between leaves and oil from Lianolia Kerkyra that show a single band on Figure 3.23. Other species are soya (Figure 3.24) and sesame (Figure 3.25). The comparison of soya leaves and oil amplicons shows that the amplified fragments have the same size in both of them. The same results were found in sesame leaves and oil amplicons.

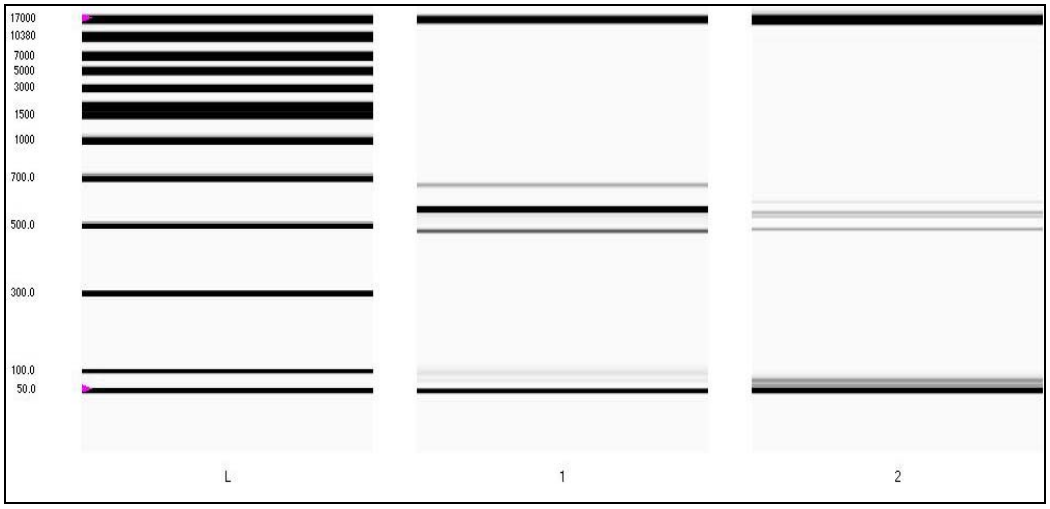


Figure 3.24 Capillary electrophoresis gel. Comparison between PCR products, produced with Plant-A1/A2 primers: 1) soya leaves and 2) soya oil.

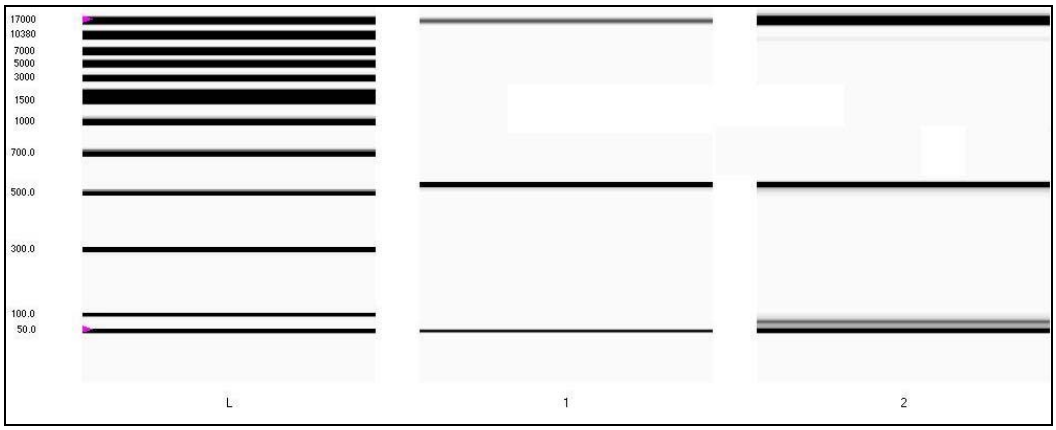


Figure 3.25 Capillary electrophoresis gel. Comparison between PCR products, produced with Plant-A1/A2 primers: 1) sesame leaves and 2) sesame oil (Haitoglou).

The plant oils from Dianthos were then compared with the same plant leaves in Figure 3.14. The analysis was carried out on capillary electrophoresis (Figure 3.26).

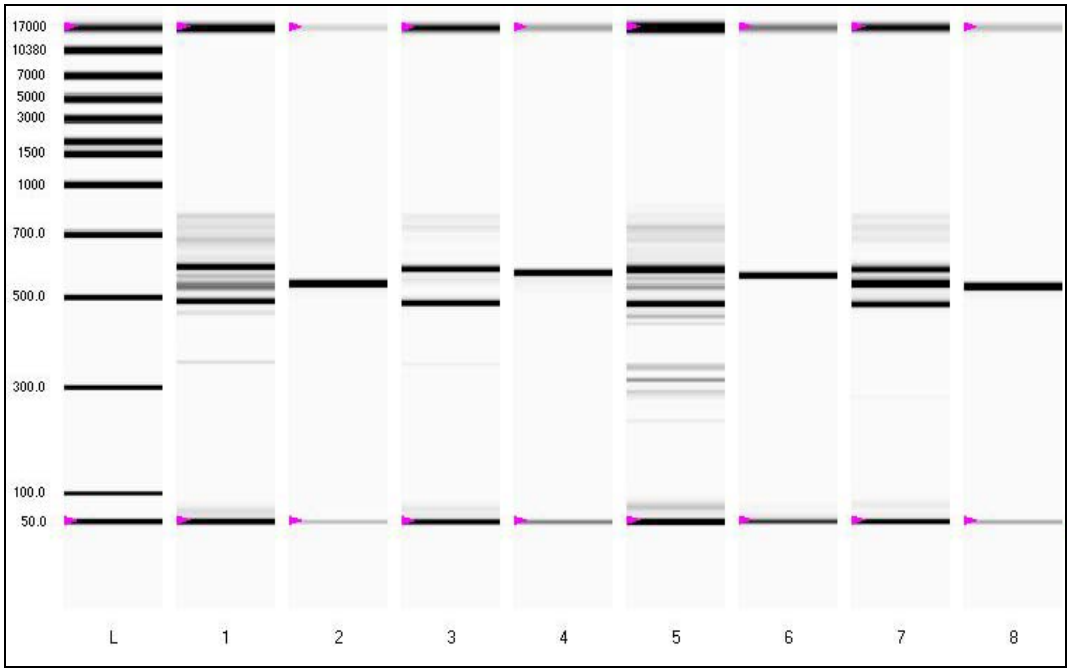


Figure 3.26 Capillary electrophoresis gel. PCR amplicons of DNA from leaves and oils produced with Plant-A1/A2 primers: L) Ladder; 1) Sesame oil (Dianthos); 2) Sesame leaves; 3) Walnut oil (Dianthos); 4) Walnut leaves; 5) Almond oil (Dianthos); 6) Almond leaves; 7) Avocado oil (Dianthos); 8) Avocado leaves.

Figure 3.26 shows more than one band on each Dianthos oil. This is probably a sign that these oils are adulterated. Dianthos buys the plant oils in bottles of 1 liter and then the plant oils are divided into small bottles of 50ml by the market salesman. One possible explanation for the adulteration of the above oils is the use of the same cone-shaped tool by the salesman for the transfer of all the oils. Moreover, the non-similarity between the leaves and oils fragment size is likely because of the heterodimers formation, due to the presence of many different plant DNA's.

CHAPTER 4

CONCLUSION AND FUTURE WORK

4.1 CONCLUSION

A preliminary work towards the authenticity of olive oil using SNPs has been carried out. Initially, the effect of olive oil storage on the stability of residual DNA and the formation of PCR inhibitors were studied. In this study, amplification signal was gradually reduced throughout the whole storage period. These results indicate a likelihood of DNA degradation, along with the production of PCR inhibitors. However, the former seems to happen at such a rate which is probably not enough to degrade DNA molecules of approximately 100 bp length, whereas the latter was further confirmed through the PCR experiment on yeast DNA.

After that many different extraction kits were tested with the intention of choosing the most appropriate for DNA extraction from vegetable oils. The extraction kits were compared for the quality of the PCR product and their price.

Then, a large number of 43 important Greek olive varieties were analyzed with the purpose of varietal discrimination. The differentiation was not completed but 19 out of 43 were totally discriminated. Some of the main oil-producing Greek varieties are 9 out of the above 19 varieties (Koroneiki, Kothreiki, Agouromanakolia, Koutsourelia, Asprolia Lefkados, Adramytini, Valanolia, Throumbolia) (Therios, 2005).

Also, a preliminary work was carried out on authenticity of olive oil against other vegetables oils like walnut oil, soya oil, sesame oil, almond oil and avocado oil using universal primers. This method showed that it works not only with samples of vegetable leaves but also with samples of soya, sesame and olive oil. The most important factor in the improvement of this method is the collection of 100% pure samples for the standardization of the method.

4.2 FUTURE WORK

In this work the effect of olive oil storage on the stability of residual DNA has been studied but the precise mechanism of DNA route from fruit-to-oil remains to be elucidated and further experiments on DNA integrity have to be carried out.

The Greek SNP database developed throughout this study has several SNP positions, the allelic variants of which could not be identified due to bad sequencing signals. In the future, the identification of these blank SNP positions, along with the in-depth screening of extra gene sequences, should be carried out for the completion of the database.

The most well-known Greek olive varieties have been analyzed but there are also many Greek varieties that are not included in this database. In addition, varietal olive oil authenticity using SNPs as a molecular marker demands the analysis of varieties not only from Greece but also from foreign varieties.

Moreover, the adulteration of olive oil with other vegetable oils using universal primers (Plant-A1/A2) needs improvement and confirmation by the use of PCR-RFLP.

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APPENDIX

A. Buffers and solutions for DNA extraction using a standard cetyltrimethylammonium bromide-based.

- CTAB buffer

CTAB	2% w/v
NaCl	1,4M
EDTA	20mM
Tris-HCl (pH=8)	100mM
PVP-40	2% v/v
2-mercaptoethanol	1% v/v
ddH_2O	

- 1M Tris-Cl (Tris (hydroxymethyl)aminomethane) (pH=8)
  - Dissolve 121.1 gr of Tris base in 800 ml of hhH_2O;
  - Adjust the pH to 8 by adding concentrated HCl;
  - Adjust the volume of the solution to 1litre by adding ddH_2O;
  - Autoclave.

- EDTA 0.5M (pH=8)
  - Add 186.1 g of disodium EDTA$\bullet 2H_2O$ to 800 ml of ddH_2O;
  - Stir vigorously on a magnetic stirrer;
  - Adjust the pH to 8 by adding NaOH;
  - Autoclave

- EtOH 76% with 10mM $AcNH_4$ (50ml)
  - Add 38ml EtOH
  - Add 11.933 ml ddH_2O
  - Add 67µl 7.5M $AcNH_4$

- NaCl 5M
  - Dissolve 292.2gr NaCl in 800 ml of hhH_2O;
  - Adjust the volume of the solution to 1litre by adding ddH_2O;
  - Autoclave.

- Ethidium bromide (10mg/µl)
  - Add 1g of ethidium bromide to 100ml of H_2O;
  - Stir on a magnetic stirrer for several hours to ensure tha the dye has been dissolved;
  - Wrap the container in aluminum foil and store at room temperature

- 50X TAE buffer
  - Prepare a 50x stock solution in 1 liter of H2O:
  - 242 g of Tris base
  - 57.1 ml of glacial acetic acid
  - 100 ml of 0.5 M EDTA (pH 8.0)

B. DNA Ladder

- A) 100bp DNA Ladder: Eleven fragments that range in size from 100bp to 1,000bp in 100bp increments with an additional band at 1,500bp. The 500bp fragment is present at increased intensity for easy identification.

- B) 1Kb DNA Ladder: Thirteen blunt-ended fragments with sizes ranging from 250bp to 10,000bp. The 1,000bp and 3,000bp fragments have increased intensity relative to the other bands on ethidium bromide-stained agarose gels for easy identification. All other fragments are of equal intensity.

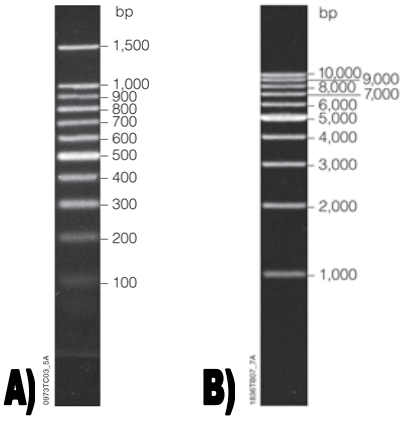

Figure 5.1 DNA Ladders: A) 100bp DNA Ladder; B) 1Kb DNA Ladder

C. An example of employing the generated SNP database to discriminate olive varieties.